Inclusion of a pH-responsive amino acid-based amphiphile in methotrexate-loaded chitosan nanoparticles as a delivery strategy in cancer therapy

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Abstract

The encapsulation of antitumor drugs in nanosized systems with pH-sensitive behavior is a promising approach that may enhance the success of chemotherapy in many cancers. The nanocarrier dependence on pH might trigger an efficient delivery of the encapsulated drug both in the acidic extracellular environment of tumors and, especially, in the intracellular compartments through disruption of endosomal membrane. In this context, here we reported the preparation of chitosan-based nanoparticles encapsulating methotrexate as a model drug (MTX-CS-NPs), which comprises the incorporation of an amino acid-based amphiphile with pH-responsive properties (77KS) on the ionotropic complexation process. The presence of 77KS clearly gives a pH-sensitive behavior to NPs, which allowed accelerated release of MTX with decreasing pH as well as pH-dependent membrane-lytic activity. This latter performance demonstrates the potential of these NPs to facilitate cytosolic delivery of endocytosed materials. Outstandingly, the cytotoxicity of MTX-loaded CS-NPs was higher than free drug to MCF-7 tumor cells and, to a lesser extent, to HeLa cells. Based on the overall results, MTX-CS-NPs modified with the pH-senstive surfactant 77KS could be potentially useful as a carrier system for intracellular drug delivery and, thus, a promising targeting anticancer chemotherapeutic agent.

Introduction

Cancer is one of the main causes of mortality worldwide and the World Health Organization (WHO) estimates that there will be 15 million new cases of cancer worldwide in 2020 (ETPN 2015). The clinical efficacy of the conventional treatments is often compromised by the acquisition of resistance in cancer cells and/or by the generation of several side effects to the patients (Banerjee et al. 2002; Dong and Mumper 2010). In this context, the nanotechnology-based pharmaceutical products might provide a wide range of new tools and possibilities in cancer therapy, from earlier diagnostics to more efficient and more targeted treatments (Chen et al. 2014; Das and Sahoo 2011; Li et al. 2012).

Methotrexate (MTX) is a cytotoxic drug used in the therapy of solid tumors and leukemias. Its main pharmacological target is the competitive inhibition of dihydrofolate-reductase (DHFR), an intracellular enzyme that reduces folic acids to key intermediates in several important biochemical pathways of DNA and RNA synthesis (Gonen and Assaraf 2012). Unfortunaltely, the resistance in cancer cells often compromises the efficacy of the anticancer therapy with MTX (Zhao and Goldman 2003). In addition, the conventional MTX delivery strategies often lead to undesirable shortcomings, such as the severe side effects associate with the treatment, including neurologic toxicity, renal failure and mucositis (Banerjee et al. 2002; Rubino 2001).

Chitosan (CS) is a naturally occurring polymer that has been attracting increasing attention in pharmaceutical and biomedical applications because of its biocompatibility, biodegradability, non-toxicity, cationic properties and bioadhesive characteristics (Bao et al. 2008; Fan et al. 2012). Therefore, CS nanoparticles (NPs) are of great interest as drug delivery systems, including applications for cancer therapy (Chen et al. 2014; Deng et al. 2014; Trapani et al. 2011). The ionotropic gelation technique is particularly suitable for the incorporation of pharmaceuticals, as it can be achieved in aqueous conditions (Agnihotri et al. 2004; Berger et al. 2004; Fan et al. 2012). Moreover, the formation process is solely based on the electrostatic interaction of oppositely charged compounds and, thus, it is not necessary any chemical modification. CS-based NPs containing MTX were already reported in the literature, but most of them with drawback of using a cross-linked agent (Chen et al. 2014; Jia et al. 2014; Luo et al. 2014; Wu et al. 2009). Likewise, there is considerable progress already achieved regarding the mechanisms underlying drug release from the nanostructures, being most of the current release methods based on reactions that commonly occur in response to environmental factors,

i.e. the acidic conditions of subcellular compartments (Li et al. 2012). Therefore, the knowledge about this mechanism gave basis for the research of pH-responsive nanocarriers.

Upon this sense, and in an attempt to develop an efficient drug delivery device for cancer therapy, here we reported a simple strategy for preparation of pH-responsive MTX-loaded CS-based NPs by using the ionic gelation method. Without crosslinkers and/or organic solvents, a bioactive amino acid-based surfactant with pH-sensitive properties was incorporated in the complexation process during NP preparation. This compound was added as a modifier into the formulation in order to achieve a greater antitumor activity of the encapsulated drug. The anionic amphiphile derived from N^{α},N^{ε}-dioctanoyl lysine with a sodium counterion (77KS) have pH-sensitive membrane-lytic behavior and low cytotoxicity (Nogueira et al. 2011a), suggesting that it may be a specific adjuvant with ability to destabilize the endosomal membrane in the mildly acidic environment and, thus, release more specifically and efficiently the drug inside the malignant cells (Lee et al. 2008). Our group have previously published a study on the design of NPs based on medium molecular weight (MMW) CS and incorporating a lysine-based surfactant with a different counterion (77KL, with lithium counterion) (Nogueira et al., 2013). This study showed promising results, which therefore provided us the basis to continue the research in this field, seeking for important progress in delivery strategies.

Finally, besides the preparation and characterization of CS-based NPs incorporating the surfactant 77KS, we evaluated the role of pH in the membrane-lytic activity of NPs and also in the *in vitro* drug release profile. In addition, to gain insight into the potential antitumor activity of NPs, the antiproliferative activity of both free and encapsulated drug was assessed using *in vitro* tumor cell models.

Experimental

Chemicals and reagents

Chitosan (CS) of low molecular weight (LMW) (deacetylation degree, 75-85%; viscosity, 20-300 cP according to the manufacturer's data sheet), pentasodium tripolyphosphate (TPP), 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and trypsin-EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA • 4Na per liter of Hanks′ Balanced Salt Solution) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methotrexate (MTX, state purity 100.1 %) was purchased from SM Empreendimentos Farmacêuticos Ltda. (São Paulo, SP, Brazil).

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Acetonitrile was purchased from Tedia (Fairfield, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM), supplemented with L-glutamine (584 mg/l) and antibiotic/antimicotic (50 mg/ml gentamicin sulphate and 2 mg/l amphotericin B), were purchased from Vitrocell (Campinas, SP, Brazil). All other reagents were of analytical grade.

Surfactant included in the Nanoparticles

An anionic amino acid-based surfactant derived from $N^{\alpha}, N^{\varepsilon}$-dioctanoyl lysine and with an inorganic sodium counterion (77KS) was included in the NP formulation. It has a molecular weight of 421.5 g/mol, a critical micellar concentration (CMC) of 3×10^3 µg/ml and its chemical structure is formed by two alkyl chains, each one with eight carbon atoms (Sánchez et al. 2006, 2007). This surfactant was synthesized as previously described (Vives et al. 1999).

Nanoparticle preparation

CS-NPs were spontaneously obtained by ionotropic gelation technique, according to the methodology previously developed by Calvo et al. (1997) but with some modifications. The NPs were prepared with a selective CS:TPP:77KS ratio of 5:1:0.5 (w/w/w). Likewise, NPs omitting the surfactant and, thus, with CS:TPP ratio of 5:1 (w/w), were also prepared in order to perform comparative studies. Unloaded CS-NPs (unloaded-CS-NPs) were prepared by dropwise addition of a premixed solution containing the cross-linker TPP and the surfactant 77KS (both at 0.1%, w/v) over the CS solution under magnetic stirring. Agitation was maintained for 20 min, under room temperature and dark conditions, to allow the complete formation of NPs. The CS solution was prepared at a concentration of 0.1% (w/v) and was dissolved in acetic acid solution (1%, v/v). The pH of the CS final solution was adjusted to 5.5 with 1 M NaOH (Gan et al. 2005). Similarly, MTX-loaded CS-NPs (MTX-CS-NPs) were prepared following the same procedure, but adding MTX to a premixed TPP and 77KS solution, providing a final concentration of 0.07% (w/v) of the antitumor drug (final ratio TPP:77KS:MTX 1:0.5:1, w/w/w).

Nanoparticle characterization

The mean hydrodynamic diameter and the polydispersity index (PDI) of the NPs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK), without any dilution of the samples. Each measurement was performed using at least three sets of ten runs. The zeta potential (ZP) values of the NPs were assessed by determining electrophoretic mobility with the Malvern Zetasizer ZS equipment. The measurements were performed after dilution of the formulations in 10 mM NaCl aqueous solution (1:10 volume per volume), using at least three sets of 10 runs. The pH measurements were determined directly in the NP suspensions, using a calibrated potentiometer (UB-10; Denver Instrument, Bohemia, NY, USA), at room temperature. Finally, the spectral properties of the model drug were assessed before its encapsulation and also after extraction from the NP structure. This assay was performed in order to verify the stability of MTX after entrapment into the NP matrix. The experiments were performed on a double-beam UV-VIS spectrophotometer (Shimadzu, Japan) model UV−1800, with a fixed slit width (2 nm) and a 10 mm quartz cell was used to obtain spectrum and absorbance measurements. The diluent optimized was 0.1 M sodium hydroxide.

Drug entrapment efficiency

The drug association efficiency was determined by the ultrafiltration/centrifugation technique using Amicon Ultra-0.5 Centrifugal Filters (10000 Da MWCO, Millipore). The entrapment efficiency (EE%) was calculated by the difference between the total concentration of MTX found in the NP suspension after its complete dissolution in methanol, and the free MTX concentration determined in the ultrafiltrate after separation of the NPs. The EE% of MTX in the NPs was measured using a previously validated reversed-phase high performance liquid chromatography (RP-HPLC) method (Nogueira et al. 2014a). The chromatographic analyses were carried out on a Shimadzu LC system (Shimadzu, Kyoto, Japan), using a Waters XBridgeTM C18 column (250 mm x 4.6 mm I.D., 5μm), a mobile phase consisted of potassium phosphate buffer (0.05 M, pH 3.2): acetonitrile (86:14, v/v) and UV detection set at 303 nm. The analytical method showed acceptable results for the specificity test (no interference of the NP components on the quantitative analysis), and also provided good linearity in the 1 − 30 μg/ml concentration range (r = 0.9999), precision (with a relative standard deviation lower than 1.5%) and accuracy (mean recovery of 99.39%).

In vitro release study

In vitro release assessments from MTX-loaded CS-NPs were carried out for 480 min in PBS at pH 7.4, 6.6 and 5.4. An aliquot of NPs (1 ml) was placed in a dialysis bag (Sigma-Aldrich, 14000 MWCO) and suspended in 50 ml of PBS at 37ºC under gentle magnetic stirring (100 rpm). The withdrawal of 2 ml of the external medium from the system was done at predetermined time interval and was filtered through a 0.45-µm membrane. An equal volume of fresh medium was replaced in order to maintain the sink conditions. The release of the free drug was also investigated in the same way. The cumulative release percentage of MTX at each time point was estimated by the RP-HPLC method described previously (Nogueira et al. 2014a), using analytical curves obtained with the release medium (PBS at pH 7.4, 6.6 and 5.4) as diluents.

pH-dependent membrane-lytic activity of nanoparticles

The pH-dependent membrane-lytic activity of NPs was assessed using the hemolysis assay, with the erythrocytes as a model of the endosomal membrane (Akagi et al. 2010; Wang et al. 2007). Erythrocytes were isolated from human blood, which was obtained from discarding samples of the Clinical Analysis Laboratory of the University Hospital of Santa Maria. Tubes containing EDTA as anticoagulant were used for blood collection. Erythrocytes (25 µl of the suspension prepared in PBS) were incubated with increasing concentrations of unloaded-CS-NPs and MTX-CS-NPs suspended in PBS buffer of pH 7.4, 6.6 or 5.4. The extent and kinetics of hemolysis were assessed as reported earlier (Nogueira et al. 2011a). Absorbance of the hemoglobin released in supernatants was measured at 540 nm by a Shimadzu UV–1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Cytotoxicity assays – antitumor activity of free and encapsulated drug

The tumor cell lines HeLa (human epithelial cervical cancer) and MCF-7 (human breast cancer) were used as *in vitro* models to study the antitumor activity of MTX in its free and nanoencapsulated forms. Both cells were grown in DMEM medium (4.5 g/l glucose), supplemented by 10% (v/v) FBS, at 37ºC with 5% CO_2. These cells were routinely cultured in 75 cm^2 culture flasks and were harvested using trypsin-EDTA when the cells reached approximately 80% confluence.

HeLa (7.5 x 10^4 cells/ml) and MCF-7 (1 x 10^5 cells/ml) cells were seeded into the 60 central wells of 96-well cell culture plates in 100 µl of complete culture medium. Cells were incubated for 24 h under 5% CO_2 at 37ºC and the medium was then replaced with 100 µl of fresh medium supplemented by 5% (v/v) FBS containing the treatments. Unloaded-CS-NPs were assayed in the 25-300 µg/ml concentration range, being these concentrations based on the total composition of the formulation. In contrast, MTX-CS-NPs and free MTX were assessed in the 1-50 µg/ml concentration range and, in this case, the concentrations were based on the total amount of MTX in each sample. Untreated control cells were exposed to medium with 5% (v/v) FBS only. The cell lines were exposed for 24 h to each treatment, and their viability was assessed by two different assays, MTT and NRU (NR uptake).

The MTT endpoint is a measurement of cell metabolic activity and the assay is based on the first protocol described by Mossmann (1983). On the other hand, the NRU assay is based on the protocol described by Borenfreund and Puerner (1985), and reflects the functionality of the lysosomes and cell membranes. After complete the treatment time (24 h), the medium was removed, and 100 µl of MTT in PBS (5 mg/ml) diluted 1:10 in medium without FBS was then added to the cells. Similarly, 100 µl of 50 µg/ml NR solution in DMEM without FBS was added to each well for the NRU assay. The microplates were further incubated for 3 h under 5% CO_2 at 37ºC, after which the medium was removed, and the wells of the NRU assay were washed once with PBS. Thereafter, 100 µl of DMSO was added to each well to dissolve the purple formazan product (MTT assay) or, similarly, 100 µl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water was added to extract the NR dye (NRU assay). After 10 min shaking at room temperature, the absorbance of the resulting solution was measured at 550 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA) microplate reader. Cell viability for MTT and NRU was calculated as the percentage of tetrazolium salt reduced by viable cells in each sample or as the percentage of uptake of NR dye by lysosomes, respectively. The viability values were normalized by the untreated cell control (cells with medium only).

Hemocompatibility studies

Erythrocytes were isolated from human blood, as described for the experiments of pH-dependent membrane-lytic activity. Twenty-five microliter aliquots of erythrocyte suspension were exposed to unloaded-CS-NPs at concentrations of 100, 250 and 500 µg/ml (concentrations based on the total composition of the formulation), and to MTX-CS-NPs or free MTX at

concentrations of 10, 25 and 50 μg/ml (concentrations based on the total amount of MTX). The samples were incubated at room temperature for 10 minutes or 1 h and then centrifuged at 10000 rpm for 5 min to stop the reaction. Absorbance of the hemoglobin released in supernatants was measured at 540 nm by a double-beam UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan), model UV–1800.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SE) and statistical analyses were performed using one-way analysis of variance (ANOVA) to determine the differences between the datasets, followed by Tukey's post-hoc test for multiple comparisons, using SPSS® software (SPSS Inc., Chicago, IL, USA). All *in vitro* experiments were performed at least three times, using three replicate samples for each formulation concentration tested. $p < 0.05$ was considered significant.

Results and discussion

The ionotropic gelation between CS, as the polycation, and TPP, as the polyanionic partner, leads to the formation of stable colloidal systems (Trapani et al. 2011). Therefore, using the optimized operating parameters, this simple and solvent-free procedure was chosen to prepare the NPs in this work. The 5:1 chitosan to TPP ratio was confirmed as a stable and suitable ratio to carry out the following studies, as also done by Calvo et al. (1997). Decreasing the chitosan to TPP ratio provokes an increasing turbidity, indicating a shift in the size variation of the particles to the larger end of the scale. Likewise, an excess of TPP may culminates in NP aggregation, once all the surface charges of CS have been annulled by excess polyanion (Fan et al. 2012).

Before NP preparation, the pH of CS solution was set at 5.5, as at this pH the NPs were smaller and have higher ZP (Gan et al. 2005), suggesting their greater stability. Additionally, about 90% of the amino groups of CS (pKa = 6.5) are protonated at pH 5.5 (Mao et al. 2010), which ensures that the crosslinking process takes place to form CS-NPs. Likewise, a pH value of 5.5 would ionize most of the carboxylic groups of MTX, which allows attractive electrostatic interactions between the negatively charged drug molecules and positively charged CS molecules. The MTX molecule is a polyelectrolyte carrying two carboxyl groups (α-carboxyl and γ-carboxyl with pKa of 3.4 and 4.7, respectively), and a number of potentially protonated

nitrogen functions, being the most basic with pKa of 5.7 (Rubino 2001). Finally, it is worth mentioning that due to its low water solubility, MTX was dissolved in the basic solution of TPP (pH ~ 9.0) before crosslinking CS.

The choice of 77KS as a bioactive excipient in the NP formulation was based on our previous studies, which showed its pH-sensitive membrane-disruptive activity, together with improved kinetics in the endosomal pH range and low cytotoxic potential (Nogueira et al. 2011a, 2011b). 77KS was incorporated into the NPs at a concentration below its CMC, thereby indicating that this surfactant is presented in the formulation in its monomer form. Moreover, our group has already demonstrated that the inclusion of another surfactant from the same family (77KL) in the composition of polymeric NPs improved their *in vitro* antitumoral activity and also gave them a pH-responsive behavior (Nogueira et al. 2013). It is noteworthy that this previous study gave us the basis to continuous the research in the field of nanocarriers with pH-dependent properties.

Characterization of Nanoparticles

Firstly, the stability of the drug after its encapsulation was assessed through the spectral analysis, as shown in Fig. 1. The UV-vis spectrum of the drug extracted from NPs was similar to that obtained for MTX solution before encapsulation, which proved the integrity of MTX molecule after its entrapment into the NP matrix. In addition, the NPs were characterized for the mean particle size, PDI and ZP. Table 1 shows the characterization parameters of the NPs obtained in the presence and in the absence of MTX and/or 77KS. The hydrodynamic size of all NPs was in the range of 162 to 183 nm, with no considerable variations due to the presence of the drug. The presence of 77KS is accompanied by an increase in the size of NPs, but without significant differences. It is worth pointing out that these NPs with LMW-CS and 77KS have a diameter almost 2-fold lower than the NPs prepared with MMW-CS and 77KL (Nogueira et al. 2013). The small size of nanocarriers may constitute a great feature to improve the drug delivery to the tumor tissues and, more specifically, to the intracellular compartments of tumor cells (Elbakry et al. 2012; Huang et al. 2012). Concerning the NP surface charge, positive ZP values (> 22 mV) were detected for all formulations. ZP is a measurement of the electric charge at the surface of the particles and indicates the physical stability of colloidal systems. It should be noted that there was a slight decrease in the surface charge due to the presence of the drug. This reduced ZP value can be attributed to the electrostatic complexation of MTX with the free amino groups of CS, which, therefore, diminishes the net positive charge in the NP surface.

Likewise, the entrapment efficiency of MTX showed a clear dependence on the presence of the surfactant in the formulation. In the absence of 77KS, the EE% obtained (~ 68%) was significantly higher than the value obtained in the presence of 77KS (~ 38%). The high incorporation capacity of CS-NPS without 77KS (CS:TPP 5:1, w/w) is probably related to the chemical nature of the drug and to its interaction with the NP structure at the pH value of the experimental conditions. On the other hand, the lower encapsulation efficiency of the CS-NPs containing the surfactant (CS:TPP:77KS 5:1:0.5, w/w/w) might be attributed to the competition between MTX and 77KS, both negatively charged, for the electrostatic complexation with the positively charged CS molecules.

In vitro release study

The release profiles of MTX from the CS-NPs showed a first phase with an initial burst release of 57% in the first 60 min at pH 7.4, as shown in Fig. 2. This is possibly due to the drug molecules dispersing close to the NP surface. Considering the electrostatic attraction existing between the negatively charged drug and the positively charged polymer, we can assume that the MTX molecules are both adsorbed at the particle surface (Gan and Wang 2007) and entrapped/embedded into CS nano-matrix by hydrogen bonding and hydrophobic forces (Calvo et al. 1997). Therefore, the way by which the drug is associated with the carrier (adsorption or encapsulation) might determine its release rate from the matrix. In the second phase, a constant release of the drug was observed up to 480 min, which can be a result of the diffusion of MTX through the polymer wall. A control experiment using free MTX was also carried out under similar conditions and complete diffusion across the dialysis membrane was found to occur within 240 min.

When the studies were performed at acidic environment, the release rate was accelerated, which confirms the pH-dependent release pattern of this nanostructure. The triggered release of payload under acidic conditions is probably attributed to the pH-responsive activity of 77KS (Nogueira et al. 2011a). The cumulative MTX release at pH 6.6 and 5.4 was in general significantly faster ($p < 0.05$) than at pH 7.4. At 480 min, 96 e 100% of MTX was released at pH 6.6 and 5.4, respectively, while only 89% of drug release was reached at pH 7.4. The total drug release at acidic conditions might be attributed to swelling and degradation of the compact NPs (Gan and Wang 2007). Additionally, noteworthy that the accelerated release at acidic conditions might promote an improved drug delivery in the acidic extracellular pH of tumors ($pH_e \sim 6.6$) (Na et al. 2003). Nevertheless, the pH-dependent pattern observed here was not as

effective as was in our previous work (Nogueira et al. 2013), in which it was used MMW-CS and the surfactant 77KL as bioactive adjuvant in the NP formulation. The faster release achieved here could be attributed to the formulation of NPs with LMW-CS, as it was previously described that the total drug release decreased significantly with increasing CS molecular weight (Gan and Wang 2007). Furthermore, these overall results could be attributed to the lower encapsulation efficiency obtained herein with the surfactant 77KS.

pH-dependent membrane-lytic activity of nanoparticles

The hemolysis assay, with the erythrocyte as a model for the endosomal membrane, was applied herein to assess whether the presence of 77KS provides to the NPs a pH-responsive membrane-lytic behavior. Firstly, it is worth mentioning that strong correlations have been reported between hemolytic activity and endosomal disturbance induced by membrane-disruptive agents (Chen et al. 2009; Lee et al. 2010; Plank et al. 1994). This biological correlation justifies, thus, the use of erythrocytes as a model for endosomal membrane.

The membrane-lytic activity of the NPs as a function of concentration, with varying pH and incubation time, is shown in Fig. 3. Primarly, we evaluated the membrane lysis induced by unloaded and MTX-loaded CS-NPs incorporating 77KS (Fig. 3a, b, respectively). At physiological pH, negligible hemolysis was observed after 10 min incubation, while 8.5 and 25% of hemolysis was observed after 60 min incubation with NPs loading or not the drug, respectively. As the pH decreased to 6.6 or 5.4, the membrane-lytic activity of unloaded and MTX-loaded CS-NPs increased significantly ($p < 0.05$) in a dose-dependent manner. After 60 min incubation, unloaded-CS-NPs were 2.81- and 3.17-fold more hemolytic at pH 6.6 and 5.4 than at pH 7.4, respectively. Likewise, and even more efficiently, MTX-CS-NPs were 4.06- and 9.35-fold more membrane-lytic active in environments simulating early and late endosomal compartments, respectively. Noteworthy that these results demonstrated that entrapment of MTX into NPs did not change their pH-responsive behavior. Finally, in order to confirm that the pH-sensitive membrane-lytic activity of NPs was really attributed to the surfactant, unloaded and MTX-loaded CS-NPs were prepared without 77KS and submitted to the same assay procedure. Negligible or low hemolysis rates were obtained in all tested conditions, proving the lack of pH-responsive behavior of these NPs in the absence of 77KS (Fig. 3c, d, respectively). The hemolysis of non-encapsulated MTX was also assessed, but insignificant membrane lysis was obtained in the entire pH range (data not shown).

Therapeutics agents, including antitumor drugs, act at intracellular sites and, thus, their clinical efficacy depends on efficient intracellular trafficking (Hu et al. 2007; Plank et al. 1998). As cells usually take up drug nanocarriers via endocytosis, an important challenge for the intracellular delivery of therapeutic compounds is to circumvent the non-productive trafficking from endosomes to lysosomes, where degradation may occur (Stayton et al. 2000). Therefore, the design of pH-responsive nanostructures is considered a great approach to facilitate the drug release into the cytosol by destabilizing endosomal membranes under mildly acidic conditions (Chen et al. 2009). Agents commonly used to promote NP escape/release from endosomes include pH-sensitive peptides and surfactants, pH-buffering polymers, and fusogenic lipids (Li et al. 2012; Nogueira et al. 2014b).

Upon this sense, we prepare polymeric NPs incorporating a surfactant that showed high efficiency at disrupting cell membranes within the pH range characteristic of the endosomal compartments (Nogueira et al. 2011a). As expected, the NPs containing 77KS presented a clear membrane-lytic behavior dependent on pH, suggesting their potential ability to promote triggered release of entrapped drug in a pH-responsive fashion. The enhanced hemolysis induced by NPs at acidic conditions could be explained firstly, by the electrostatic attraction of the NPs to the negatively charged cell membranes and, secondly, by a modification in the hydrophobic/hydrophilic balance of 77KS at the pH range of endosomes. The carboxylic group of 77KS seems to become protonated at acidic conditions, which makes it non-ionic and enhances its hydrophobicity. These hydrophobic segments can insert into lipophilic regions of lipid bilayers, causing membrane solubilization or altering the permeability of the membrane, hence, inducing cell lysis.

In vitro assessments of antitumor activity

The *in vitro* antitumor activity of MTX-CS-NPs and free MTX were performed using two different tumor cell lines, HeLa and MCF-7. We chose the *in vitro* model systems to perform this study, as they provide a rapid and effective means to assess NPs for a number of toxicological endpoints and mechanism-driven responses. Primarily, we evaluated if the unloaded-CS-NPs have any cytotoxic effect, which can be attributed especially to the presence of surfactant (Nogueira et al. 2011b). Fig. 4a demonstrated that unloaded-CS-NPs have low or negligible cytotoxicity against both cell lines and displayed at least 80% of cell viability.

As a second step, we studied the antiproliferative activity of the NPs containing the drug, and our results clearly proved that the cytotoxicity of MTX-CS-NPs incorporating 77KS was

greater than that of free drug against both tumor cells (Fig. 4b-e). By increasing drug concentration, antitumor activity of NPs became higher in comparison to the same concentration range of free MTX. Low to moderate cytotoxic effects were achieved for free MTX, whereas MTX encapsulated into NPs significantly reduced the cell growth. For example, it can be evaluated from Fig. 4d that, by MTT assay, the MCF-7 cell viability at the 50 μg/ml drug concentration was decreased from 71.14% for free MTX to 40.80% for MTX-CS-NPs (30.34% decrease in cell viability, $p < 0.05$). Likewise, but with low intensity, the viability of HeLa cells was decreased from 79.87% for free MTX to 61.57% for MTX-CS-NPs (18.30% decrease in cell viability, $p < 0.05$) (Fig. 4b). On the other hand, the NRU assay displayed lower sensitivity to detect the cytotoxic effects of free and encapsulated drug in both cell lines. No significant differences were observed in HeLa cells (Fig. 4c), but this assay also detected greater activity of NPs in MCF-7 cells at the highest concentrations assayed (Fig. 4e). Noteworthy that MTT was the most sensitive viability assay for detecting the cytotoxic effects of the NPs, regardless of the cell line. The MTT assay determines cell metabolic activity within the mitochondrial compartment, while NRU endpoint measures membrane integrity. This implies that NPs primarily exerted toxicity on the mitochondrial compartment after cellular internalization, while the cell membranes were affect only at a later stage. This finding could be related to the mechanism of action of the drug. MTX is a potent inhibitor of the enzyme DHFR, which plays a key role in the synthesis of purines and thymine and, thus, leads to a blockage of DNA and RNA synthesis, and eventually to cell death (Rubino 2001). Therefore, MTX is a chemotherapeutic agent that mainly inhibits cell proliferation by acting in the intracellular sites, which justifies its lower effects on cell membrane integrity.

Due to its high polarity (at the neutral pH of biological fluids it is present mainly in the doubly anionic species), MTX can cross the cell membrane only with difficulty and, therefore, is transported into the cells by a specific, high-affinity carrier system, or by a passive diffusion mechanism, which can be limited by the high ionization state of MTX at physiological pH (Rubino 2001). This mechanism could justify the general low activity of free MTX in comparison with its encapsulated form. Likewise, MTX does not exert its cytotoxic activity only towards neoplasic cells, but also targets other tissues, thus determining a range of side effects. Therefore, in order to overcome the undesirable toxicity of MTX and to circumvent the risks associated with the administration of the free drug, considerable research efforts have been directed toward novel nanotechnology-based MTX delivery approaches (Chen et al. 2007; Kuznetsova et al. 2012; Thomas et al. 2012). Here, the enhanced cytotoxicity of MTX via the pH-responsive NPs means that a significant reverse effect of drug resistance could has occurred.

Moreover, nanoparticulated systems may reduce the resistance effects that characterize many anticancer drugs by a mechanism of cell internalization of the drug by endocytosis, by lowering drug efflux from the cells and, in the case of pH-responsive NPs, by allowing an effective drug release from the endosomes to cell cytosol (Dong and Mumper 2010). Therefore, the pH-dependent membrane-lytic activity of the MTX-CS-NPs (see the previous section) seems to be directly related with their enhanced therapeutic efficacy compared to the native drug.

Finally, we can highlight that during this study we observed differences in the sensitivity of cell lines and viability assays to each treatment. In view of that, we support the idea that complementary assays based on various cell mechanisms, as well as a combination of cell culture models, are need to raise the information output and the reliability of the results.

Hemocompatibility studies

The assessment of the hemocompatibility of a nanocarrier is indispensable for frequent intravenous dosing (Kuznetsova and Vodovozova 2014). Therefore, as the proposed nanocarriers were intended for intravenous administration, we evaluated if they cause any toxicological reaction to the erythrocytes. The hemocompatibility of unloaded and MTX-loaded CS-NPs, both containing the surfactant 77KS, was studied by hemolysis experiments (Fig. 5). The release of hemoglobin was used to quantify their erythrocyte-damaging properties. The unloaded-CS-NPs were non-hemolytic (less than 5%) at the lower concentrations, and showed slight hemolysis at the highest concentration and after 1 h incubation. In contrast, the NPs containing MTX were almost non-hemolytic regardless of the concentration and the incubation time (maximum 8% hemolysis). Likewise, free MTX was non-hemolytic in all the conditions (data not shown). Fortunately, these results were in contrast with those previously reported for other nanoformulations containing MTX (Kuznetsova and Vodovozova 2014; Nogueira et al. 2013), in which the encapsulated drug displayed higher hemoreactivity than its free solution. Altogether, the obtained results suggest the hemocompatibility of the designed pH-sensitive NPs.

Conclusions

In summary, we have demonstrated a simple and solvent-free approach to prepare pH-responsive CS-NPs by incorporating a biocompatible amino acid-based surfactant in the ionotropic complexation process. The obtained NPs presented a suitable nanosize and displayed

accelerated release of MTX by decreasing pH from 7.4 to 5.4. The inclusion of 77KS on the formulation clearly gives it a pH-sensitive membrane-lytic behavior, which suggests these NPs as a potential intracellular delivery system. Outstandingly, pH-responsive CS-NPs significantly improved the *in vitro* antitumor activity of MTX with respect to the free drug in MCF-7 cell culture and, to a lesser extent, in HeLa cells. Based on the overall results, the conclusion that may be reached from this study is that this nanocarrier system represents a promising strategy for the administration of antitumor drugs in cancer therapy, which merits further investigations under *in vivo* conditions to confirm this premise.

Conflict of interest statement

The authors state that they have no conflict of interest.

Acknowledgments

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Table 1. Characterization parameters of the different CS-based NPs obtained in the presence and in the absence of MTX and/or the surfactant 77KS

	CS-NPs (CS:TPP:77KS)	MTX-CS-NPs (CS:TPP:77KS)	CS-NPs (CS:TPP)	MTX-CS-NPs (CS:TPP)
Hydrodynamic size (nm) ± SD*	183.0 ± 1.234	182.7 ± 3.449	162.9 ± 1.405	174.4 ± 3.027
PDI ± SD*	0.206 ± 0.015	0.225 ± 0.022	0.221 ± 0.02	0.223 ± 0.013
Zeta potential (mV) ± SD*	30.7 ± 1.21	22 ± 0.569	29.4 ± 2.08	23.5 ± 1.29
pH	5.55	5.53	5.52	5.53
Entrapment Efficiency (EE%)	-	38.4%	-	68.02%

* SD = standard deviation

Figure captions:

Fig. 1 UV-vis spectra of (1) MTX free solution and (2) MTX extracted from CS-NPs.

Fig. 2 pH-dependent *in vitro* cumulative release of MTX from NPs in PBS buffer at pH 7.4, 6.6 and 5.4. Results are expressed as the mean ± SE of three independent experiments. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. [a] Significantly different from PBS pH 7.4 ($p < 0.05$) and [b] significantly different from PBS pH 6.6 ($p < 0.05$).

Fig. 3 pH-responsive membrane-lytic activity of CS-NPs with and without 77KS: (a) unloaded-CS-NPs with 77KS, (b) MTX-CS-NPs with 77KS, (c) unloaded-CS-NPs without 77KS and (d) MTX-CS-NPs without 77KS. NP-induced membrane-lysis was expressed as a function of pH, concentration and incubation time. Results are expressed as the mean ± SE of three independent experiments. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. [a] Significantly different from pH 7.4 ($p < 0.05$), [b] significantly different from pH 7.4 ($p < 0.005$), [c] significantly different from pH 6.6 ($p < 0.05$) and [d] significantly different from pH 6.6 ($p < 0.005$).

Fig. 4 The effect of varying concentrations of free drug and NPs incorporating 77KS on the survival rates of HeLa and MCF-7 cell lines by using two different viability assays: (a) unloaded-CS-NPs; (b) and (c) represent free MTX and MTX-CS-NPs in HeLa cells by MTT and NRU assays, respectively; (d) and (e) represent free MTX and MTX-CS-NPs in MCF-7 cells by MTT and NRU assays, respectively. Data are expressed as the mean of three independent experiments ± SE. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. [a] Significantly different from HeLa cells ($p < 0.05$). * $p < 0.05$ and ** $p < 0.01$ denote significant differences from free MTX.
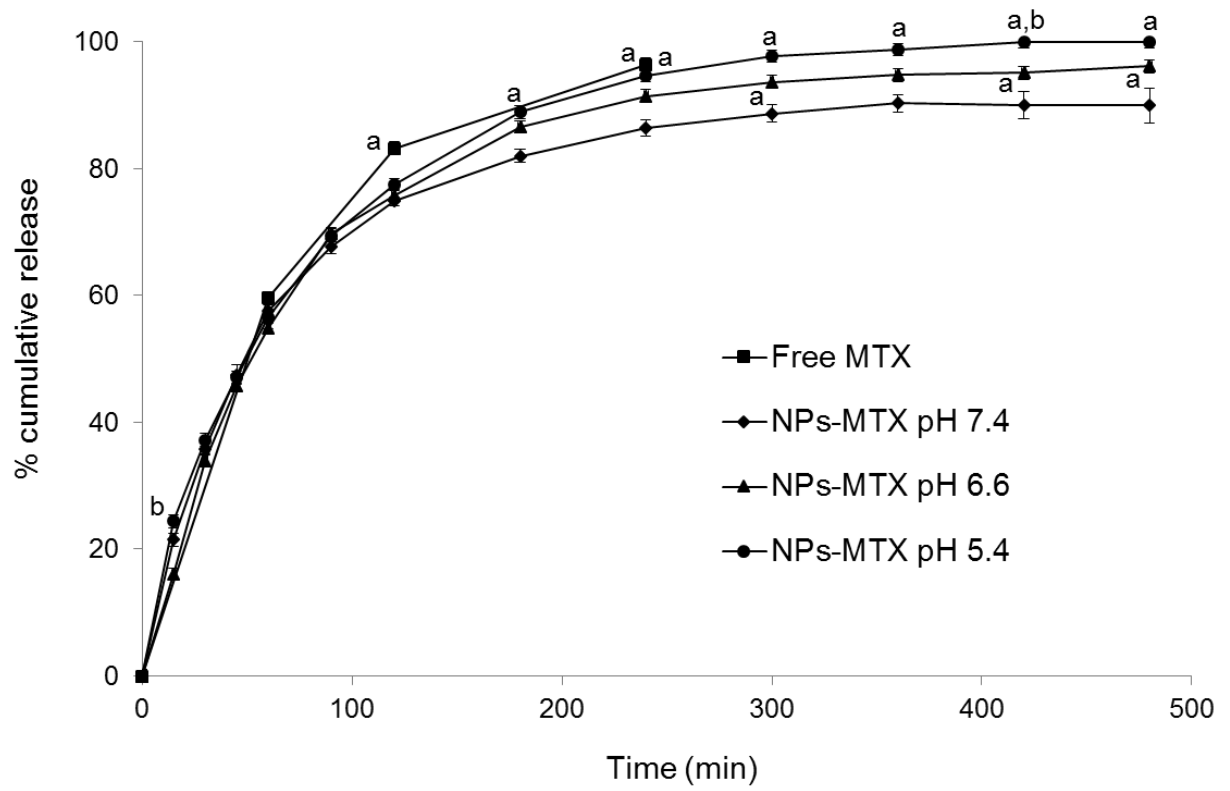
Fig. 5 Percentage of hemolysis caused by NPs incorporating 77KS after 10 and 60 min of incubation with human erythrocytes: (a) unloaded-CS-NPs and (b) MTX-CS-NPs. Each value represents the mean ± SE of three experiments.
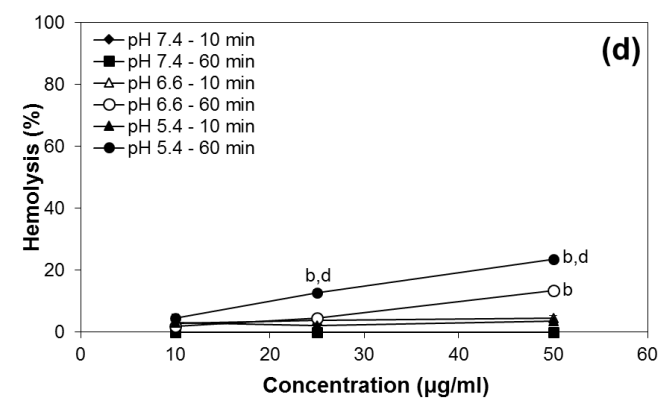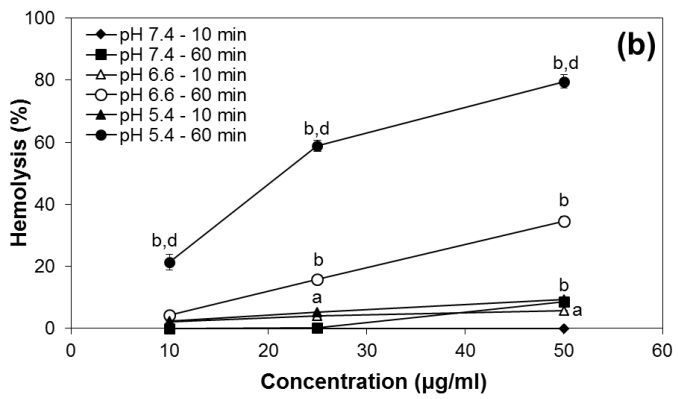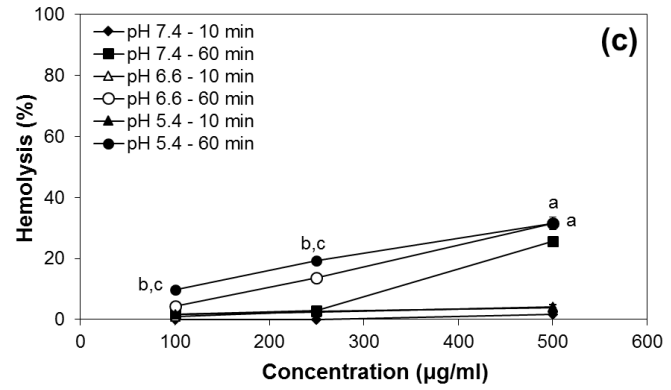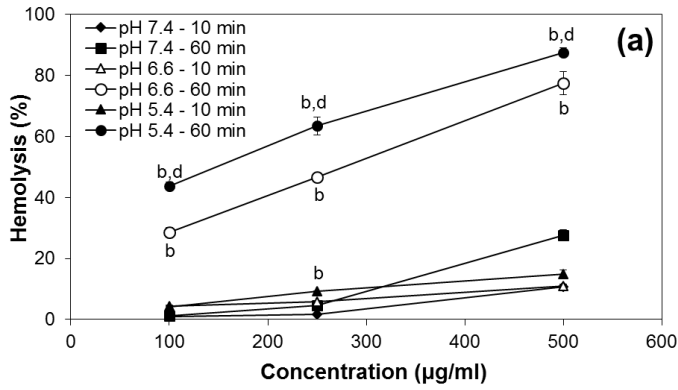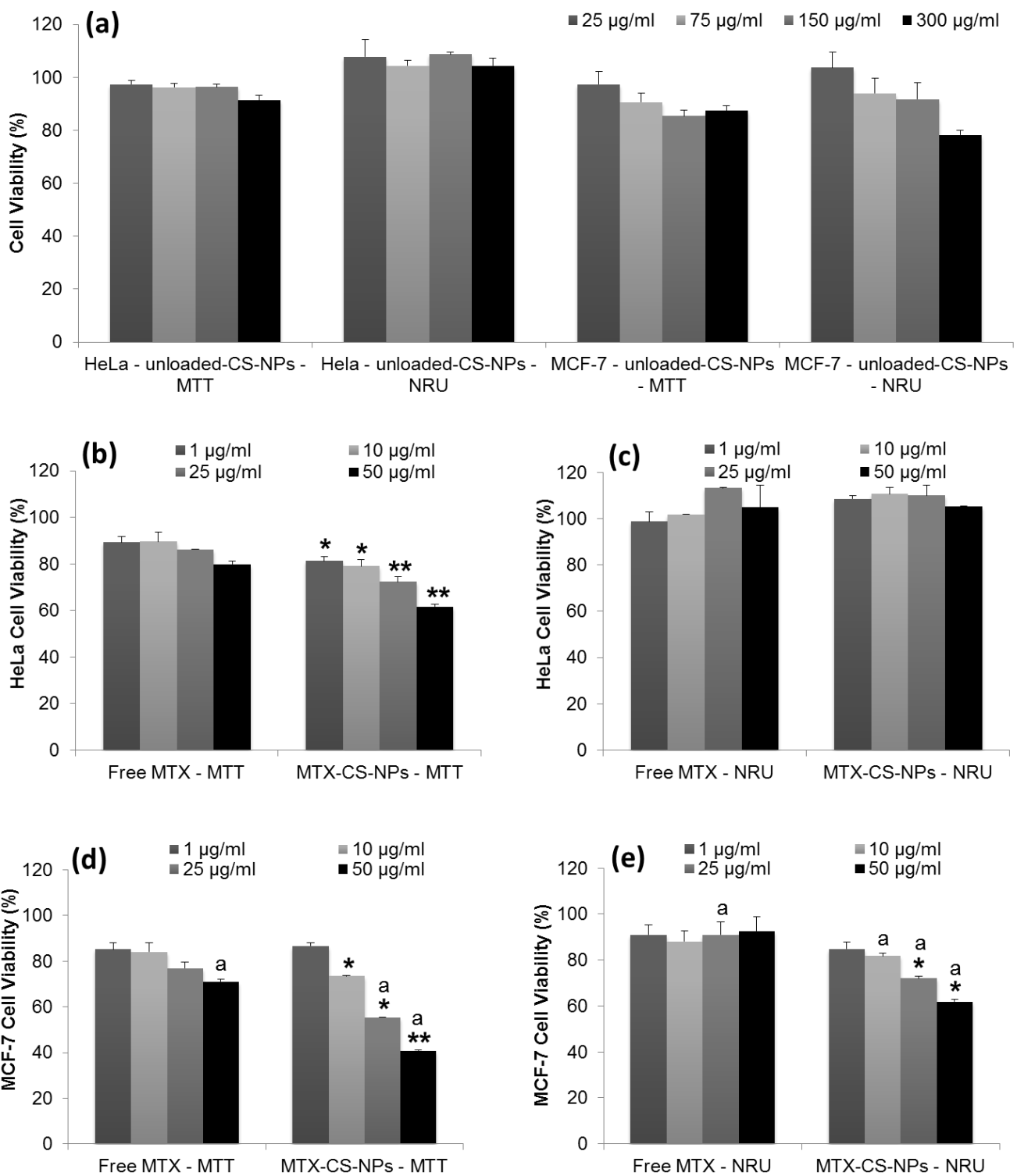
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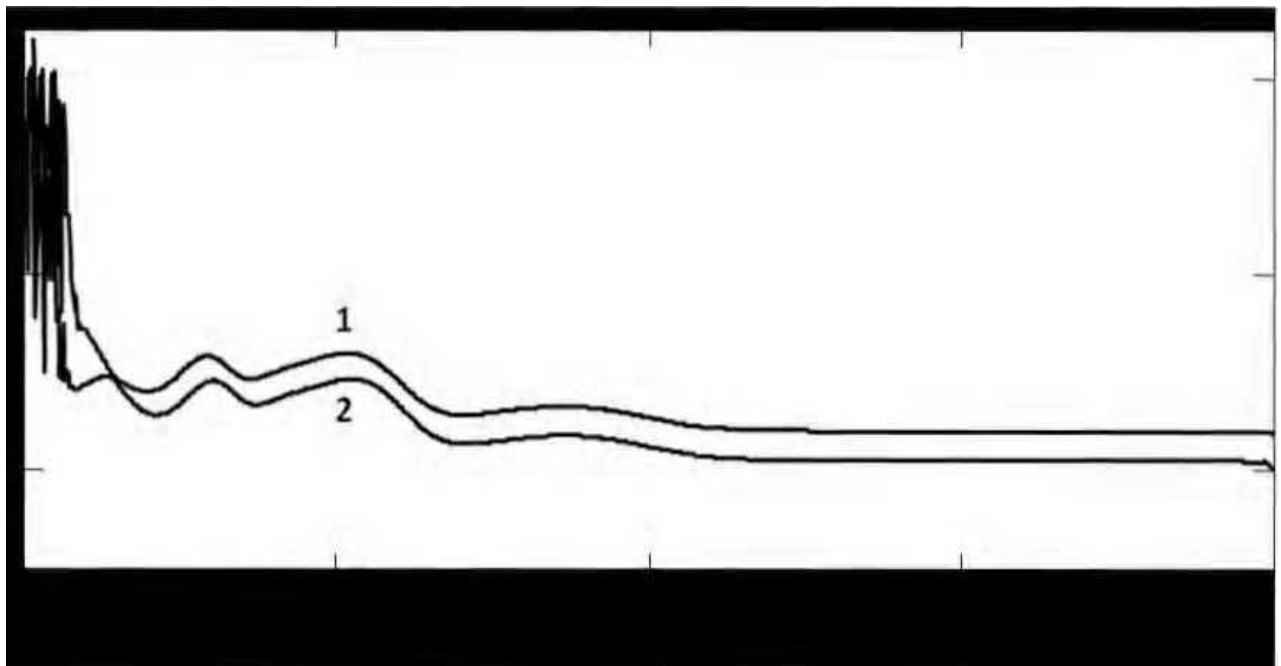
Figure 2

Figure 3.

Figure 4.

Figure 5.