Carbohydrate Polymers 94 (2013) 669–676

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

A comparative study of chitosan shielding effect on nano-carriers hydrophilicity and biodistribution

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ARTICLE INFO

Article history:
Received 22 September 2012
Received in revised form 4 December 2012
Accepted 23 January 2013
Available online 8 February 2013

Keywords:
Chitosan
Polylactic acid (PLA)
Nanoparticles
Stealth
Pharmacokinetics
Biodistribution

ABSTRACT

Engineering polymer surfaces reduces nanoparticles (NPs) aggregation and phagocytosis due to effective shielding, hindering recognition by the reticuloendothelial system (RES). The shielding of NPs is complex and affected by the type of groups used in terms of charge and hydrophilicity. This will, in turn, affect NPs biodistribution which will determine the length of activity of the drug. Polysaccharides are nowadays recognized for decreasing the uptake of particulate carriers by the mononuclear phagocytic system (MPS). Chitosan is considered as an attractive candidate due to its biocompatibility, biodegradability, non-toxicity and low cost. In this study clozapine (CZP)-loaded NPs were coated with chitosan, pluronic F-68, polyethylene glycol (PEG) 4000 and polysorbate 80. The factors affecting drug encapsulation efficiency, particle size, surface charge, surface hydrophilicity, pharmacokinetics and biodistribution were studied. The results proved that although a similarity in surface hydrophilicity, chitosan-stealth NPs showed different pharmacokinetic profile and biodistribution behavior compared to polysorbate-stealth NPs.

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1. Introduction

Nanoparticulate drug carriers should be present in the blood circulation long time enough to reach their site of action. However, the removal of nano-systems from the body, known as opsonization, by the reticuloendothelial system (RES), is a prime barrier. Opsonization of hydrophobic particles has been shown to occur more promptly than hydrophilic ones due to the adsorption of serum proteins on these surfaces (Carstensen, Muller, & Muller, 1992). The macrophages usually located in the liver, kidney and spleen, have the capability to eradicate these particles from the circulation within a few seconds after intravascular administration (Owens & Peppas, 2006; Panagi et al., 2001); a significant biological obstacle facing the use of nanoparticles as a mean for controlling drug delivery (Kumari, Yadav, & Yadav, 2010).

Evading nanoparticles (NPs) recognition by the mononuclear phagocytic system (MPS) and increasing their blood circulation half-life are generally done by several methods for camouflaging (Kaul & Amiji, 2002). The use of surface groups can block electrostatic and hydrophobic interactions responsible for opsonins binding to particle surfaces. These groups tend to be hydrophilic polymer chains and non-ionic surfactants. Some examples of such surface groups include polysaccharides, poly(vinyl alcohol), poly(N-vinyl-2-pyrrolidone), polysorbates, poloxamers and polyethylene glycol (PEG) (Gref et al., 1994; Owens & Peppas, 2006; Schipper et al., 2009; Zahr, Davis, & Pishko, 2006).

Polysaccharides are currently documented for developing long-circulating systems. Hydrophilic polysaccharide coatings, such as heparin and dextran, have been reported to decrease particulate carriers uptake by the MPS (Jaulin, Appel, Passirani, Barratt, & Labarre, 2000; Lemarchand, Gref, & Couvreur, 2004). Specific consideration has been given to chitosan due to its biocompatibility, biodegradability and low cost (Illum, 1998).

The shielding of nanoparticles is complex and affected by the type of groups used in terms of charge and hydrophilicity. This will in turn affect the biodistribution of the nanoparticles which will determine the length of activity of drug.

Clozapine (CPZ), a model drug chosen in this work, is an effective atypical antipsychotic used in the treatment of both positive and negative symptoms of resistant schizophrenia. It has an oral bioavailability as low as 27% due to first-pass effect (Lous, Richard, Hans, & Henk-Jan, 2003). Taking into consideration chitosan advantages, the goal of this work is to prepare shielded CZP NPs and to compare the effect of chitosan on NPs hydrophilicity, charges, pharmacokinetics and biodistribution with other coating materials.

2. Materials and methods

2.1. Materials

Clozapine (CZP) was kindly supplied by Apex Pharma (Cairo, Egypt). 50:50 capped (esterified carboxyl end groups)

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http://dx.doi.org/10.1016/j.carbpol.2013.01.072

poly(DL-lactide-co-glycolide) (DL-PLGA 5004A) and capped (esterified carboxyl end groups) poly(DL-lactide) (DL-PLA 04), both with weight average mole mass = 44,000 g/mol, determined by Gel Permeation Chromatography in chloroform at 35 °C relative to polystyrene standards, and inherent viscosity = 0.4 dL/g, were supplied by PURAC Biochem (Gorinchem, The Netherlands). Polyvinyl alcohol (PVA) 'Mowiol', with average molecular weight 31,000 g/mol 86.7–88.7 mol% hydrolysis and viscosity of 3.5–4.5 mPa s, 4% in H_2O (20 °C), Chitosan (low Mw) with a degree of deacetylation ≥75%, trehalose and solvents of HPLC grade; methanol, ethyl acetate, glacial acetic acid and triethylamine were purchased from Sigma–Aldrich Company (St. Louis, USA). Pluronic F-68 was purchased from BASF AG (Ludwigshafen, Germany). Polyethylene glycol (PEG) 4000 PhEur, with molecular weight 4000 g/mol and linear formula $H(OCH_2CH_2)_nOH$, was purchased from Riedel-De Haën AG, Seelze, Hannover, Germany. Potassium dihydrogen phosphate, disodium hydrogen phosphate, methyl cellulose, hydrochloric acid (HCl), sodium hydroxide (NaOH), Polysorbate 80, dichloromethane (DCM) and methyl alcohol were purchased from El Nasr Pharmaceutical Chemicals (ADWIC) (AbouZaabal, Cairo, Egypt). Celecoxib was kindly supplied by Sedico Co (6th October City, Egypt). Sterile highly purified water for HPLC and normal saline (0.9% NaCl) were purchased from Otsuka Co. (Egypt).

2.2. Preparation of CZP-loaded NPs

The CZP-loaded NPs were prepared by an O/W emulsion–solvent evaporation method (Pillai, Somayaji, Rabinovich, Hudso, & Gonsalve, 2008). Known amounts of PLA-04 or capped PLGA-5004 were dissolved in dichloromethane (DCM) containing CZP and mixed with 20 mL of aqueous PVA solution. Mixture homogenization was done at 26,000 rpm using a high shear homogenizer (Heidolph Diax900, Germany) to produce an oil-in-water emulsion. The organic phase was allowed to evaporate, during 3 h, using a magnetic stirrer at room temperature. The NPs were recovered by centrifugation at 15,000 rpm (21,380 × *g*) at 4 °C for 15 min (Hermie Labortechnik GmbH, type Z216MK, Germany) and washed twice. The washing solutions were eliminated by further centrifugation as described above. The purified NPs were freeze-dried (Christ Alpha 1-2 LD plus, Germany) using trehalose as a cryoprotectant at a w/w ratio of NPs/trehalose (2:1).

A 2^3 factorial design experiment was built up to study the effect of three factors, each at two levels, namely: polymer type (PLA 04 and PLGA 5004), polymer concentrations (1 and 2% (w/v) in DCM) and PVA concentrations (2 and 4% (w/v) in water). The responses studied were the drug entrapment efficiency (EE), the zeta potential (ζ) and the time required for the release of 80% of the drug ($T_{80\%}$). The experimental factorial design is shown in Table 1.

Table 1
Composition of formulae of CZP-loaded NPs used in the factorial design.

Polymer type	Polymer conc. (%, w/v)	PVA conc. (%, w/v)	
		2	4
PLA	1	F-1	F-5
	2	F-2	F-6
PLGA	1	F-3	F-7
	2	F-4	F-8

N.B.:
F-1: NPs formula prepared with 1% PLA and 2% PVA.
F-2: NPs formula prepared with 2% PLA and 2% PVA.
F-3: NPs formula prepared with 1% PLGA and 2% PVA.
F-4: NPs formula prepared with 2% PLGA and 2% PVA.
F-5: NPs formula prepared with 1% PLA and 4% PVA.
F-6: NPs formula prepared with 2% PLA and 4% PVA.
F-7: NPs formula prepared with 1% PLGA and 4% PVA.
F-8: NPs formula prepared with 2% PLGA and 4% PVA.

2.3. Preparation of coated CZP-loaded NPs

Various concentrations of chitosan were dissolved in 0.25 N HCl to yield 0.2, 0.4 and 0.8%. The pH of chitosan solutions was then adjusted to 5 using 1 N NaOH solution. NPs were suspended in chitosan solutions and incubated for 1 h at room temperature (Kim, Kim, & Lee, 2008). After incubation and to remove excess coating materials, centrifugation and washing steps were repeated. For comparison, other coating materials were used by adopting the immersion method; the prepared NPs suspensions were incubated overnight at room temperature with variable concentrations (1, 2.5 and 5% (w/v)) of one of the following: pluronic F-68, PEG 4000 and polysorbate 80 (Gelperina et al., 2010).

2.4. Characterization of coated and uncoated NPs

2.4.1. Determination of encapsulation efficiency (EE)

Encapsulated CZP was determined by dissolving 10 mg of the freeze-dried of coated and uncoated NPs in 100 ml DCM and the drug content was determined spectrophotometrically (Shimadzu UV visible 1601 PC, Kyoto, Japan) at λ_{max} = 297 nm. The components of the NPs did not interfere with CZP at this wavelength.

The encapsulation efficiency (EE) of CZP was calculated according to the following equation:

$$EE = D_m \times \frac{100}{D_t} \quad (1)$$

where D_t is the total amount of CZP loaded in PLGA solution and D_m is the amount of CZP in the prepared NPs (Zhang & Feng, 2006). Experiments were run in triplicate.

2.4.2. In vitro drug release

An accurately weighed amount of the freeze-dried NPs, equivalent to 2 mg CZP, was placed into dialysis bags (Mw cut off 12,000–14,000 Da) containing phosphate buffer pH 7.4 (D'Souza & DeLuca, 2006). The bags were placed into 50 ml phosphate buffer pH 7.4, containing 25% (v/v) methanol to achieve the sink conditions (Panwar, Pandey, Lakhera, & Singh, 2010), under continuous rotation (50 rpm) in a water bath on a magnetic stirrer adjusted at 37 ± 0.5 °C. At predetermined time intervals, 2 ml samples (replaced by fresh medium) were withdrawn and assayed UV-spectrophotometrically at λ_{max} = 293 nm. The *in vitro* release studies were performed in triplicate for each formula

2.4.3. Particle size and zeta potential ζ measurements

Particle size was determined by dynamic light scattering (DLS) technique (Zetasizer Nanoseries, Malvern Instruments Ltd., Malvern, UK). Before measurement, the lyophilized NPs were suspended in distilled water using bath sonicator (Crest Ultasonics, model 575DAE, Cortland, NY) for 30 s. All measurements were performed at 25 ± 0.5 °C. The *Z*-average of each sample was measured at least 20 times (Manchanda, Fernandez-Fernandez, Nagesetti, & McGoron, 2010) and the size distribution was expressed by the polydispersity index (PDI).

For ζ measurements, Laser Doppler Anemometry (LDA) technique (Zetasizer Nanoseries, Malvern Instruments Ltd., Malvern, UK) was employed (Ma et al., 2010). The freeze-dried NPs were suspended in deionized water under magnetic stirring. About 1 ml of dispersion was injected into the capillary of the Zeta cell. All measurements were performed at 25 ± 0.5 °C. Each sample was measured at least ten times.

2.4.4. Transmission electron microscopy (TEM)

The NPs shape and size were observed by TEM (Jeol Electron Microscope, JEM-1010, Tokyo, Japan). Samples were first dispersed in deionized water, then dropped on copper grids stained with

phosphotungstic acid solution (2%, w/v) and dried at room temperature before visualization (Zhang, Hou, Zhang, Hu, & Wang, 2010).

2.4.5. Determination of surface hydrophilicity

Various concentrations of the Rose Bengal dye solutions were incubated with the nanosuspensions for 3 h at 25 ± 0.5 °C. The NPs were separated by centrifugation, and the dye content in the separated liquids was determined photometrically at 542 nm. Correction for possible dye adsorption on centrifuge tubes and pipette tips was done by running a control experiment (Sahooa, Panyama, Prabhaa, & Labhasetwar, 2002). The adsorption data were analyzed according to the Langmuir equation (Egbaria & Friedman, 1992):

$$\frac{x}{m} = \frac{k_1 k_2 C_{eq}}{1 + k_1 C_{eq}} \quad (2)$$

where x/m is the amount of dye adsorbed per mass of NPs, C_{eq} is the concentration of unadsorbed dye at equilibrium, k_1 is the dye affinity constant for the NPs and the Langmuir-capacity constant, k_2, indicates the apparent maximum amount of adsorbate that can be adsorbed per unit weight of adsorbent.

Eq. (2) can be rearranged as follows:

$$\frac{C_{eq}}{x/m} = \frac{1}{k_1 k_2} + (1|k_2) C_{eq} \quad (3)$$

The slope of the line obtained by plotting $C_{eq}/(x/m)$ *versus* C_{eq} equals $1/k_2$ and the intercept is $1/(k_1 k_2)$.

2.4.6. Sterilization by gamma irradiation

Selected lyophilized formulae were sterilized by gamma irradiation after packing in dry ice inside polyurethane container (Kamel, Awad, Geneidi, & Mortada, 2009) using cobalt-60 Gamma Chamber 4000-A, at a dose of 25 KGy (European Pharmacopoeia, 6th ed., 2006). The sterilized NPs were re-evaluated for their EE and particle size. The CZP release profiles of sterilized and non-sterilized NPs were compared using the difference factor (f_1) and the similarity factor (f_2) according to the following equations (Moore & Flanner, 1996):

$$f_1 = \left[\frac{\sum_{t=1}^{n} |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right] \times 100 \quad (4)$$

$$f_2 = 50 \times \log \left\{ \left[1 + \left(\frac{1}{n} \right) \sum_{t=1}^{n} (R_t - T_t)^2 \right]^{-0.5} \times 100 \right\} \quad (5)$$

where R_t and T_t are the cumulative percentage drug dissolved at each of the selected n time points of the reference and test product, respectively.

Similar dissolution profiles should have f_1 values 0–15 and f_2 values 50–100 (Costa & Lobo, 2001).

2.5. In vivo study

2.5.1. Animals

Animal experiments were conducted according to protocols approved by the Experiments and Advanced Pharmaceutical Research Unit (EAPRU), Faculty of Pharmacy, Ain Shams University on the use of the animals. Male albino rats (200 ± 20 g) and Swiss albino mice (25 ± 2 g) were used in pharmacokinetic and biodistribution studies, respectively. During the whole study, uniform feed and free water were supplied.

2.5.2. Pharmacokinetic study

After a 7-day acclimatization period, twenty-four white male albino rats were divided into four groups (each of six animals) were used for the pharmacokinetic study. Group I received CZP solution prepared at a concentration of 4 mg/ml in 0.25 N HCl adjusted to pH 5 with 1 N NaOH. Group II received CZP-loaded PLA NPs coated with 0.8% chitosan (0.8%F-2C-CH) while Groups III and IV received CZP-loaded non-coated PLA NPs (F-2) and PLA NPs coated with 5% polysorbate 80 (5%F-2C-P80), respectively. Coated and uncoated NPs were dispersed in 1.5% methyl cellulose and 0.9% NaCl, before administration. All the rats were injected into their tail vein with a 1 cm^3-U100 insulin syringe equipped with 28 G needle at a dose level of 10 mg/kg (Manjunath & Venkateswarlu, 2005). Samples of 0.5 ml blood were withdrawn from the retro-orbital venous plexus puncture, at different time intervals after drug administration, put in sterile tubes containing EDTA-K3 and centrifuged at 3500 rpm for 15 min. The plasma samples were stored at −20 °C until assayed.

2.5.3. Biodistribution study

After a 7-day acclimatization period, sixty-three Swiss albino mice were divided into three groups each of 21 mice as follows; Group A received F-2C-0.8%CH while Groups B and C received CZP F-2 and F-2C-5%P80, respectively. The animals were injected intravenously as described above but at a dose level of 20 mg/kg (Manjunath & Venkateswarlu, 2005). At each time point (15, 30, 45, 60, 120, 240 and 360 min), three animals from each group were sacrificed. Tissues of interest (liver, spleen and kidney) were collected immediately after cervical dislocation and they were blotted dry with tissue paper and frozen at −20 °C until analysis.

2.5.4. Plasma and tissue sample analysis

Both drug and internal standard (celecoxib) were extracted from plasma with 4 ml ethylacetate after addition of 200 μl 2 M NaOH (Manjunath & Venkateswarlu, 2005). After vortexing, the mixture was centrifuged at 3500 × *g* for 15 min and the separated organic phase was evaporated under reduced pressure. The residue was then reconstituted in the mobile phase and injected into HPLC column. On the day of analysis, thawed tissue samples were weighed accurately and homogenized for 1 min after mixing with normal saline. Volume equivalent to 0.2 g (in case of liver and kidney) and 0.1 g (in case of spleen) of tissue were processed similarly as that of plasma samples for HPLC analysis. The chromatographic system consisted of Agilent Technologies 1200 series LC – G 1311A solvent delivery pump equipped with a 20-μl loop and rheodyne sample injector and G1315D diode array detector. The column used was Agilent TC-C_{18} analytical column (5 μm particle size; 250 mm × 4.6 mm ID). The mobile phase consisted of methanol–water–triethylamine (75:25:0.5, v/v/v), pH was adjusted to 6.5 with glacial acetic acid and was used at a flow rate of 1 ml/min (Manjunath & Venkateswarlu, 2005). The eluate was monitored at 254 nm. The data was recorded and calculated using ChemStation B.04.01 software.

2.5.5. Pharmacokinetic data

Plasma CZP concentration *versus* time data of the administered NPs in individual rats were analyzed by non-compartmental estimations using Thermo Kinetica software (version 5.0.11). C_{max} and T_{max} were taken directly from the observed concentration *versus* time profiles. The areas under the curve from zero to last sampling time after drug administration (AUC_{0-t}) and the area under the first moment curve (AUMC) were determined by the linear trapezoidal rule. The ratio between AUMC and AUC gave residence time (MRT). The area under the curve from time zero to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/K_e$, where C_t is the last detectable plasma concentration (Owens & Peppas, 2006).

Tissue concentration data of CZP obtained from mice were pooled to provide mean concentration data. The relative bioavailability (F_r) is defined as ratio of AUC of CZP-loaded coated NPs to the AUC of CZP-loaded non-coated NPs when same doses were

Table 2
F-values for EE, zeta potential and $T_{80\%}$ responses of CZP-loaded NPs according to the factorial design.

Source of variation	*F*-value[a]		
	EE	Zeta potential	$T_{80\%}$
Polymer type	255.69[b]	11.45	534.58[b]
Polymer conc.	427.95[b]	95.68[b]	822.56[b]
PVA conc.	389.52[b]	276.97[b]	1543.95[b]
Polymer type–polymer conc.	2.33	0.78	11.64
Polymer type–PVA conc.	23.65[b]	1.57	104.73[b]
Polymer conc.–PVA conc.	244.66[b]	13.41	94.64[b]
Polymer type–polymer conc.–PVA conc.	2.22	1.34	189.66[b]

[a] F-value = between-group variance/within-group variance.
[b] Significant at $p < 0.001$.

administered. All data were expressed as mean ± standard error of the mean (S.E.).

2.6. Statistical analysis

MINITAB for Windows (release 15.1.30.0, 2007) computer program was used to analyze statistically the data obtained from the factorial design. The results are expressed as mean ± S.D. The significance of differences was assessed using ANOVA test.

3. Results and discussion

3.1. Effect of formulation variables on particle size, EE, ζ and $T_{80\%}$ of CZP-loaded NPs prepared according to the factorial design

The particle size distributions of all prepared NPs were unimodal. The Z-average of NPs particle size ranged between 319 and 393 nm (data not shown).

The 'main effect' of an independent variable is the effect of the variable averaging over all levels of other variables in the experiment. The higher the significance effect of any of the variables (or factors) under study, the higher the *F*-value is. The ANOVA test (Table 2) shows that the polymer type and concentration and the PVA concentration affected significantly CZP EE ($p < 0.001$) in a descending order as follows: the polymer concentration > PVA concentration > the polymer type ($p < 0.001$). Increasing polymer concentration, decreasing PVA concentration and shifting from PLA to PLGA decreased drug EE ($p < 0.001$).

Two independent variables interact if the effect of one of the variables differs depending on the level of the other variable. Only two significant interactions were noticed: between PVA and polymer concentration on one side, and polymer concentration and type on the other side ($p < 0.001$). In other words, the effect of PVA concentration differs significantly on EE depending on the level of polymer concentration and *vice versa.* The effect of polymer concentration differs also significantly on EE depending on the level of its type and *vice versa*. Accordingly, combining 2% concentration of PLA with the lowest concentration (2%) of PVA yielded NPs with the highest EE.

PVA concentration showed a significantly higher effect than polymer concentration on ζ ($p < 0.001$) with the effect of the polymer type being non-significant. Thus, NPs with high ζ can be obtained by using low PVA concentration (2%) and higher polymer concentration (2%), whatever the type of polymer used.

The three factors under study have significant effects on CZP $T_{80\%}$ ($p < 0.001$). PVA concentration was the highest and the polymer type the lowest ($p < 0.001$). The interactions between PVA concentration and polymer concentration and type were significant ($p < 0.001$) and the three-way interactions between the three variables were also significant. The NPs with extended $T_{80\%}$ arranged in an ascending manner were as follows: (F-6 with 15.60 h) < (F-1 with 25.20 h) < (F-4 with 27.20 h) < (F-2 with 33.60 h). The goal of this study was a possible once daily or every other day administration of CZP-loaded NPs. Hence $T_{80\%}$ values lower than 24 h were excluded from further studies.

From the factorial design data analysis, it could be concluded that formula CZP NP (F-2), prepared from 2% (w/v) PLA and 2% (w/v) PVA shows the highest EE (43.98%), optimum zeta potential (−28.10 mV) (Al-Hallak et al., 2010; Guo & Gemeinhart, 2008), acceptable particle size (380 nm) and extended $T_{80\%}$ (33.6 h). Therefore, this formula (F-2) was chosen for coating with different chitosan concentrations, in addition to different concentrations of conventional shielding materials namely: pluronic F-68, PEG 4000 and polysorbate 80.

3.2. Evaluation of chitosan-coated NPs in comparison with other shielding materials

3.2.1. Transmission electron microscopy (TEM)

Fig. 1 shows representatives of the TEM images of chitosan-coated CZP NPs along with those of NPs coated with different coating materials, namely; pluronic F-68, PEG 4000 and polysorbate 80.

3.2.2. NPs surface hydrophilicity

Plots of the amount of rose bengal adsorbed on CZP-loaded NPs (F-2) before and after coating, with different concentrations of chitosan and other coating materials, as a function of equilibrium concentration of the dye were all linear with R^2 values ranging from 0.9707 to 0.9905. They showed higher amounts of adsorbed dye onto the non-coated F-2 than on coated NPs (data not shown). The hydrophobic dye with log *P* = 11.39 was attracted to the hydrophobic surface of non-coated NPs (Egbaria & Friedman, 1992). The affinity and capacity constants of the dye to NPs (k_1 and k_2) obtained from typical Langmuir plot of $C_{eq}/(x/m)$ *versus* C_{eq} were higher in case of F-2 than with chitosan-coated particles and any of the conventional shielded particles (Table 3). In contrast, polysorbate-coated NPs (F-2C-P80) showed the lowest dye affinity. Polysorbate 80 possesses one unsaturated carbon–carbon double bond in the alkyl moiety, forced the molecule to lie more nearly parallel to the hydrophobic substrate (Graca, Bongaerts, Stokes, & Granick, 2007). It has been reported that the coating (or protective) effect of a coating polymer depends on a balance between its hydrophilicity and its interaction with the surrounding hydrophilic medium and the energy of its hydrophobic interaction at the particles surface (Torchilin & Trubetskoy, 1995). In other words, the more hydrophobic character of the coating material as supported by its HLB, the higher the anchoring and coating effect on the NPs surface will be. The coating substances used can be arranged according to their hydrophilicity as follows; PEG > pluronic (HLB = 29) > polysorbate (HLB = 15). Hence a higher energy of hydrophobic surface anchor interaction can be expected in case of polysorbate.

Chitosan-coated NPs (F-2C-CH) showed lower k_1 and k_2 than those calculated in case of pluronic-coated (F-2C-P68) and PEG-coated (F-2C-P40) particles. The spontaneous chitosan adsorption from solution governed by the electrostatic interactions of the polyamine with the negatively charged PLA particle surface might had enhanced the coating properties of chitosan (Messai & Delair, 2005).

3.2.3. Entrapment efficiency (EE)

Table 3 shows the EE of the selected formula (F-2) before and after coating with chitosan and other coating materials where non-significant changes in the EE of the selected formula (F-2) were seen due to coating (ANOVA test, $p > 0.05$).

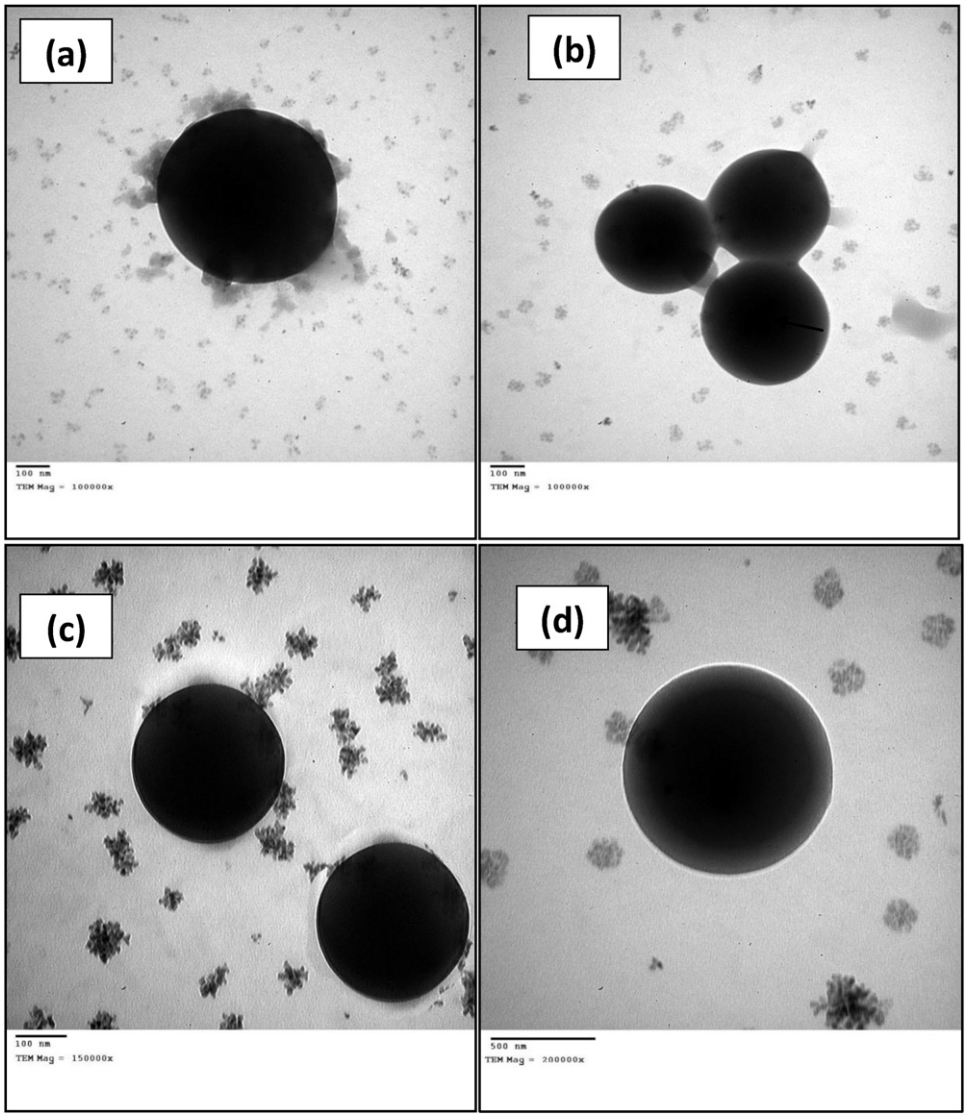

Fig. 1. TEM of coated CZP-loaded PLA NPs with pluronic (a), PEG (b), polysorbate (c) and chitosan (d).

Table 3
Adsorption constants, EE, *Z*-average and zeta potential of the selected CZP-loaded NPs formula (F-2) before and after coating.

Formula code	Coat type	Coat. conc. (%)	k_1 [a] (ml/μg)	k_2 [b] (μg/mg)	EE (%) ± S.D. (n = 3)	*Z*-average (nm) ± S.D. [PDI] (n = 20)	Zeta potential (mV) ± S.D. (n = 10)
F-2	Uncoated	–	0.1266	67.568	43.98 ± 0.55	380 ± 8.00 [0.38]	−28.10 ± 0.88
F-2C-P68	Pluronic F-68	1	0.0702	33.898	42.95 ± 0.46	411 ± 10.00 [0.30]	−24.87 ± 1.03
		2.5	0.0632	22.124	43.11 ± 0.35	416 ± 5.00 [0.35]	−23.09 ± 0.89
		5	0.0542	20.367	43.55 ± 0.64	430 ± 9.00 [0.34]	−22.20 ± 1.13
F-2C-P40	PEG 4000	1	0.0822	29.326	43.78 ± 0.62	406 ± 8.00 [0.33]	-24.12 ± 0.98
		2.5	0.0437	21.142	43.67 ± 0.59	415 ± 8.00 [0.31]	−24.15 ± 0.63
		5	0.0260	13.550	43.88 ± 0.54	426 ± 11.00 [0.35]	−24.09 ± 0.58
F-2C-P80	Polysorbate 80	1	0.0117	17.153	42.50 ± 0.45	380 ± 5.00 [0.29]	-27.00 ± 0.95
		2.5	0.0095	13.870	42.23 ± 0.36	380 ± 7.00 [0.30]	-25.12 ± 0.73
		5	0.0054	14.514	42.15 ± 0.58	378 ± 6.00 [0.32]	-24.40 ± 0.64
F-2C-CH	Chitosan	0.2	0.0655	19.342	42.85 ± 0.22	691 ± 10.00 [0.36]	−17.70 ± 0.92
		0.4	0.0328	15.175	42.87 ± 0.48	703 ± 11.00 [0.44]	−6.60 ± 0.73
		0.8	0.0161	13.141	42.95 ± 0.15	742 ± 11.00 [0.37]	+9.52 ± 1.00

N.B.: F-2: CZP NPs prepared with 2% (w/v) PLA and 2% (w/v) PVA. EE: entrapment efficiency. S.D.: standard deviation. PDI: polydispersity index.
[a] k_1: the affinity constant of the dye for the NPs.
[b] k_2: a constant indicating the capacity of the NPs for the dye.

Table 4
Pharmacokinetic parameters of CZP solution, uncoated NPs and selected coated NPs in rats ($n = 6$) after IV administration.

Parameters	Mean value ± S.E.			
	CZP solution	Uncoated CZP-loaded NPs (F-2)[a]	Coated CZP-loaded NPs (5%F-2C-P80)[b]	(0.8%F-2C-CH)[c]
C_{max} (ng/ml)	622.48 ± 55.26	565.46 ± 46.34	583.44 ± 90.96	466.75 ± 35.54
T_{max} (h)	0.25 ± 0.00	0.50 ± 0.00	0.50 ± 0.10	0.50 ± 0.09
AUC_{0-12} (μg h/ml)	2.03 ± 0.21	–	–	–
AUC_{0-48} (μg h/ml)	–	3.42 ± 0.28	6.78 ± 0.68	9.15 ± 0.79
$AUC_{0-\infty}$ (μg h/ml)	2.51 ± 0.23	5.17 ± 0.55	8.91 ± 1.29	16.99 ± 1.38
$AUMC_{0-12}$ (μg h^2/ml)	7.72 ± 0.95	–	–	–
$AUMC_{0-48}$ (μg h^2/ml)	–	27.72 ± 2.18	117.57 ± 10.08	194.62 ± 16.06
$AUMC_{0-\infty}$ (μg h^2/ml)	17.59 ± 2.94	116.38 ± 14.78	298.33 ± 23.58	1089.86 ± 98.58
$t_{1/2}$ (h)	5.47 ± 0.64	18.48 ± 1.98	24.41 ± 2.70	45.88 ± 3.62
Clearance (L/h/kg)	4.05 ± 0.08	1.93 ± 0.04	1.12 ± 0.06	0.59 ± 0.05
MRT (h)	6.97 ± 0.93	22.50 ± 3.30	33.13 ± 3.14	64.16 ± 5.88

[a] (F-2): non coated CZP-loaded NPs prepared with 2% PLA and 2% PVA.
[b] (5%F-2C-P80): F-2 coated with 5% polysorbate solution.
[c] (0.8%F-2C-CH): F-2 coated with 0.8% chitosan solution

3.2.4. Particle size and zeta potential ζ

Table 3 shows that the mean diameter of chitosan-PLA NPs increases significantly with the increase in chitosan concentrations (ANOVA test, $p < 0.05$) with the largest one being of 742 nm obtained at 0.8% chitosan. Similar findings were obtained previously (Guo & Gemeinhart, 2008; Yuan et al., 2010). Moderate increase in particle size was observed after coating with pluronic F-68 and PEG 4000 (ANOVA test, $p < 0.05$). Coating with different polysorbate80 concentrations causes no significant change in particle size (ANOVA test, $p > 0.05$) conforming with a previous study (Al-Hallak et al., 2010).

Table 3 also shows that significant reduction in ζ after coating with chitosan and other shielding materials (ANOVA test, $p < 0.05$) in agreement with previous works (Mei et al., 2009; Sun, Xie, Wang, & Hu, 2004). Increasing chitosan concentration above 0.4% caused charge reverse. Spontaneous adsorption of the polymer on the particle surface occurs mainly due to electrostatic interaction between the particles charges and the positively charged chitosan. The reversal of particle charge noticed with 0.8% F-2C-CH suggested the presence of residual amino group after neutralization of NP polymer surface (Messai & Delair, 2005). On the other hand, the significant reduction in ζ in case of other shielding materials can be attributed to the presence of the non-ionic coating layer on the surface, which shifts the shear plane of the diffusive layer to a larger distance (Dong & Feng, 2004). The variation in these coating materials concentration did not have an obvious effect on ζ values.

From the previous results, it can be concluded that CZP-loaded PLA NPs coated with 0.8% chitosan as well as those coated with 5% polysorbate 80 showed the least surface hydrophobicity with positive and negative surface charges, respectively. A decrease in surface hydrophobicity is known to be accompanied by a decrease in the quantitative amount of adsorbed hydrophobic opsonic protein segments on NPs surface (Gessner et al., 2000) and their subsequent uptake by macrophages. Thus the two latter formulae were selected for further *in vitro* and *in vivo* studies.

3.2.5. In vitro CZP release from NPs coated with chitosan- and polysorbate 80

The *in vitro* release profiles of CZP from the selected non-coated (F-2) NPs and the corresponding coated NPs show a slower drug release rate from the coated ones than from F-2. The recorded $T_{80\%}$ of F2, 0.8%F-2C-CH and5%F-2C-P80 were 33.60, 50.75 and 36.11 h, respectively. The initial drug release is mainly due to desorption and diffusion of the drug from the surface and through minute pores present on particle surface. This observation was based on the data given in Table 3 where the particle size of formula (0.8%F-2C-CH) was larger than that of (5%F-2C-P80) with 742 and 378 nm, respectively.

3.2.6. Sterilization by gamma irradiation

The EE and particle size of both selected coated NPs were not significantly affected by sterilization (ANOVA test, $p < 0.05$). The release profiles of the selected formulae before and after gamma irradiation were similar with f_1 value of 0.05 and 0.07 and f_2 value of 76.55 and 67.78, for NPs coated with chitosan and polysorbate 80, respectively. Gamma irradiation in presence of dry ice prevented polymer degradation that may occur as a result of the increase in temperature taking place during the sterilization. Same observations were reported by Fernández-Carballido, Herrero-Vanrell, Molina-Martínez, and Pastoriza (2006).

3.3. Pharmacokinetic study

The comparative pharmacokinetic parameters after IV administration of CZP formulae (0.8%F-2C-CH, 5%F-2C-P80 and F-2) are reported in Table 4. The $AUC_{0-\infty}$ of 0.8%F-2C-CH was 16.99 μg h/ml while that of drug solution, F-2 and 5%F-2C-P80 were 2.51, 5.17 and 8.91 μg h/ml, respectively. The respective $t_{1/2}$ were 45.88, 5.47, 18.48 and 24.41 h. According to Sheng et al. (2009) and Kamel et al. (2009), chitosan and polysorbate80 are considered effective in reducing the uptake by RES hence increasing blood circulation time. The clearance of CZP did not differ significantly ($p > 0.01$) between the prepared NPs whether coated or uncoated, but were lower than that of drug solution ($p < 0.01$). The MRT of (0.8%F-2C-CH) increased significantly ($p < 0.01$) by 9.21-fold in plasma compared to solution. Meanwhile the MRT of (F-2) and (5%F-2C-P80) increased by about3.23 and 4.75-fold, respectively. The significantly highest MRT noticed with (0.8%F-2C-CH) could be interpreted on the basis of a better coating quality and the positively charged nature of the particles. Aiming to confirm the obtained results, further studies concerning the biodistribution of the three tested NP formulae in the RES containing organs were studied.

3.4. Biodistribution study

The major pathway for the removal of NPs from blood appears to be the NP capture in RES organs, especially in liver, kidney and spleen. The level of increased blood retention is positively correlated with a reduced sequestration by RES organs. The distributions

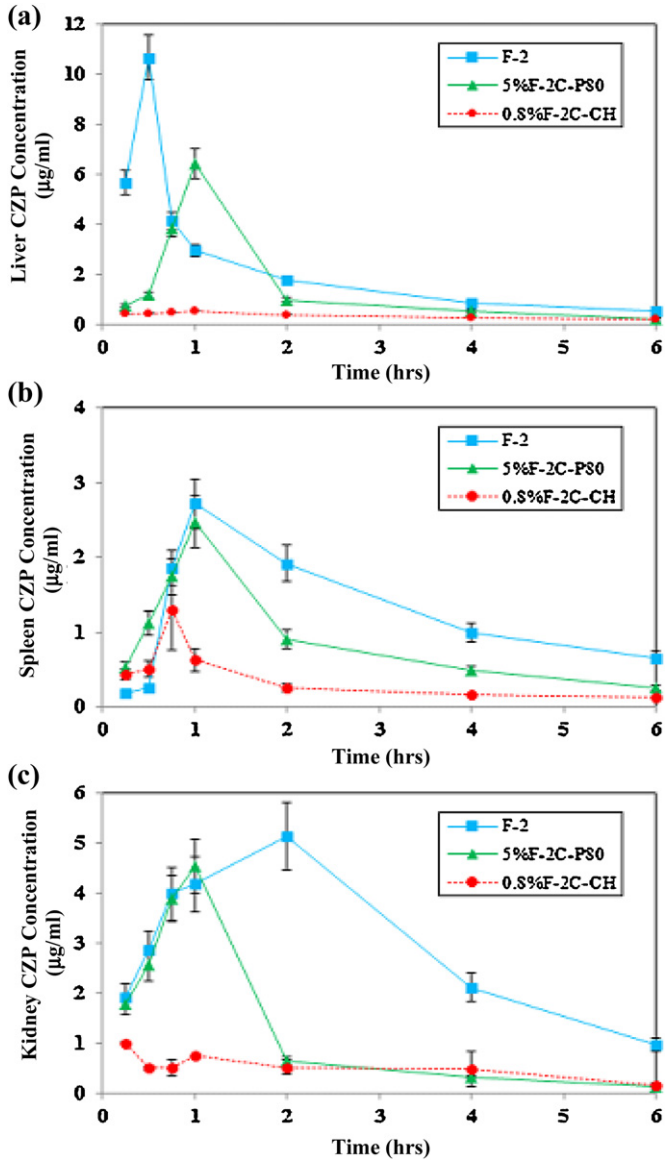

Fig. 2. Mean CZP concentration–time profiles in different organs after IV administration of Uncoated NPs (F-2), F-2 coated with polysorbate 80 (5%F-2C-P80) and chitosan (0.8%F-2C-CH) in (a) liver, (b) spleen and (c) kidney. Each point represents mean ± S.E. ($n = 3$).

of non-coated NPs and coated NPs with either chitosan or polysorbate in various RES organs for a 6-h period post-administration in mice are illustrated in Fig. 2. In the case of coated NPs, the tissue CZP concentrations, in terms of AUC, compared with those of non-coated NPs, was intensely reduced in liver and kidney and decreased to a lesser extent in spleen. Within the three organs under study, F-2 displayed a fast accumulation starting from the first 0.5 h, with the highest AUC, followed by progressive clearance out to 6 h. On the other hand, chitosan-coated NPs (0.8%F-2C-CH) showed the most pronounced decrease in uptake amounting by 3.85, 4.17 and 5-fold decrease in liver, spleen and kidneys respectively. Furthermore, a dramatic decrease in the kidney CZP concentration was observed for 0.8%F-2C-CH indicating a slow excretion. The relative bioavailability (F_r) of CZP-loaded NPs coated with chitosan to uncoated ones was computed as 0.26, 0.24 and 0.18, in liver, spleen and kidney, respectively.

Polysorbate-coated NPs (5%F-2C-P80) showed a decrease in uptake but less than 0.8%F-2C-CH. This reduction in stealth NPs uptake correlated well with the noticed retention of higher circulatory level in the blood. The relative bioavailability (F_r) of CZP-loaded NPs coated with polysorbate 80 to uncoated ones was computed as 0.60, 0.56 and 0.32, in liver, spleen and kidney, respectively.

Surface treatment of NPs with chitosan or polysorbate 80 exhibited substantial positive effect on surface hydrophilicity and blood circulation prolongation. However the extended circulation in case of 0.8%F-2C-CH was explained by a different *in vivo* biodistribution when compared with 5%F-2C-P80. The correlation between surface charge and opsonization is controversial. It has been reported that neutrally charged particles have a much lower opsonization rate than charged ones of the same size (Zahr et al., 2006). Others reported that particles with charged surface showed reduced uptake by organs of RES where amino-bearing polystyrene particles stimulate the phagocytic uptake mechanism to a lesser extent than those deprived from them (Moghimi, Hunter, & Murray, 2001). By its positive charge, chitosan-stealth NPs lacked binding with opsonins hence evading phagocytosis and prolonging blood circulation. However, polysorbate-modified NPs bearing negative charges would have higher affinity for opsonins, explaining a certain uptake in the RES system.

The surface chain density and molecular conformation of polymer was reported to affect the coat properties on the particle surface (Kenworthy, Simon, & Mcintosh, 1995). Hydrophilic polymers coated on the particle surface display either a 'mushroom' or a 'brush' arrangement according to polymer surface density. The 'brush' conformation establishes more powerful repulsion of opsonins than the 'mushroom' one (Vonarbourg, Passirani, Saulnier, & Benoit, 2006). While chitosan will most likely not form a brush-like structure, it is likely that the combination of the density of the chitosan layer along with its particular chemical composition leads to greater opsonins repulsion.

4. Conclusion

A great improvement in surface hydrophilicity was brought by chitosan and polysorbate 80 coatings. However, the *in vivo* particle uptake by the RES was less pronounced with positively charged chitosan-stealth NPs than with polysorbate 80.

Acknowledgements

The authors wish to express their thanks to the staff members of the pharmacology and toxicology department, Faculty of Pharmacy, Ain Shams University (Cairo, Egypt) for their assistance in performing the *in vivo* study. The authors would also like to acknowledge PURAC Biochem, Apex Pharma, Sedico companies for supplying PLGA/PLA, clozapine and celecoxib, respectively, for the current work.

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