Loading studies of the anticancer drug Camptothecin into dual stimuli-sensitive nanoparticles. Stability scrutiny

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Abstract

In recent years, the preparation of valuable drug delivery systems (DDS) from self-assembled amphiphilic copolymers has attracted much attention since these nanomaterials provide new opportunities to solve problems such as the lack of solubility in water of lipophilic drugs, improve their bioavailability, prolong their circulation time and decrease the side effects associated with their administration. In the current study two types of biocompatible pH-responsive nanoparticles derived from poly(2-hydroxyethyl methacrylate) (pHEMA) have been used as drug nano-carriers, being one of them core cross-linked to circumvent their instability upon dilution in human fluids. The present paper deals with the optimization of the loading process of

the labile, hydrophobic and highly active anticancer drug, Camptothecin (CPT) into the nanoparticles with regard to four independent variables: CPT/polymer ratio, sonication, temperature and loading time. Forty experiments were carried out and a Box–Behnken experimental design was used to evaluate the significance of the independent variables related to encapsulation efficiency and drug retention capacity. The enhanced drug loading and encapsulation efficiency values (58% and >92%, respectively) of CPT were achieved by the core cross-linked NPs in 2 hours at 32 ºC at CPT/polymer ratio 1.5:1 w/w and 14 min of sonication. The optimized CPT-loaded NPs were studied by dynamic light scattering and scanning electron microscopy, and an increase in size of the loaded-NP compared to the unloaded counterparts was found. Other twenty experiments were conducted to study the enability to retain CPT into the conjugates at different ionic strength values and times. The stability studies demonstrated that the core cross-linked nanocarriers displayed an excellent drug retention capacity (> 90%) at 25ºC for 15 days in every ionic-strength environments whereas the non-cross-linked ones were more stable at physiological ionic strength. The optimized systems proved to be a major step forward to encapsulate and retain CPT in the NP nuclei, what makes them ideal devices to control the delivery of CPT upon the triggered acidic conditions of solid tumors.

Keywords: Kinetic drug loading, polyHEMA, pH-responsive nanoparticles, drug delivery systems, anticancer therapy, core cross-linked nanoparticles, experimental design.

1. Introduction

Cancer is a major public health problem worldwide and is the second leading cause of death in the developed countries, which accounts for more than 8.8 million deaths per year. The lifetime probability of developing an invasive cancer is currently above 40% for men and close to it for women and in 2018, 1,735,350 new cancer cases and 609,640 cancer deaths are projected to occur in the United States (Siegel et al., 2018). Treatment is dictated by the cancer type, stage at diagnosis, and the patient's tolerance to the prescribed therapy (Wolinsky et al., 2012). Thus, for solid tumors, surgery is the local treatment of choice as the damage is confined in a limited area of the body. However, most patients require the use of two or more treatments due to the potential spread of the disease as well as to effectively prevent the evolution of the disease from early to advanced stages. Chemotherapy may be combined with surgery or radiotherapy to increase the effectiveness of these treatment modalities (Gavhane et al., 2011). On the other hand, conventional chemical treatments with proven effectiveness in early stages may lack of success in advanced stages. Related to drug administration, the methods used has been traditionally limited to making the drug accessible to the blood stream, relying on the irrigation and the drug affinity for the tissues for the access to the target. In fact, bioavailability is still measured from drug levels in the bloodstream, not in the target surroundings. As a consequence, cancer treatments generally involve the administration of relatively high doses of the drug in the hope that a portion, although minor, will go to damaged tissues (Alvarez-Lorenzo and Concheiro, 2014). Therefore, there is a need to increase the drug concentration in the cancerous tissue while reducing the side effects associated with chemotherapeutic molecules. This requirement is even more compelling in the case of highly toxic anticancer drugs, which may also present too-deficient physico-chemical and stability features such as the labile camptothecin.

Twenty-(S)-camptothecin is a strong cytotoxic molecule with excellent antitumor activity in a broad spectrum of human cancers. However, its clinical use is currently limited by its poor solubility in water, low plasma stability due to the cleavage of its lactone ring at physiological pH and severe toxicity. Nowadays, one of the most effective research strategies to achieve a safe and efficient release of camptothecin to target cells is the use of nano-vehicles and a recently published review includes the most innovative approaches (Botella and Rivero-Buceta, 2017).

Nanoparticulate drug delivery systems (DDS) have the potential to improve current disease therapies because of their ability to overcome multiple biological barriers and releasing a therapeutic molecule within the optimal dosage range. Angiogenesis during tumor growth results in a defective hypervascularization and a deficient lymphatic drainage system, which has given rise to the concept of passive targeting of nanoparticles (NPs) to tumors through the “enhanced permeability and retention” (EPR) effect (Vivek et al., 2013; Wolinsky et al., 2012). The EPR is a unique feature which allows drug delivery nanocarriers (cutoff size of >400 nm) to accumulate and diffuse preferentially in tumor tissues. Some factors such as composition, size, charge and targeting ligand functionalization, can substantial and positively affect the biodistribution and blood circulation half-life of circulating NP by reducing the level of non-specific uptake, thus delaying opsonization, and increasing the extent of tissue specific accumulation (Alexis et al., 2008). Once the NPs are concentrated in the damage tissues, the drug can be release by the trigger of a specific stimulus and, therefore, boosting the drug concentration where it is required. One of these stimuli is a decrease in pH, feature commonly encountered in the cancerous tissues of solid tumors with regards to the pH of healthy tissues.

Regarding the composition of the polymeric NPs, the presence of a highly hydrophilic corona layer makes the micelles very stable in aqueous media (Li et al.,

2006; Zhao and Liu, 2015). Among the most common hydrophilic blocks, poly(ethylene glycol) (PEG) (Salmaso et al., 2007; Zhao and Liu, 2015), chitosan (Chang and Xiao, 2010), and some polymers obtained from methacrylate esters such as 2-hydroxyethyl methacrylate [HEMA, (Cheng et al., 2012)] and *N,N*-dimethylaminoethyl methacrylate [DMAEMA, (Dinu et al., 2016)] can be found. In the hydrophobic blocks, the presence of the biocompatible polycaprolactone [(PCL, (Zhao and Liu, 2015)], poly(lactic acid) [PLA, (Karimi et al., 2016)], poly(lactic glycolic acid) [PLGA, (Doppalapudi et al., 2014)], poly(propylene oxide) [PPO, (Biswas et al., 2016)], polydimethylsiloxane [PDMS, (Dinu et al., 2016)], ciclodextrins (Alvarez-Lorenzo and Concheiro, 2014; Salmaso et al., 2007), and other polymers based on methacrylate derivatives such as *N,N*-diethylaminoethyl methacrylate [DEAEMA, (Liu et al., 2002)] are the most common options. Our research group have designed biocompatible pH-sensitive NPs capable of loading lipophilic molecules and releasing them upon acidic environments (Galbis et al., 2018, 2017). As far as we are aware, there is no precedent in the literature of a finely-tuned design to find the optimal loading conditions of lipophilic molecules into NPs.

In the present paper, we aim to investigate the loading of an anticancer drug, CPT, in freshly prepared non- and cross-linked pHEMA-based NPs under different experimental conditions in order to find the optimal values for the four variables studied. For the optimization of the CPT-loading conditions in the pH-responsive systems, we study the effect of CPT/polymer concentration, sonication time, temperature and loading time. Furthermore, stability experiments were carried out to stablish the influence of the ionic strength of the medium in the drug retention capacity of the uploaded conjugates.

2. Experimental Section

2.1. Materials

Camptothecin was purchased from TCI Europe (Tokyo Chemicals Industry). All other chemicals used were purchased from Sigma-Aldrich and used as received. The 1kDa cut-off mini-dialysis tubes used in the present work were purchased from GE Healthcare.

2.2. General Methods

Measurements of UV and visible light absorbance were carried out at an Agilent 8453 UV-visible spectrophotometer (Palo Alto, USA), which presents diode array detection (DAD). The data were the result of, at least, three measurements.

A 0.4 kW ultrasonic processor (UP400S, Hielscher Ultrasonics GmbH, Teltow, Germany) with 3 mm of diameter probe was used for sonication. Samples were processed at a constant frequency of 24 kHz. The energy input was controlled by setting the amplitude of the sonicator probe. Extrinsic parameters of amplitude (50%) and time (0, 7 and 14 min), were varied with total duration per pulse cycle On/Off 1 second. Corresponding ultrasonic intensity level was 230 W/cm^2. Micelle samples of 12.5 mL were placed in a 100 mL jacketed vessel through which water at 25 ± 1.0 ºC with a flow rate of 0.5 L/min was circulated. The ultrasound probe was submerged to a depth of 12 mm in the sample.

The morphology and distribution of the NPs were characterized by scanning electron microscopy (SEM). Before SEM observations, the dispersions were deposited and allowed to dry on a carbon coated grid usually used for transmission electron microscopy. Then, the images were performed by SEM using a field emission HITACHI S5200 microscope operating at 5 kV at the CITIUS Service (University of Seville).

The average diameter (D_h) and size distribution (polydispersity index, PdI) of the samples were determined with a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25 °C, with a particle size analysis range of 0.6 nm to 6 μm. The intensity of the scattered light (expressed in kilo counts per second) was measured by dynamic light scattering (DLS). The instrument was provided with 4 mW He–Ne laser (λ = 633 nm), digital correlator ZEN3600, and non-invasive backscatter (NIBS®) technology. Measurements were performed with a scattering angle of 173º to the incident beam, and data analyzed using CONTIN algorithms (Malvern Instruments). Data for each dispersion were collected from at least three runs.

2.3. Synthetic procedures and micelle formation

The nanoparticles were synthesized following a modified procedure recently described by us (Galbis et al., 2018) and can be summarized as follows:

For the preparation of the non cross-linked micellar dispersions, the freshly prepared amphiphilic block-copolymer poly[(DMAEMA$_{31\%}$-HEMA$_{19\%}$)-*block*-(DEAEMA$_{45\%}$-FMA$_{5\%}$)] (DMAEMA: *N,N*-dimethylaminoethyl methacrylate; HEMA: 2-hydroxyethyl methacrylate; DEAEMA: *N,N*-diethylaminoethyl methacrylate; FMA: furfuryl methacrylate. M_n = 34,700; M_w = 45,100; M_w/M_n = 1.3, Figure 1) was dissolved in tetrahydrofuran (THF, 10 mL, polymer concentration = 20 mg/mL), and then the solution was added dropwise into double-distilled water (790 mL, final polymer concentration 0.25 mg/mL) at 30 ºC and stirred for 12 hours. The dispersion adopted the slightly bluish appearance typical of nanosized particle suspensions. The DLS studies revealed that quasi-monodisperse systems were achieved at a polymer concentration of 0.25 mg/mL (PdI = 0.14; D_h = 210 nm).

For the synthesis of the core cross-linked micellar dispersions, the cross-linker used was 1,8-dimaleimide-3,6-dioxaoctane (DMDOO, Figure 1) and was freshly prepared following the procedure described by us elsewhere (Galbis et al., 2014). Two separated solutions of the polymer and the crosslinker in THF were prepared. The solution (A) was obtained by dissolving the amphiphilic *block*-copolymer poly[(DMAEMA$_{31\%}$-HEMA$_{19\%}$)-*block*-(DEAEMA$_{45\%}$-FMA$_{5\%}$)] in THF (final polymer concentration: 2.04 %w/v); the solution (B) was prepared so that the concentration of the crosslinker in THF was 0.1 %w/v. The solution (A) (0.062 mequiv of FMA units) and the solution (B) (0.0064 mequiv of maleimide units) were mixed and gently stirred at 25 ºC for 5 min (mol ratio FMA/maleimide = 5:1; degree of cross-linked aimed, based on FMA residues: 20%), and then added dropwise into double-distilled water (790 mL, final polymer concentration 0.25 mg/mL) at 30 ºC and stirred for 24 hours. As previously, the dispersion adopted the typical slightly-bluish appearance of NP suspensions. DLS studies revealed that nano-sized systems were achieved (PdI = 0.33; D_h = 130 nm).

2.4. Preparation of camptothecin-loaded NPs

The encapsulation studies of camptothecin (CPT, Figure 2) by the freshly prepared non- and core cross-linked NPs were conducted by means of UV spectroscopy varying several experimental parameters such as CPT/polymer ratio, sonication time, temperature and loading time. The optimized CPT-loaded NPs were also studied by dynamic light scattering (DLS) and scanning electron microscopy (SEM).

Optimization of the experimental parameters in the loading assays

To stablish the optimal experimental conditions to prepare CPT–loaded NPs (drug/polymer ratio, sonication time of NP samples, temperature, and time), well-defined assays were designed and carried out as described next.

To study the influence of sonication time and CPT/polymer ratio in drug loading, 20 CPT-loaded NP systems, 10 of them named Non-CPT/POL_x-S_y and the other 10 systems named Xr-CPT/POL_x-S_y were prepared from non cross-linked NPs and cross-linked NP, respectively. The final polymer concentration was 0.1 mg/mL, the final targeted CPT/polymer ratios in mass were 0.5:1, 1:1, or 1.5:1 and the sonication time of the NP samples were set at 0, 7 or 14 min. In Table S1 (Supplementary Information document) and along the text “x” denotes final CPT/polymer ratio (in mass) and “y” denotes the sonication time (in minutes) of NPs before CPT uptake.

The general procedure was conducted as follows (Figure 3): the selected micellar dispersion was sonicated for either 0, 7 or 14 minutes and then introduced into a mini-dialysis tube (1kDa cut-off, GE Healthcare). The latter was placed into a sealed tube containing a freshly prepared aqueous-based CPT solution (2:3 v/v DMSO-water) at a predetermined CPT/polymer ratio and gently stirred for 1, 2, or 3 h at 25 ºC. The NP suspension and the CPT solution were in contact through the dialysis membrane allowing the circulation of CPT through it, but still keeping the NPs into the dialysis tube. Once the loading time expired, the mini-dialysis tube was removed and the absorbance of the remaining CPT in the supernatant solution was measured by UV spectroscopy at 369 nm.

The concentration of CPT in the medium was determined by UV spectroscopy, and making use of the calibration curve prepared *ex professo* (Figure S1, Supplementary Information document).

Drug loading (DL) and encapsulation (or entrapment) efficiency (EE) of CPT embedded into the nanoparticles were calculated according to Equations (1) and (2)

$$\boldsymbol{DL} = \frac{mass\ of\ CPT\ loaded\ into\ NP}{total\ mass\ of\ CPT\ loaded\ NP}\ x\ 100 \qquad \text{(Eq. 1)}$$

$$\boldsymbol{EE} = \frac{mass\ of\ CPT\ loaded\ into\ NP}{mass\ of\ CPT\ at\ t_0\ in\ the\ incubation\ tube}\ x\ 100 \qquad \text{(Eq. 2)}$$

Once the optimal conditions regarding sonication time and CPT/polymer ratio were established from the previous studies (14 min and 1.5, respectively), the influence of time and temperature on EE was evaluated next. Twenty new CPT-loaded NP systems, 10 of them named Non-T_m-t_n and the other 10 systems named Xr-T_m-t_n were prepared from non cross-linked NPs and cross-linked NP, respectively (NP suspensions were previously sonicated for 14 minutes; final CPT polymer ratio = 1.5:1, Table S2 in Supplementary Information document). In Table S2 and along the text “m” denotes the final temperature of the loading process (°C) and “n” denotes the loading time (in hours).

Similarly to the general procedure described above, a mini-dialysis tube containing the previously sonicated NP suspension was immersed into the CPT solution and the temperature of the mixture was set at 25 °C, 32 °C or 39 °C, depending on the system. After 1, 2 or 3 hours, the dialysis tube was removed and the absorbance of the remaining CPT in the supernatant CPT-solution was measured at 369 nm.

2.5. Drug retention assays of CPT-loaded nanoparticles

In order to check the drug retention capacity (DRC) of the CPT-loaded NPs suspended in aqueous media at different values of ionic strength and time, 20 samples were prepared under optimum uploading conditions (CPT/polymer ratio: 1.5:1;

sonication time: 14 minutes; temperature: 32 ºC, loading time: 2 hours) as described above. The 20 CPT-loaded dispersions were centrifugated and the supernatants were removed and replaced with a NaCl solution of concentration 0.1, 0.5 or 0.9% w/v (Table S3, Supplementary Information document). The dispersions were then gently stirred at 25 °C for either 1, 8 or 15 days. After centrifugation, the CPT released during the trials were determined by UV spectroscopy at 369 nm. In the 10 stability tests named Non-NA_p-t_q and the other 10 systems named Xr-NA_p-t_q, CPT-loaded non cross-linked NPs and cross-linked NP, respectively were the systems to study. In Table S3 and along the text "p" denotes NaCl concentration in the media (%w/v) and "q" denotes the time (in days) the systems were stirred at 25 °C.

The drug retention capacity (DRC) in percentage was determined using Equation (3):

$$DRC = \frac{m_{entrapped(0)} - m_{supernatant(t)}}{m_{entrapped(0)}} \times 100 \qquad \text{(Eq. 3)}$$

where $m_{entrapped(0)}$ is the weight of initial entrapped CPT into the NPs; $m_{supernatant(t)}$ is the weight of CPT released from the nanocarriers to the medium at time *t*.

2.6. Experimental design to study the effect of loading conditions on CPT encapsulation and the effect of ionic strength and time in drug retention capacity

In order to obtain optimized conditions for the loading step, a Box–Behnken experimental design (CSS Statistica, StatSoft Inc., Tulsa, UK) was used to evaluate the significance of the independent variables (firstly sonication time and CPT/polymer ratio; secondly temperature and time), as well as the interactions among them in the non- and core cross-linked NPs. The number of experiments (N) is defined by the Equation (4):

$$N = k^2 + k + cp \qquad \text{(Eq. 4)}$$

where k represents the number of factors (variables) involved in the study and cp is the number of replicates of the central point. Box–Behnken could be seen as a cube, consisting of a central point and the middle points of the edges.

Sonication treatment (S) was performed for NP samples during 0, 7 or 14 min following the procedure described in the *Experimental Section: General methods*. The CPT/polymer ratios used were 0.5:1, 1:1 or 1.5:1. The factorial design was used for 20% cross-linked NPs and non-crosslinked NPs in which encapsulation efficiency [EE, Equation (2)] was stated as dependent variable.

Regarding drug retention assays, the analyses were conducted in a similar way. In those trials, the independent variables were time (1, 8 or 15 days) and NaCl concentration (0.1, 0.5 or 0.9 %w/v). The dependent variable was drug retention capacity (DRC) and it has been described in Equation (3).

The total number of experiments required for our considered independent variables at three levels was 10. The values of the selected pair of independent variables were normalized from -1 to +1 by using Equation (5) in order to facilitate direct comparison of the coefficients and visualization of the effects of the individual independent variables on the response variable.

$$X_n = \frac{X - \bar{X}}{(X_{max} - X_{min}) / 2} \qquad \text{(Eq. 5)}$$

where X_n is the normalized value of independent variables; X is the absolute experimental value of the variable concerned; $\overline{X}$ is the mean of all fixed values for the variable in question; and X_{max} and X_{min} are the maximum and minimum values of the variable, respectively.

The data, analyzed by multiple regression analysis following polynomial equation, were derived to represent EE (%) (or DRC, in percentage, depending on the studied trials) as a function of the independent variables tested [Equation (6)],

$$y = \beta + \sum_{i=1\ to\ 3} \beta_i x_i + \sum_{i<j} \sum_{i=1\ to\ 3} \beta_{ij} x_i x_j + \sum_{i=1\ to\ 3} \beta_i x_i^2 \qquad \text{(Eq. 6)}$$

where y is the predicted EE (or DRC), β, β_i, and β_{ij}, denotes the regression coefficients and x_i, x_j are the normalized values between pairs of independent variables (for EE: sonication time and CPT/polymer ratio, temperature and time; for DRC: time and NaCl concentration). Only the estimated coefficients with significant levels higher than 95% ($p<0.05$) were included in the final models. The differences between the experimental values and those that were calculated using the previous equations never exceeded 10% of the former.

3. Results and Discussion

The target of the present work is to find the optimal experimental conditions to prepare pH-responsive CPT-loaded NPs and study the stability of the prepared conjugates over time and at several values of ionic strength. Methacrylate derivatives have been chosen due to the highly efficient polymerization techniques available for these monomers such as oxyanionic polymerization, atom transfer radical polymerization (ATRP) or reversible addition−fragmentation chain-transfer polymerization (RAFT). They allow the preparation of tailor-made amphiphilic block-polymers with varied functionalities and compositions to meet the ongoing-research needs. Thus, for example, the presence of tertiary amino groups in the block-copolymers will impart pH-responsive properties to the final micelles, as has recently been confirmed (Galbis et al., 2018).

3.1. Preparation of core cross-linked and non cross-linked NPs from the auto-assembly block-copolymer poly[(DMAEMA$_{31\%}$ *random*-HEMA$_{19\%}$)-*block*-(DEAEMA$_{45\%}$-*random*-FMA$_{5\%}$)]

The preparation method of the amphiphilic *block*-copolymer used in the present work has been published elsewhere (Galbis et al., 2017) and the experimental copolymer composition (in mole percentage), was found to be poly[(DMAEMA$_{31\%}$ HEMA$_{19\%}$)-*block*-(DEAEMA$_{45\%}$-FMA$_{5\%}$)] (Figure 1). The hydrophobic block was mainly constituted by the pH sensitive poly(*N,N*-diethylaminoethyl methacrylate) (pDEAEMA), which turns into hydrophilic mopolymer at acid pH; the second monomer incorporated in this block, furfuryl methacrylate (FMA), is a reactive monomer in Diels-Alder reactions with a key role in the cross-linking of the NPs.

This lipophilic block was responsible for the encapsulation of the hydrophobic CPT in the prepared NPs. The hydrophilic block composition is of crucial relevance in the behavior of the NP in biological fluids (Chouhan and Bajpai, 2009). In our case, the shell of the NPs was constituted by the biocompatible and widely used poly(2-hydroxyethyl methacrylate) (pHEMA) and the pH sensitive poly(*N,N*-dimethylaminoethyl methacrylate) (pDMAEMA). The hydrophilicity of this block ensures the stability of the NP in an aqueous medium.

The choice of the hydrophilic components was made to guarantee the biocompatibility of the resulting NPs as well as to ensure the ability to respond to pH changes useful in the drug release of CPT in acidic environments such as solid tumors (Wu et al., 2010). Thus, it is well known that pHEMA is a non-toxic and biocompatible hydrophilic material and is particularly attractive for biomedical engineering applications (Chouhan and Bajpai, 2009). Moreover, because of its abundant hydroxyl functional groups content, pHEMA can be easily functionalized by covalent conjugation with, for example, targeting ligands and fluorescent molecules (Cheng et al., 2012).

PDMAEMA is another biocompatible polymer used in the co-delivery of paclitaxel and DNA (Guo et al., 2010) with pH responsive behavior (Dinu et al., 2016). This polymer forms part of graft co-polymers or block-copolymers with other biocompatible blocks such as polycaprolactone (PCL) or polyethylene glycol (PEG).

An major challenge in the design of smart NPs is to avoid the instability associated to the micelle-unimers equilibrium due to dilution phenomena in the human fluids and the serious side effects concomitant with the premature release of the drug in normal tissues (Zhao and Liu, 2015). That is the reason why one of the nano-sized samples studied was core cross-linked to circumvent not only its potential disintegration but also some inter-micellar cross-linking (Galbis et al., 2017). The presence of furfuryl methacrylate (FMA) moieties in the polymer structure would enable the stabilization of the NPs formed by cross-linking reaction when required.

The chemical stabilization of the core cross-linked micelles was carried out by a Diels-Alder coupling reaction between the furan rings in the core with the maleimide rings of the crosslinker. The amount of DMDOO added was set so that 20% of furan rings in the NP core reacted in pairs with maleimide rings of DMDOO, leading to a degree of cross-linking of 20%. This degree of cross-linking was chosen based on our previous findings related to the stability and size of the 20% reticulated core NPs (Galbis et al., 2017), which displayed enhanced features (Figure 4).

To optimize the loading of CPT into the freshly prepared NPs, the variables investigated were those that most frequently display a marked effect on drug loading in the literature, i.e., drug/polymer ratio, sonication time, temperature and loading time. The experimental part was carried out by choosing two out of the four variables

mentioned above and their influence on the encapsulation efficiency of CPT was studied.

3.2. Modelling and influence of Sonication time and CPT/polymer ratio on the encapsulation efficiency of the NPs

Sonication is a technique widely used in the preparation of NP suspensions to obtain samples with consistent properties. Thus, this method has been used for the preparation of graphene oxide NPs as anticancer drug carrier (sonication time: 3 h) (Hashemi et al., 2017) and some magneto-fluorescent nanoparticles based on carboxymethyl chitosan have been sonicated during the preparation procedure (sonication time: 1 h) (Bhattacharya et al., 2011). The sonication procedure can be part of the one-pot micelle-formation / drug-loading as for example in the preparation of chitosan nanoparticles loaded with oxaliplatin, an anticancer agent (sonication time: 30 minutes) (Vivek et al., 2014). However, in general terms, the effect of sonication time on drug loading has been scarcely investigated. One exception is the simultaneous preparation of poly lactic-co-glycolic-acid (PLGA) NPs and load of rifampicin by an optimized method in which sonication time was one of the variables involved (from 4 min to 24 min). The authors found that the best conditions for obtaining stable NPs with maximum percentages of drug loading was achieved after sonicating the formulation for 20 minutes (Tripathi et al., 2010).

When methacrylate derivatives are involved, as is the case of the present work, necessary sonication times to guarantee the formation of stable NPs suspensions are, in general terms, quite short. For example, during the preparation of some methacrylate-based cross-linked NP by miniemulsion polymerization, sonication times of 10 minutes were adequate (Griset et al., 2009). When the micelle formation of pHEMA-based co-polymers was attempted by the solvent exchange method,

sonication times of 30 minutes were used (Cheng et al., 2012). In contrast, in the case of pDMAEMA-based copolymers, stable NP suspensions were obtained in aqueous media with sonication times as short as just 1 min (Dinu et al., 2016). In the present work, we decided to check the effect that the sonication of the prepared methacrylate NPs could exert onto their CPT load and encapsulation efficiency. The time range chosen in this experimental design for the independent variable sonication time (0, 7 or 14 minutes, depending on the trial) was selected taking into account that our systems are DMAEMA- and HEMA-based NPs.

On the other hand, one of the main parameters with a weight influence on drug loading and encapsulation efficiency of hydrophobic molecules into polymer nanoparticles is the drug/polymer ratio in the feed. For example, tamoxifen-loaded chitosan NPs were prepared at various feeding ratios of drug to polymer (Vivek et al., 2013). The load of the anticancer drug honokiol into poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) micelles increased in line with the honokiol/micelle mass ratio (Gou et al., 2009). Moreover, the loading of rifampicin in poly lactic-co-glycolic-acid (PLGA) nanoparticles was studied at several drug/polymer ratios (Tripathi et al., 2010) as well as was investigated the encapsulation of levofloxacin in poly(lactic-co-glycolic acid) (PLGA) NPs, displaying maximum drug loading (86%) at drug/polymer ratio1:10 (Gupta et al., 2011). In general terms, it was observed that the bigger the drug concentration in the feed, the higher was its EE (Chouhan and Bajpai, 2009). This is especially accurate as long as the drug was soluble in the media. For example, Friedrich *et al.* (Friedrich et al., 2008) observed this trend in the encapsulation of dexamethasone up to a certain drug concentration, in which a drop in EE was observed due to the insolubility of the therapeutic molecule. This was attributed to the overload of the drug (lack of solubility) followed by its crystallization in the external aqueous phase. To confirm this hypothesis, the authors observed the presence of

dexamethasone crystals in the suspension by optical microscopy. Hence, the initial drug contents should be in its solubility range to prevent drug precipitation/crystallization. Regarding the choice of CPT concentration range, we took into account the concentrations in which a lineal relationship between drug concentration and absorbance at 369 nm were found (Figure S1, Supplementary Information document). In that way, Table 1 shows the values of independent variables and the experimental values of DL and EE obtained for both, non and cross-linked NPs.

Table 1. Experimental drug loading and encapsulation efficiency values of CPT-loaded NPs prepared at 25 °C at different CPT/polymer ratios and sonication times following the experimental design[1].

Non cross-linked loaded NPs				Cross-linked loaded NPs			
Sample	**Formulation code**	**DL (%)**	**EE (%)**	**Sample**	**Formulation code**	**DL (%)**	**EE (%)**
1	**Non-CPT/Pol$_{0.5}$-S$_0$**	14.05	32.70	**11**	**Xr-CPT/Pol$_{0.5}$-S$_0$**	24.87	66.21
2	**Non-CPT/Pol$_{0.5}$-S$_7$**	9.66	21.39	**12**	**Xr-CPT/Pol$_{0.5}$-S$_7$**	22.95	59.56
3	**Non-CPT/Pol$_{0.5}$-S$_{14}$**	8.45	18.46	**13**	**Xr-CPT/Pol$_{0.5}$-S$_{14}$**	20.01	50.03
4	**Non-CPT/Pol$_1$-S$_0$**	32.77	48.75	**14**	**Xr-CPT/Pol$_1$-S$_0$**	40.33	67.58
5	**Non-CPT/Pol$_1$-S$_7$**	31.50	45.98	**15**	**Xr-CPT/Pol$_1$-S$_7$**	41.85	71.97
6	**Non-CPT/Pol$_1$-S$_7$**	33.64	50.69	**16**	**Xr-CPT/Pol$_1$-S$_7$**	42.20	73.00
7	**Non-CPT/Pol$_1$-S$_{14}$**	32.73	48.66	**17**	**Xr-CPT/Pol$_1$-S$_{14}$**	38.26	61.97
8	**Non-CPT/Pol$_{1.5}$-S$_0$**	47.82	61.10	**18**	**Xr-CPT/Pol$_{1.5}$-S$_0$**	52.83	74.66
9	**Non-CPT/Pol$_{1.5}$-S$_7$**	46.06	56.93	**19**	**Xr-CPT/Pol$_{1.5}$-S$_7$**	53.23	75.89
10	**Non-CPT/Pol$_{1.5}$-S$_{14}$**	49.02	64.11	**20**	**Xr-CPT/Pol$_{1.5}$-S$_{14}$**	54.20	78.90

[1]Each value is the average of three samples ($p<0.05$). DL = drug loading; EE = encapsulation efficiency; CPT/Pol = CPT/polymer ratio (0.5, 1.0 or 1.5); S = sonication time (0, 7 or 14 minutes).

In Table 2, the equations obtained using polynomial regression and statistical parameters (R^2, df and F) are shown.

Table 2. Equations yielded for the dependent variable (EE) as a function of the independent variables (CPT/polymer ratio and sonication time, normalized values) for the first experimental design.

Equation	R^2	df	F
$XrEE = 67.99 + 8.94\,C - 2.93\,S + 5.10\,C\,S$	0.91	3.6	29.11
$NonEE = 48.52 + 18.26\,C - 6.07\,C^2 + 4.31\,C\,S$	0.97	3.6	57.04

C = CTP/polymer ratio normalized value; S = sonication normalized value;
Xr-EE = encapsulation efficiency in percentage for core cross-linked NPs;
Non-EE = encapsulation efficiency in percentage for non cross-linked NPs

Concerning the response equation, in most cases an acceptable R^2 (>0.90) and F (>29) values have been found. Both equations contain complex terms that involve interactions between the independent variables. Identifying the influence of the relative independent statistical variables on the dependent variable in the displayed equations is not straightforward, nor are the calculations to obtain the two values of independent variables at which the maximum percentage of drug absorption could be achieved. To overcome this drawback, the response surface for the dependent variable in each system studied is shown in Figure 5.

As can be seen in Figure 5, the relative influence of the cross-linked treatment is readily visible. The EE percentages obtained for cross-linked NPs are, in the studied ranges, higher than those achieved by the non cross-linked ones. This is in line with our findings related to the therapeutic molecule pilocarpine which is used clinically as co-drug in glaucoma and xerostomia and presents low water solubility (Galbis et al., 2018).

Among the independent variables studied, the CPT/polymer ratio is the most influential and positive variable on EE percentage for the two systems investigated, being more relevant in the non reticulated NPs. In addition, the lower the CPT/polymer ratio tested, the more marked were the differences in EE encountered between the two types of NPs. The use of higher drug/polymer ratios resulted in an increase in load values under every experimental conditions, similar to the observations found by other research teams in other drug-polymer systems (Biswas et al., 2016). Conversely, sonication time not only exerted a slighter influence on CPT loading than that exerted by the CPT/polymer ratio, but also displayed a dual effect on CPT load depending on the drug/polymer ratio. Thus, for example, sonication showed a negative influence on the loading of the drug in non- and cross-linked NPs at low and medium CPT/polymer ratios, whereas this trend was reversed when the CPT/polymer ratio used was the highest one (1.5:1 w/w); to the latter concentration, the longer the sonication time the higher the EE of CPT. This is probably due to concomitant enhancement of the total micellar surface with the reduction of aggregates in the NP suspensions so that the encapsulation of the drug at a high drug content was facilitated.

In summary, the best DL and EE values of CPT were achieved by core cross-linked NPs at the highest CPT/polymer ratio (1.5:1 w/w) and sonication time (14 min).

3.3. Modelling and influence of Temperature and Time on NP Uptake

This second experimental design was tailored to investigate the influence of temperature and CPT loading time in DL and EE under the optimized conditions disclosed in the first batch of trials, i.e., CPT/polymer ratio =1.5:1 w/w and sonication time = 14 min. For this experimental design, Table 3 shows the values of independent variables —temperature and loading time— and DL and EE of CPT for the two nano-carriers studied.

Table 3. Experimental drug loading and encapsulation efficiency values of CPT-loaded NPs at different temperatures and loading times following the experimental design[1].

Non cross-linked loaded NPs				Cross-linked loaded NPs			
Sample	**Formulation code**	**DL (%)**	**EE (%)**	**Sample**	**Formulation code**	**DL (%)**	**EE (%)**
21	**Non-T_{25}-t_1**	50.05	66.8	**31**	**Xr-T_{25}-t_1**	54.48	79.78
22	**Non-T_{25}-t_2**	50.06	66.82	**32**	**Xr-T_{25}-t_2**	54.56	80.05
23	**Non-T_{25}-t_3**	49.90	66.39	**33**	**Xr-T_{25}-t_3**	54.46	79.72
24	**Non-T_{32}-t_1**	55.50	83.13	**34**	**Xr-T_{32}-t_1**	57.76	91.18
25	**Non-T_{32}-t_2**	55.50	83.15	**35**	**Xr-T_{32}-t_2**	57.77	91.21
26	**Non-T_{32}-t_2**	55.81	84.19	**36**	**Xr-T_{32}-t_2**	57.90	91.67
27	**Non-T_{32}-t_3**	56.58	86.87	**37**	**Xr-T_{32}-t_3**	57.85	91.48
28	**Non-T_{39}-t_1**	41.19	46.69	**38**	**Xr-T_{39}-t_1**	44.50	53.45
29	**Non-T_{39}-t_2**	41.43	47.16	**39**	**Xr-T_{39}-t_2**	43.97	52.32
30	**Non-T_{39}-t_3**	42.95	50.2	**40**	**Xr-T_{39}-t_3**	44.25	52.92

[1]Each value is the average of three samples ($p<0.05$). DL = drug loading; EE = encapsulation efficiency; T = temperature (25, 32 or 39 °C); t = time (1, 2 or 3 hours).

Similar to those obtained in the previous section, the estimates of the model coefficients and statistical parameters (R^2, df and F) were calculated by means of a polynomial regression between the analytical response and the independent variables (Table 4).

Table 4. Equations yielded for the dependent variable (EE) as a function of the independent variables (temperature and loading time, normalized values) for the second experimental design.

Equation	R^2	df	F
$XrEE = 91.02 - 13.4\,T - 24.90\,T^2 + 0.37\,t^2$	0.99	3.6	747.2
$NonEE = 84.51 - 9.33\,T - 27.17\,T^2 - 0.93\,t$	0.99	3.6	443.4

T = temperature normalized value; ..... t = time normalized value;
Xr-EE = encapsulation efficiency in percentage for core cross-linked NPs;
Non-EE = encapsulation efficiency in percentage for non cross-linked NPs.

Regarding the response equations, suitable R^2 (0.99) and F (>400) values have been found. As can be seen in the equations recorded in Table 4, temperature turned to be the most influential variable on EE for both systems (non- and cross-linked NPs) and a low statistical effect of time was found. To obtain the optimal values of EE for the independent variables in the systems studied, the response surfaces for this dependent variable were plotted against temperature and time (Figure 6).

Similar to the observations found in the first experimental design, the influence of the cross-linked treatment is fairly visible and the EE obtained for cross-linked NPs are, in the ranges studied, higher than those achieved by the non cross-linked ones.

The interval of temperature chosen at the present experimental design is established based on the most common laboratory working temperature (25 ºC) and to avoid reaching the lower critical solution temperature (LCST) of polyDMAEMA (42 ºC (Dong

et al., 2013)), the main component of the shell of the prepared NPs, in order to keep the hydrophilicity of the HEMA-DMAEMA-based blocks. The trials carried out at 32 ºC for non and cross-linked NPs, exhibited the best CPT DL and EE; good results were also found at room temperature. Conversely, once the temperature was set at 39 ºC, the found up values dropped significantly. To explain this effect, it is necessary to take into account that polyDEAEMA, the main component of the hydrophobic core in both systems, shows phase separation at elevated temperatures (displaying a LCST behavior) (Thavanesan et al., 2014). It has been stated that polyDEAEMA exhibits a pronounced change in polarity with phase separation and provides non-polar surroundings for the uptake of hydrophobic molecules. The cloud point found for this polymer ranges from 31 ºC to 60 ºC at a low polymer concentration, depending on the pH and the technique used to determine it (by turbidimetry and fluorescence spectroscopy). Thavanesan *et al.* (Thavanesan et al., 2014) stated that when working below the cloud point, only minor amounts of dehydrated DEAEMA moieties (aggregates of diethylaminoethyl groups) are sufficient to incorporate the first hydrophobic molecules, although there is no macroscopic precipitation at this stage. This is the reason why even at the lowest temperature studied (25 ºC) the systems displayed high EE (from 66% to 80%). Moreover, when the temperature used was 32 ºC, the dehydrated hydrophobic domains augmented, which allowed an enhanced drug uptake by the NPs, reaching the maximum values of DL and EE in the non- and cross-linked systems (Table 3 and 4, Figure 6). However, once the temperature approached towards polyDEAEMA cloud point, the NP cores became mostly-dehydrated and the drug could not enter the collapsed core with the consequent drop in DL and EE at 39 ºC. The other independent variable, time, as previously observed in the equations in Table 4, displayed a small influence, with DL and EE data almost steady in the studied loading time, findings that are in line with those observed for the

encapsulation of pilocarpine (Galbis et al., 2018). Consequently, the highest DL and EE values were achieved at 32 ºC for the cross-linked NPs in the very first hours.

To sum up, for the uploading of CPT by the nano-carriers studied, the cross-linked NPs displayed the best performance at every experimental condition tested in contrast with their non-crosslinked counterparts. For both types of NPs, the most critical experimental parameters in the CPT loading ended up being the CTP/polymer ratio (the higher ratio, the enhanced DL and EE percentage values achieved) and temperature, being 32 °C the optimum one.

3.4. Size and shape morphology of optimized camptothecin-loaded NPs

Z-average, polydispersity index (PdI), and hydrodynamic diameter (D_h) of the initial unloaded NPs (samples S-01 and S-02) and of the NPs loaded with CPT at the optimum loading conditions (samples Non-T_{32}-t_2 and Xr-T_{32}-t_2) were determined by dynamic light scattering (DLS, Table 5). In general terms, non-crosslinked NPs exhibited larger hydrodynamic diameters (for example 210 nm against 130 nm for CPT-free samples) and the loading procedure cause a substantial increase in the micellar diameter as has been observed by other authors (Gou et al., 2009).

Table 5. Comparison of Z-average, polydispersity index (PdI), and hydrodynamic diameter (D_h, determined by DLS) of non-cross-linked NP (Non-Xr) and stabilized NP at 20% of cross-linking (Xr) (unloaded or loaded with CPT).

Degree of crosslinking	**Unloaded samples** (Galbis et al., 2018)				**Camptothecin-loaded NPs**			
	Sample	**Z-average (± SD) (nm)**	**PdI (± SD)**	**Size (± SD) (D_h.nm)**	**Sample**	**Z-average (± SD) (nm)**	**PdI (± SD)**	**Size (± SD) (D_h.nm)**
Non-Xr	**S-01**	177 (± 1)	0.14 (± 0.02)	210 (± 80)	**Non-T_{32}-t_2**	256 (± 20)	0.45 (± 0.04)	423 (± 10)
Xr 20%	**S-02**	108 (± 1)	0.33 (± 0.01)	130 (± 70)	**Xr-T_{32}-t_2**	230 (± 6)	0.43 (± 0.01)	394 (± 30)

The CPT-loaded NPs were prepared at pH 7.0 according to the optimized conditions found in the present study: sonication time = 14 min; CPT/polymer ratio = 1.5:1; temperature = 32 ºC; loading time = 2 h.

The representative SEM images of the CPT-loaded samples Non-T_{32}-t_2 and Xr-T_{32}-t_2 under neutral pH conditions are displayed in Figure 7, which confirmed the presence of round structures of nanometric sizes. The SEM images also reveal large differences between the appearance of the CPT-loaded cores and the NP shells, in discordance with the homogeneous nature of the unloaded NPs as evidenced in a previous work (Galbis et al., 2017). The drug-loading systems formed stable micellar suspensions without the presence of aggregates.

3.5. Drug retention capacity modelization

After determining the optimal conditions for the maximum CPT uptake, the study of the influence of time and ionic strength on retaining CPT into the prepared nanoparticle systems was then addressed. Bench stability studies were conducted in order to corroborate the utility of those NPs as pH-responsive CPT nano-carriers. Thus, the NPs were loaded with CPT under the optimized conditions and the systems were centrifugated so that the supernatants could be decanted. The CPT-loaded NPs were then suspended in NaCl solutions at different salt concentrations and gently stirred for predetermined periods of time. The NPs were analyzed to quantify the percentage of CPT retained in the systems at 25 °C.

In general terms, the usefulness of polymeric nanocarriers can be limited by its sensitivity to the environment such as dilution and ionic strength (Jaturanpinyo et al., 2004). The prepared non cross-linked NP were reasonably stable at high dilution due to their low critical micelle concentration (CMC, 0.078 mg/mL), which was determined by UV spectroscopy and the pirene probe method (Galbis et al., 2017), whereas the core cross-linked NPs did not dissociate even at a very high dilution due to the stable covalent bonds formed during the reticulation process between the hydrophobic

segments of the polymer in the core and the cross-linker DMDOO. The range for the independent variable ionic strength has been selected in order to cover from almost unsalted aqueous solutions (NaCl at 0.1 %w/v) to the physiological ionic strength encountered in the cell nuclei (NaCl at 0.9 %w/v, (Terry et al., 2011)). For this experimental design, Table 6 shows the values of independent variables —NaCl concentration and time— and drug retention capacity of CPT (in percentage) for the two nano-carriers studied.

Table 6. Experimental drug retention capacity values of CPT-loaded NPs at 25 ºC and different NaCl concentrations and times following the experimental design[1].

Non cross-linked loaded NPs			Cross-linked loaded NPs		
Sample	**Formulation code**	**DRC (%)**	**Sample**	**Formulation code**	**DRC (%)**
41	**Non-$NA_{0.1}$-t_1**	89.21	**51**	**Xr-$NA_{0.1}$-t_1**	99.03
42	**Non-$NA_{0.5}$-t_1**	93.06	**52**	**Xr-$NA_{0.5}$-t_1**	95.90
43	**Non-$NA_{0.9}$-t_1**	92.64	**53**	**Xr-$NA_{0.9}$-t_1**	96.27
44	**Non-$NA_{0.1}$-t_8**	79.31	**54**	**Xr-$NA_{0.1}$-t_8**	98.41
45	**Non-$NA_{0.5}$-t_8**	87.82	**55**	**Xr-$NA_{0.5}$-t_8**	94.21
46	**Non-$NA_{0.5}$-t_8**	86.08	**56**	**Xr-$NA_{0.5}$-t_8**	94.42
47	**Non-$NA_{0.9}$-t_8**	87.98	**57**	**Xr-$NA_{0.9}$-t_8**	96.84
48	**Non-$NA_{0.1}$-t_{15}**	68.20	**58**	**Xr-$NA_{0.1}$-t_{15}**	92.70
49	**Non-$NA_{0.5}$-t_{15}**	76.47	**59**	**Xr-$NA_{0.5}$-t_{15}**	88.31
50	**Non-$NA_{0.9}$-t_{15}**	77.60	**60**	**Xr-$NA_{0.9}$-t_{15}**	91.04

[1]Each value is the average of three samples ($p<0.05$). DRC = drug retention capacity; NA = NaCl concentration (0.1, 0.5 or 0.9 % w/v); t = time (1, 8 or 15 days).

Similar to those calculated in the previous sections, the estimates of the model coefficients and statistical parameters (R^2, df and F) in the obtained equations are displayed in Table 7.

Table 7. Equations yielded for each dependent variable as a function of the independent variables (normalized values) for the third experimental design.

Equation	R^2	Df	F
$XrDRC = 94.50 - 3.19\,t - 0.99\,NA - 2.58\,t^2 - 2.93\,NA^2$	0.99	4.5	67.9
$NonDRC = 86.82 - 8.77\,t + 3.58\,NA - 1.92\,t^2 - 3.04\,NA^2 + 1.49\,t\,NA$	0.99	5.4	116.3

NA = NaCl concentration normalized value; ..... t = time normalized value;
Xr-DRC = drug retention capacity in percentage for core cross-linked NPs;
Non-DRC = drug retention capacity in percentage for non cross-linked NPs.

In this case, good R^2 (0.99) and F (> 67) values have been found. As can be seen in equations, time is the most influential variable on DRC percentage for both non- and cross-linked NPs.

Good drug retention capacities, under both cross-linked treatment, could be observed when using the response surfaces (Figure 8). The most relevant feature of Figure 8 is the excellent DRC (> 90%) displayed by the core cross-linked nanocarriers during 15 days at different ionic strength and under bench conditions. Conversely, a clear influence of time on DRC was observed for the non-cross-linked NPs, being this effect dimished at high salt concentrations. The equilibrium that may have been stablished between the micelles and the unimers in the aqueous media led to the unwanted partial release of the drug to the media (< 15% in 8 days). Consequently, for good DRC of the non cross-linked systems, the CPT-loading nano-vehicles could be freshly prepared and then suspended and stored under solutions with physiological ionic strength for times no longer than 8 days.

4. Conclusions

The present work have stablished the optimal conditions to upload the anticancer drug camptothecin into NPs, cross-linked or not, with hydrophilic, biocompatible and pH-responsive pHEMA-based shells. The variables investigated were drug/polymer ratio, sonication time, temperature and loading time, being temperature and CPT/polymer ratio the most influencial variables. In the global ranges studied of the four independent variables, the encapsulation efficiency percentages obtained for cross-linked NPs were higher than those achieved by the non cross-linked ones in every trial. Between sonication time and CPT/polymer ratio, the latter was the most influential and positive variable on encapsulation efficiency of CPT for the two systems investigated with an optimal value of 1.5:1 w/w. Sonication time, although not as relevant, exerted a positive effect on drug load at the highest CPT/polymer ratio, with optimized times of 14 min. When temperature and loading time were investigated, the most critical experimental parameter turned to be temperature, with 32 °C being the optimum one. Regarding time, CPT was loaded efficiently in the first 2 hours. The optimized CPT-loaded NPs were studied by dynamic light scattering and scanning electron microscopy, and an increase in size of the loaded-NP compared to the unloaded counterparts was found.

The stability studies demonstrated that the core cross-linked nanocarriers displayed excellent drug retention capacities (> 90%) at 25ºC for 15 days and in different ionic-strength environments. In addition, the non cross-linked conjugates could behave as good CPT-loaded nano-vehicles when suspended into aqueous media at physiological ionic strength and for periods of time of up to 8 days. The optimized systems proved to be a major step forward to encapsulate and retain CPT in the NP nuclei, what makes

them ideal devices to control the delivery of drugs under acidic triggering conditions such as those of solid tumors.

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Figure 1. Structure of the amphiphilic *block*-copolymer and the cross-linker used in the present work

Figure 2. Chemical structure of camptothecin (CPT)

Figure 3. General scheme of the loading process of CPT into the selected nanoparticles at 25 °C.

Figure 4. Schematic representation of non cross-linked NP (on the left), in which formulation the crosslinker has not been added, and core cross-linked NPs (on the right). In the latter samples, some of the furan rings have reacted with the bifunctional cross-linker DMDOO leading to core cross-linked NPs by means of Diels-Alder reaction.

Figure 5. Response surface for CPT encapsulation efficiency percentage on both, non- and cross-linked NPs.

Figure 6. Response surfaces for encapsulation efficiency (EE in percentage) against temperature and loading time on non- and cross-linked nanocarriers for the second experimental design.

Figure 7. Selected SEM images of the CPT-loaded NP systems at pH 7.0 prepared according to the optimized conditions found in the present study. (a) Non-cross-linked NP (**Non-T_{32}-t_2**); (b) core cross-linked NP with 20% of degree of cross-linking (**Xr-T_{32}-t_2**).

Figure 8. Response surface for drug retention capacity (DRC, in percentage) against salt concentration (NaCl, in % w/v) and time on non- and cross-linked nanocarriers for the third experimental design.

Names of chemical compounds studied in the article.

CPT: camptothecin

DMAEMA: *N,N*-dimethylaminoethyl methacrylate

HEMA: 2-hydroxyethyl methacrylate

DEAEMA: *N,N*-diethylaminoethyl methacrylate

FMA: furfuryl methacrylate

DMDOO: 1,8-dimaleimide-3,6-dioxaoctane