Comparative *In Vitro* Studies on PBN Loaded Nanoparticles Prepared by Biodegradable Chitosan, PLGA Polymers and Their PEGylated Block Copolymers

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Abstract

α-phenyl-N-tert-butyl nitrone (PBN) is a neuroprotective free radical scavenger however it has low *in vivo* stability and blood residence time. Aim of this study is to develop a nanoparticle formulation by using different polymeric system which enhance the blood residence time and in vivo stability of PBN and characterize in terms of particle size, zeta potential, morphology, encapsulation efficiency, in vitro release profiles. Chitosan (CS), poly(D,L-lactide-co-glycolide) (PLGA) and their poly(ethylene glycol) (PEG) block co-polymers were used for comparative study. Results showed that particle sizes of CS, CS-PEG, PLGA and PLGA-PEG nanoparticles are between 142-356 nm. PLGA nanoparticles and their block-copolymers' nanoparticle have greatly monodisperse distribution. CS and CS-PEG nanoparticles have zeta potential values between 17-40 mV related to amine groups, contrariwise PLGA and PLGA-PEG nanoparticles have negative zeta potential in the range of (-8) – (-19) mV. Encapsulation efficiency and loading capacity for all formulations are between 12 – 54 %, 9 – 68 % respectively. PLGA-PEG nanoparticles are promising for further studies due to their sufficient encapsulation efficiency and *in vitro* release profiles.

Key words: Chitosan, PLGA, nanoparticle, PBN, PEGylation

1. Introduction

Polymeric nanoparticles have been extensively studied as particulate carriers in the pharmaceutical and medical fields, because they show promise as drug delivery systems as a result of their controlled- and sustained-release properties, subcellular size, and biocompatibility with tissue and cells [1, 2]. Biodegradable nanoparticles based on polyester polymers such as poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide) (PLA) have been widely investigated as parenteral delivery systems. Polyester polymers, approved by the Food and Drug Administration, have raised great interest due to their physicochemical and biological properties in addition to their biocompatibility and bioresorbability properties, the possibility of modulating drug release profiles by selecting the appropriate polymer is particularly interesting for the development of parenteral drug products [3].

Chitosan (CS) based nanoparticles have received much attention for the delivery of drugs since this cationic polysaccharide, which is obtained by deacetylation of chitin, may be considered as non-toxic, biodegradable, and biocompatible material [4]. Chitosan nanoparticles are prepared by ionotropic gelation due to the simplicity and the lack of toxic solvents in this technique [5].

The originally hydrophobic particles, after intravenous administration, will become coated by blood components (opsonins) and rapidly taken up by reticuloendothelial system (RES) [6]. Therefore, nanoparticle surfaces should be modified with hydrophilic components such as PEG. The goal of surface modification is to make the particles unrecognizable by the RES and guide it to the desired site. Particle size is also a crucial factor for prolonged circulation time in the blood stream. Generally the smaller nanoparticle with more hydrophilic surface shows less RES uptake [7].

α-phenyl-N-tert-butyl nitrone (PBN) have emerged as a potent reactive oxygen species (ROS) scavenger,with neuroprotective efficacy and large therapeutic time window that was proven in several models of central nervous system injury, such as traumatic brain injury (TBI), stroke and intracerebral hematoma. PBN has a high degree of blood–brain barrier (BBB) penetration and a half-life in plasma of 3 hours [8, 9]. However the stability of PBN in blood is relatively low and the residence time in blood is also too short [10]. Pretreatment with a single intravenous (IV) dose of PBN (30 mg/kg 30 minutes before injury) recently has been shown to reduce cognitive deficits and lesion volume in a controlled cortical contusion model in rat. PBN has been shown to reduce infarct volume, ischemic/nitrative stress, restore microcircular patency and neuroprotective in rodent models of cerebral ischemia [8, 11, 12].

Herein we report formulation strategies to control the size, zeta potential, encapsulation efficiency and in vitro release properties of PLGA, Chitosan, PLGA-PEG and Chitosan-PEG nanoparticles. The aim of this study is to evaluate effect of polymer type on in vitro characterization of nanoparticles for further studies. To overcome low in vivo stability and short blood residence time of PBN, PEGylated and nonPEGylated polymers and also cationic natural chitosan and anionic surface charged synthetic PLGA polymers were used and discussed in detail.

2. Material and Methods

2.1. Materials

Chitosan was commercially available as Protasan Cl 113 (MW: <150 kD, deacetylation degree: 75–90%) and was purchased from FMC Biopolymers (Norway). Chitosan-poly(ethylene glycol) (CS-PEG) was previously synthesized at the University of Santiago de Compostela, Spain as described by Aktas et al. [13]. Tripolyphosphate (TPP) and PBN were supplied by Sigma Chemical Co. (USA). Ultrapure water was obtained with MilliQ equipment (Waters, USA). HPLC grade methanol was purchased from Merck (Darmstadt,

Germany). All other chemicals and reagents used were of analytical or pharmaceutical grade. PLGA (50:50; Resomer® RG 502 H, MW: 28000 Da) and Poly[(D,L-lactide-co-glycolide)-co-PEG] diblock (RESOMER® RGP d 50105) were purchased from Boehringer Ingelheim Pharma GmbH (Ingelheim, Germany). Polyvinyl alcohol (PVA) (MW: 30000–70000 Da) was purchased from Sigma-Aldrich Co. (St. Louis, USA). Ethyl acetate was purchased from Merck KgaA (Darmstadt, Germany). Deionized water was obtained by a Millipore Milli-Q® System (Bedford, USA). All other chemicals and reagents used were of analytical or pharmaceutical grade.

2.2. Preparation of drug loaded chitosan and chitosan-PEG nanoparticles

CS and CS-PEG nanoparticles were prepared by the ionic gelation of TPP and CS or CS-PEG according to the procedure previously developed by P. Calvo et al. for the preparation of CS nanoparticles [14, 15]. Practically, CS nanoparticles were formed upon dropwise addition of 1 mL TPP aqueous solution (0.4 mg/mL) to 1 mL of CS aqueous solution (1.75 mg/mL). Likewise, CS-PEG (1 mg/mL) nanoparticles were prepared by dropwise addition of 0.4 mL TPP aqueous solution (0.84 mg/mL) to 1 mL of each of the corresponding aqueous polymer solutions. These solutions were then stirred under magnetic stirring at medium speed (700 rpm) and room temperature. PBN-loaded nanoparticles were obtained according to the same procedure, and the ratio of polymer/TPP remaining unchanged. PBN was incorporated in the polymer solution before the addition of the TPP. Two different drug concentration was chosen for loading to the nanoparticles. The resulting mixtures were broadly characterized as either a clear solution, an opalescent suspension displaying a tyndall effect (NPs), or aggregates. Nanoparticles were isolated by ultracentrifugation (10 000 rpm, 4 °C, 60 min in the presence of 10 µl of glycerol) and then resuspended in water by manual shaking [13].

2.3. Preparation of drug loaded PLGA and PLGA-PEG nanoparticles

Nanoparticles were prepared by emulsification by homogenization-solvent evaporation (homogenization) method. Homogenization involve preparation of an organic phase consisting of polymer (PLGA or PLGA-PEG, typical concentration, 20 mg/mL) and drug (PBN,two different concentration, 1 mg/mL, 2 mg/mL) dissolved in ethyl acetate (typical volume, 10 mL). This organic phase is added to an aqueous phase containing a surfactant (PVA, typical concentration, 3%, 20 mL) to form an emulsion. This emulsion is broken down into nanodroplets by applying external energy during 2 minutes at 11 000 rpm (through a homogenizer) and these nanodroplets form nanoparticles upon evaporation of the highly volatile organic solvent. The solvent is evaporated using rotary evaporator under vacuum and 37 ° C for 45 minutes leaving behind a colloidal suspension of PLGA nanoparticles in water. After the removal of ethyl acetate, nanospheres were collected by centrifugation at 13 000 rpm for 20 min and lyophilized.

2.4. Nanoparticle characterization

Shape and morphology of nanoparticles were analysed by Scanning Electron Microscopy (SEM), using a scanning electron microscope (Nova™ NanoSEM 430, FEI, USA). Dry samples of nanospheres were mounted on carbon adhesive stubs and coated with a gold layer of appropriate thickness. The size (Z-average mean) and zeta potential of the nanoparticles were analyzed by photon correlation spectroscopy and laser doppler anemometry, respectively, in triplicate using a Zetasizer Nano Series (Nano-ZS) (Malvern Instruments, UK). Formulations were coded as presented in Table I.

2.5. Determination of PBN entrapment

Different methods were performed for Chitosan and PLGA nanoparticles. The amount of entrapped PBN was determined directly for chitosan and chitosan-PEG nanoparticles. Nanoparticles were resuspanded in methanol and extraction was performed using ultrasonic

bath for 30 min. Thus, nanoparticles were degraded and PBN extracted to the methanol phase. The solutions were passed through a membrane filter (pore size 0.22 μm, Millipore) before HPLC measurements. The amount of non-entrapped PBN was determined indirectly for PLGA and PLGA-PEG nanoparticles and also chitosan and chitosan-PEG nanoparticles. The supernatant containing non-entrapped PBN was separated from solid nanoparticles by ultracentrifugation by HPLC by UV detection set at 286 nm (Agilent Technologies 1200 Series,USA). The mobile phase consisted of methanol:water (50:50) and the flow rate was set at 1 mL/min. Separation was achieved using a Clipeus C18 column (150mm×4.6 mm, 5 μm). PBN loading capacity (LC) of the nanoparticles and their encapsulation efficiency (AE) were calculated according to the following equations [5, 16]:

$$\text{Loading Capacity (\%)} = \frac{\text{Total PBN amount} - \text{Free PBN amount}}{\text{Nanoparticle weight}} \times 100$$

$$\text{Encapsulation Efficency (\%)} = \frac{\text{Amount of PBN in nanoparticles}}{\text{Initial amount of PBN}} \times 100$$

2.6. In vitro release studies

Nanoparticles (1 mg) were resuspended in 1.5 mL of phosphate buffered saline solution (PBS) (pH 7.4) and incubated at 37 °C under light agitation. At appropriate time intervals individual samples were centrifugated and 1 mL of the supernatant was withdrawn. The amount of PBN in the release medium was determined by HPLC. The calibration curve obtained from the HPLC method was linear between 25 and 800 ng/mL ($y = 0.117x - 0.044$, $R^2 = 0.99994$). The limit of detection was 2.06 ng/mL.

3. Results

3.1. Particle size, zeta potential and morphology

The size (Z-average mean) and zeta potential of the nanoparticles were analyzed by photon correlation spectroscopy and laser doppler anemometry as mentioned previously. Mean nanoparticle size and zeta potential values are summarized in Table II and III. Chitosan/ Chitosan-PEG and PLGA/ PLGA-PEG nanoparticles were evaluated separately but the results will assess in discussion part in terms of preparation methods and polymer types. Particle size and zeta potential distribution of prepared nanoparticles were obtained from Zetasizer Nano Series (Figure 1, 2).

To monitor the morphology of the nanoparticles scanning electron microscopy (SEM) was used. SEM pictures of drug loaded and blank nanoparticles were showed in Figure 3 - 6. As shown in SEM pictures, nanoparticles have spherical shapes and monodispers distributions. SEM pictures of chitosan nanoparticles are different from other, because when scannig process was performing nanoparticles have disintegrated because of the high energy, for this reason these photos are not including scanning process.

3.2. PBN content of nanoparticles

PBN content of nanoparticles was analyzed using HPLC. Direct and indirect analyses were performed for chitosan and chitosan-PEG nanoparticles, however only indirect analyses carried out for PLGA and PLGA-PEG nanoparticles because of both of them hydrophobic drug and polymer. For each formulation encapsulation efficiency (%) and drug loading capacity (%) were calculated and summarized in Table IV and V.

3.3. In vitro drug release studies

In vitro drug release studies was performed as mentioned in section 4.6. Prepared nanoparticles using biodegradable polymers have showed different release profiles but, both

of them have got a burst release. The reason of this burst effect adsorbed drug to the surface of nanoparticle. Release profiles were indicated in Figure 7 and 8.

4. Discussion

In our study, biodegradable chitosan and PLGA polymers and their modified block copolymers were used for designing PBN loaded nanoparticle drug delivery system. Effect of polymer type, PEGylation and preparation method on particle size and zeta potential of nanoparticles, encapsulation efficiency of PBN and release behaviour of PBN were investigated. PBN constitutes the parent compound of the nitrone family of spin-trapping agents commonly used to trap free radicals. Trudeau-Lame *et al* reported that plasma concentrations after i.v. PBN (10 mg/kg) administration declined rapidly with a terminal half-life of 2.01 ± 0.35 h in male Sprague-Dawley rats and also total plasma clearance and volume of distribution at steady state averaged 12.37 ± 3.82 ml/min/kg and 1.74 ± 0.5 l/kg, respectively [17]. Whereas PBN has got considerably low in vivo stability and short blood residence time we prepared nanocarrier systems using different biodegradable polymers modified with PEG chain. Main purpose of this study is to develop and determine optimum nanocarrier system be able to increase PBN blood residence time and concentration at the therapeutic site of action for further studies.

PLGA is a hydrophobic polymer therefore nonPEGylated form of PLGA nanoparticles can be uptaken by mononuclear phagocytic system compound. PEGylated form would be provided more hydrophilic surface and also steric hindrance thus PEGylated PLGA nanoparticles present enhanced blood residence time for PBN.

Through spatial and temporal controlled drug delivery, injectable nanoparticle carriers have the ability to revolutionize disease treatment. Spatially localizing the release of toxic and other potent drugs only at specific therapeutic sites can lower the overall systemic dose and damage that these drugs would otherwise produce. Temporally controlling the release of a

drug can also help decrease unwanted side effects. The overall benefit of these improvements in disease treatment would be an increase in patient compliance and quality of life. In order for a drug delivery device to achieve these desired benefits it must be present in the bloodstream long enough to reach or recognize its therapeutic site of action. However, the opsonization or removal of nanoparticulate drug carriers from the body by the mononuclear phagocytic system (MPS), also known as the RES, is a major obstacle to the realization of these goals [6]. PEG modified polymers were used to overcome these problems.

Here, different biodegradable polymers were used and its effect was observed on morphology, particle size, zeta potential of nanoparticles, drug entrapment to the nanoparticles and in vitro release from the nanoparticles. Different polymer types and also PEGylation on the same polymer affected size of nanoparticle. It was observed that the size of CS-PEG NPs (142 ± 13.49 nm) was smaller as compared to CS NPs (319.6 ± 19 nm) (P<0.05). This may be explained by the colloid stabilization exerted by the PEG. However particle sizes of PLGA and PLGA-PEG nanoparticles were not different statistically (P>0.5). PLGA-PEG and PLGA nanoparticles have similar sizes, because of synthetic and high purified polymers are not affected by PEGylation significantly. Compared with PLGA nanoparticles, PLGA–PEG nanoparticles showed a marked decrease in the surface charge. This could be related to a shift of the hydrodynamic phase of shear to greater distances from the nanoparticles surface. The same observations have been reported for CS and CS-PEG nanoparticles [18]. Particle size of nanoparticles is very important in terms of blood residence time. Smaller particles can be stayed longer at blood circulation. CS-PEG NPs and PLGA-PEG NPs are not different concerning particle size however particle size distribution of PLGA-PEG NPs are more homogenious than CS-PEG NPs (P<0.5). Monodispers particle size distribution provides optimised formulations and it helps to get better pharmacokinetic results from in vivo studies.

The nanoparticles prepared in this study appeared to be spherical and rather homogeneous in size under the scanning electron microscope (Figure 3-6).

Encapsulation efficiency and drug loading capacity (%) have been increased at CS, CS-PEG, PLGA and PLGA-PEG nanoparticles increasing of theoretical loaded PBN amount. It can be explained by low level of drug could not reach the saturation on polymer. Steric hindrance of PEG caused low drug loading capacity at CS-PEG nanoparticles. Compared with CS and PLGA, higher encapsulation efficiency and drug loading capacity were obtained by PLGA. Lipophilicity of PBN provides stronger interaction with hydophobic PLGA polymer. Since obtained higher encapsulation efficiency, PLGA nanoparticles can be an option for in vivo studies.

For all formulations except PLGA-PEG nanoparticles, PBN release showed an initial burst release. This fast release could be relation part PBN adsorbed onto the surface of nanoparticles that would be immediately released during the initial stage. After the initial burst, PBN release profiles displayed a sustained fashion. This sustained release could result from diffusion of PBN into the polymer surface and the drug through polymer wall as well as the erosion of the polymers. In vitro release studies can be consider as a quality control test or in vitro characterization study. Obtained results showed that all formulations provide release of PBN under simulated conditions. Due to chitosan is a hydophilic biodegradable polymer release of PBN from CS NPs and CS-PEG NPs are faster than PLGA and PLGA-PEG NPs however long circulation time of PLGA-PEG nanoparticles can be provide controlled PBN release.

As a conclusion, it is observed that nanoparticles can be formulated as almost spherical in shape. They have homogeneous distribution, stability, having suitable particle sizes and effective encapsulation capacity. Moreover, they exhibit initially burst release, but then controlled release following 24-hour period, so that its half life could be increased. In vivo

experiments are needed to prove the effectiveness and safety of these nanoparticule carrier system and if they could be used for neuroprotection.

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351 Table I. Formulation codes of nanoparticles.

Formulation Code	Polymer Type	PBN Amount (mg)
CS NP	Chitosan HCl	-
CS1PBN NP	Chitosan HCl	1
CS2PBN NP	Chitosan HCl	2
CS-PEG NP	Chitosan-PEG	-
CS-PEG3.5PBN NP	Chitosan-PEG	3.5
CS-PEG7PBN NP	Chitosan-PEG	7
PLGA NP	Poly(D,L-lactide-co-glycolide)	-
PLGA10PBN NP	Poly(D,L-lactide-co-glycolide)	10
PLGA20PBN NP	Poly(D,L-lactide-co-glycolide)	20
PLGA-PEG NP	Poly[(D,L-lactide-co-glycolide)-co-PEG] diblock	-
PLGA-PEG10PBN NP	Poly[(D,L-lactide-co-glycolide)-co-PEG] diblock	10
PLGA-PEG20PBN NP	Poly[(D,L-lactide-co-glycolide)-co-PEG] diblock	20

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Table II. Particle size (nm), PDI and zeta potential (mV) values of CS and CS-PEG nanoparticles containing different concentrations of PBN.

Formulation	**Particle Size (nm)**	**PDI**	**Zeta Potential (mV)**
CS NP	319.6 ± 18.13	0.19	+38.7 ± 7.6
CS-PEG NP	142.4 ± 13.49	0.281	+17.5 ± 1.1
CS1PBN NP	340 ± 19	0.3	+20.2 ± 0.9
CS2PBN NP	356.4 ± 4.19	0.32	+18.6 ± 3.5
CS-PEG3.5PBN NP	265.6 ± 10.54	0.434	+25.9 ± 0.44
CS-PEG7PBN NP	208.8 ± 1.153	0.424	+23.45 ± 0.49

366 Table III. Particle size (nm), PDI and zeta potential (mV) values of PLGA and PLGA-PEG
367 nanoparticles containing different concentrations of PBN.

Formulation	**Particle size (nm)**	**PDI**	**Zeta Potential (mV)**
PLGA NP	269.6 ± 6.42	0.09	-18.4 ± 2.86
PLGA-PEG NP	271.1 ± 4.88	0.051	-14.4 ± 0.53
PLGA10PBN NP ***(before lyophilization)***	279.06 ± 5.71	0.063	-7.9 ± 3.2
PLGA20PBN NP ***(before lyophilization)***	285.6 ± 3.93	0.075	-15.4 ± 3.8
PLGA10PBN NP ***(after lyophilization)***	318.4 ± 10.87	0.191	-14.7 ± 0.36
PLGA20PBN NP ***(after lyophilization)***	303.1 ± 15.54	0.197	-16.3 ± 0.3
PLGA-PEG10PBN NP ***(before lyophilization)***	278.2 ± 2.177	0.07	-12.9 ± 0.34
PLGA-PEG20 PBN NP ***(before lyophilization)***	290.0 ± 2.1	0.09	-11.9 ± 0.47
PLGA-PEG10PBN NP ***(after lyophilization)***	293.6 ± 6.03	0.11	-13.3 ± 1.03
PLGA-PEG20PBN NP ***(after lyophilization)***	301.8 ± 2.82	0.098	-14.0 ± 0.7

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371 Table IV. Encapsulation efficiency and drug loading capacity of PBN-loaded CS and CS-PEG
372 nanoparticles.

Formulation	**Encapsulation Efficiency (%)**	**Loaded Drug Amount (µg)**	**Drug Loading Capacity (%)**
CS NP	-	-	-
CS-PEG NP	-	-	-
CS1PBN NP	32.67 ± 2.3	326.7	16.5 ± 0.2
CS2PBN NP	44.65 ± 2.8	1106.87	55.9 ± 0.3
CS-PEG3.5PBN NP	53.26 ± 1.8	1065.25	29.91 ± 0.11
CS-PEG7PBN NP	24 ± 3.3	1680	47.19 ± 0.35

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375 Table V. Encapsulation efficiency and drug loading capacity of PBN-loaded PLGA and
376 PLGA-PEG nanoparticles.

Formulation	Encapsulation Efficiency (%)	Loaded Drug Amount (mg)	Drug Loading Capacity (%)
PLGA NP	-	-	-
PLGA-PEG NP	-	-	-
PLGA10PBN NP	52.3 ± 2.4	5.23	34.36 ± 0.28
PLGA20PBN NP	54 ± 3.1	10.815	68.40 ± 0.29
PLGA-PEG10PBN NP	12.7 ± 1.6	1.27	9.26 ± 0.22
PLGA-PEG20PBN NP	21.21 ± 2.2	4.242	29.24 ± 0.24

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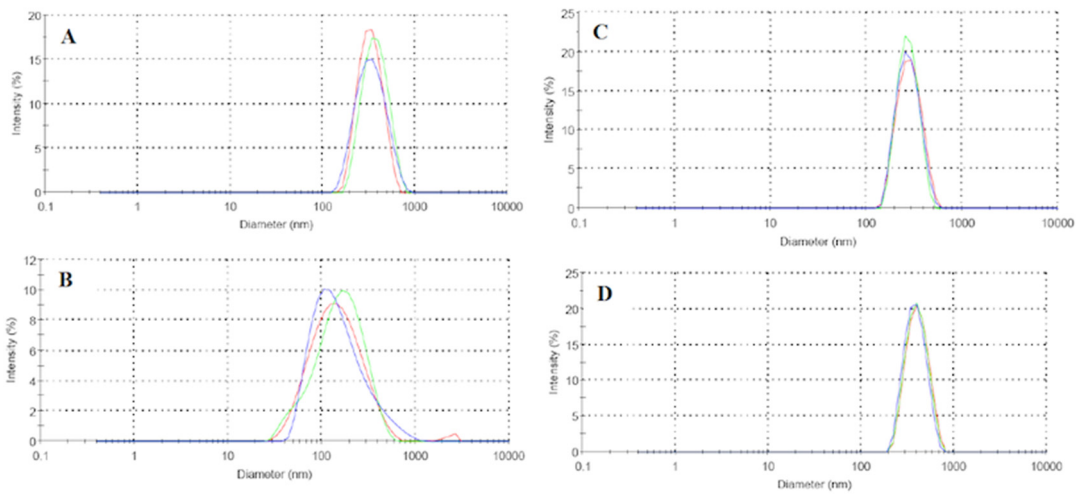

Figure 1. Particle size distribution graphics of (A) CS NP, (B) CS-PEG NP, (C) PLGA NP, (D) PLGA-PEG NPS.

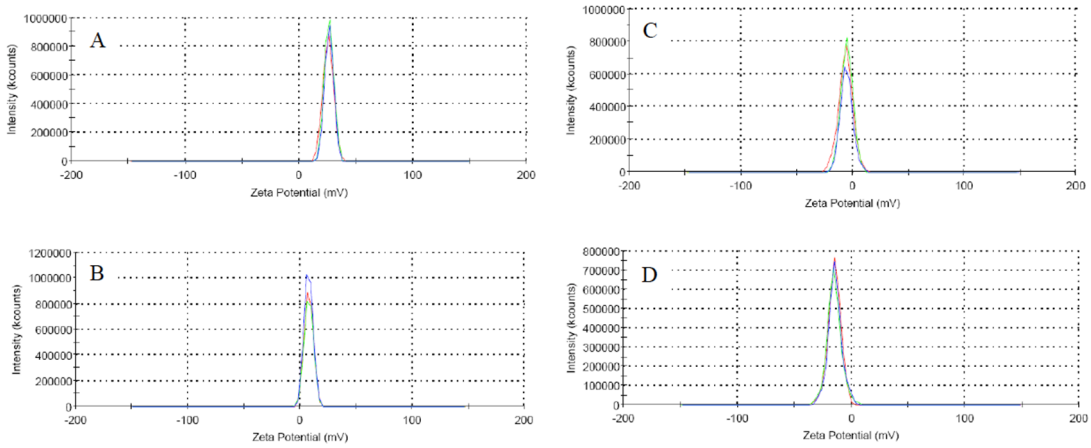

Figure 2. Zeta potential distribution graphics of (A) CS NP, (B) CS-PEG NP, (C) PLGA NP, (D) PLGA-PEG NPS.

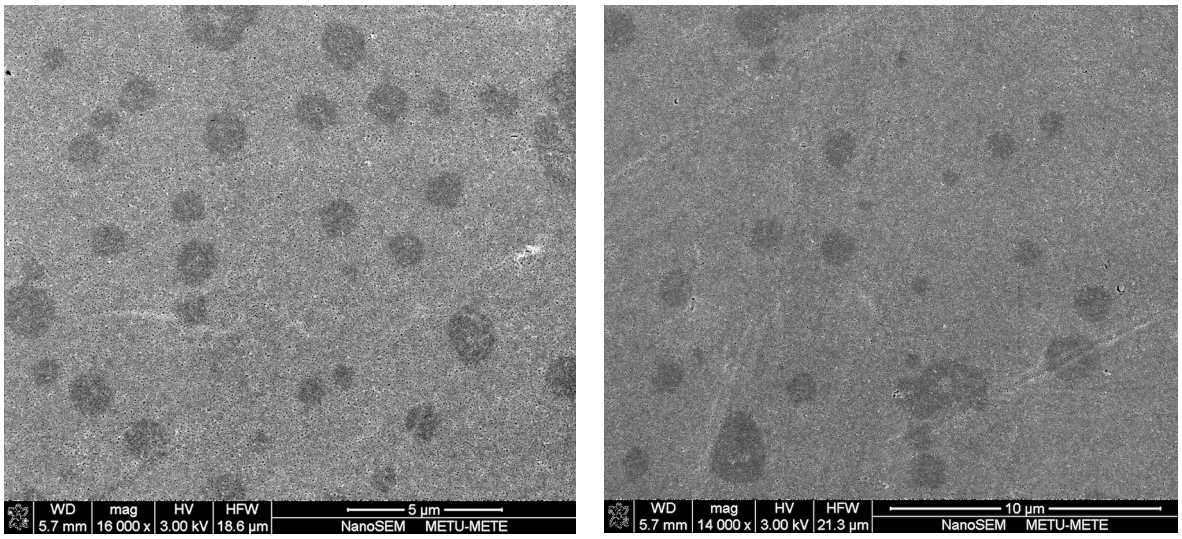

Figure 3. SEM pictures of blank (a) and drug loaded (b) Chitosan nanoparticles.

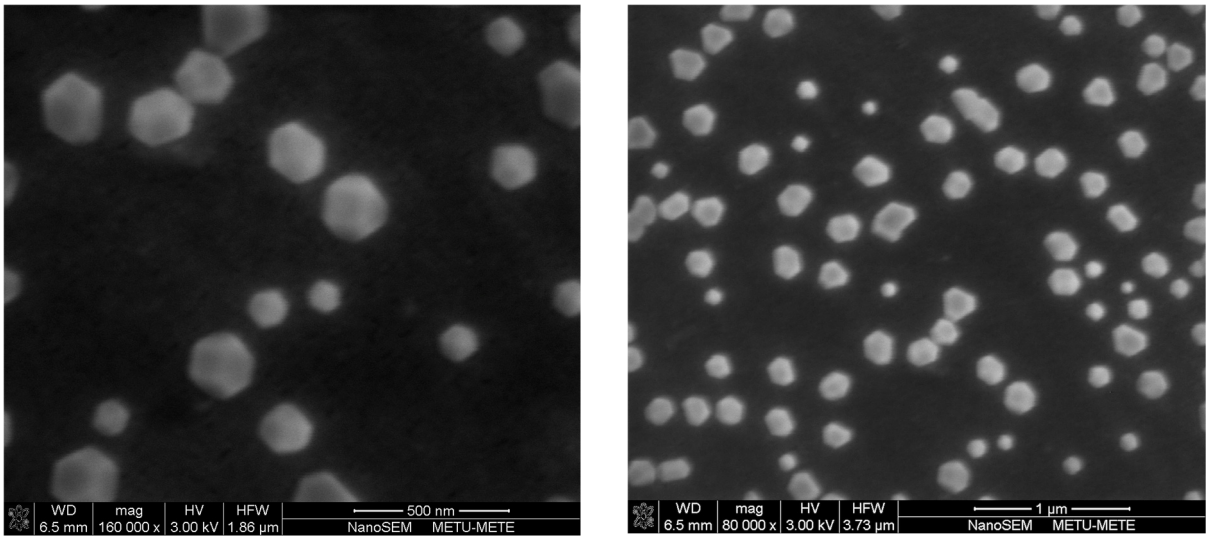

Figure 4. SEM pictures of blank (a) and drug loaded (b) Chitosan-PEG nanoparticles.

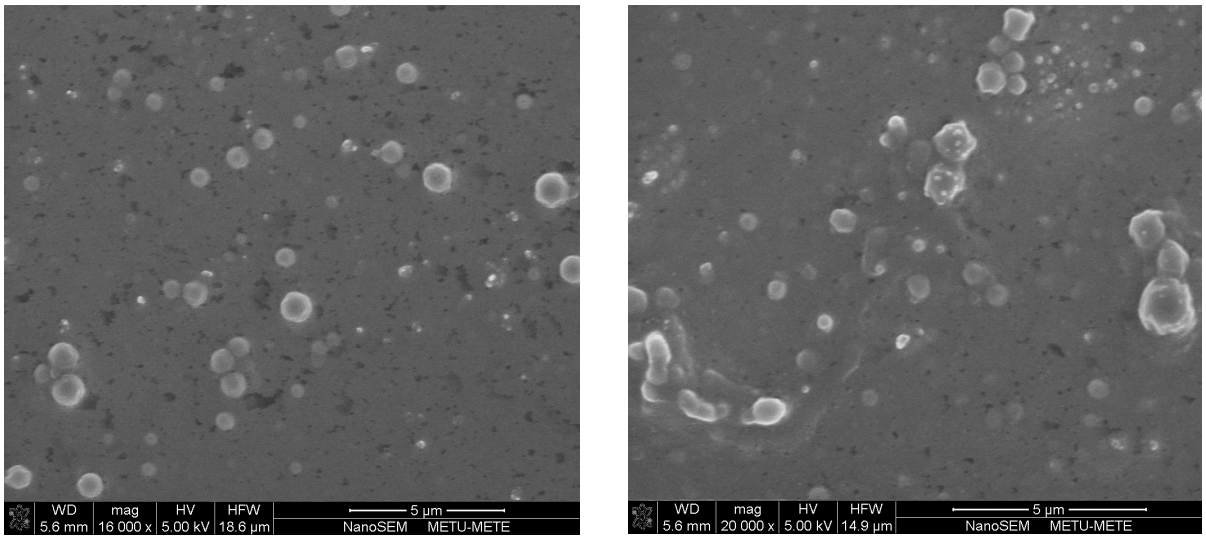

Figure 5. SEM pictures of blank (a) and drug loaded (b) PLGA nanoparticles.

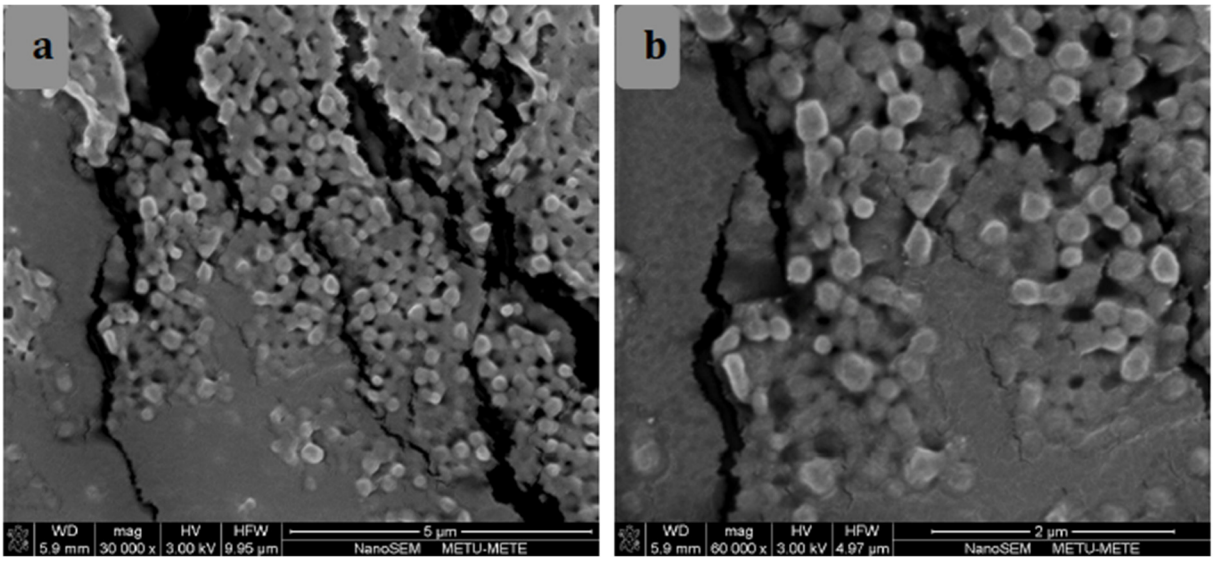

Figure 6. SEM pictures of blank (a) and drug loaded (b) PLGA-PEG nanoparticles.

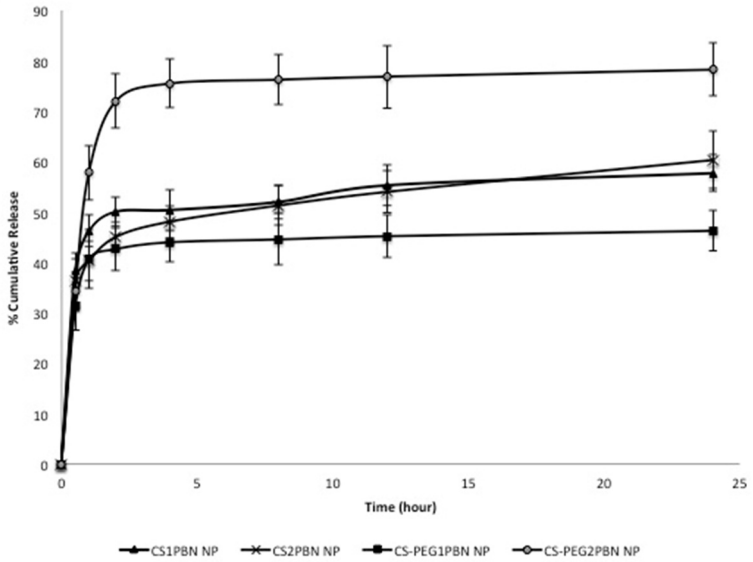

Figure 7. In vitro release profiles of PBN-loaded CS and CS-PEG nanoparticles (n=6).

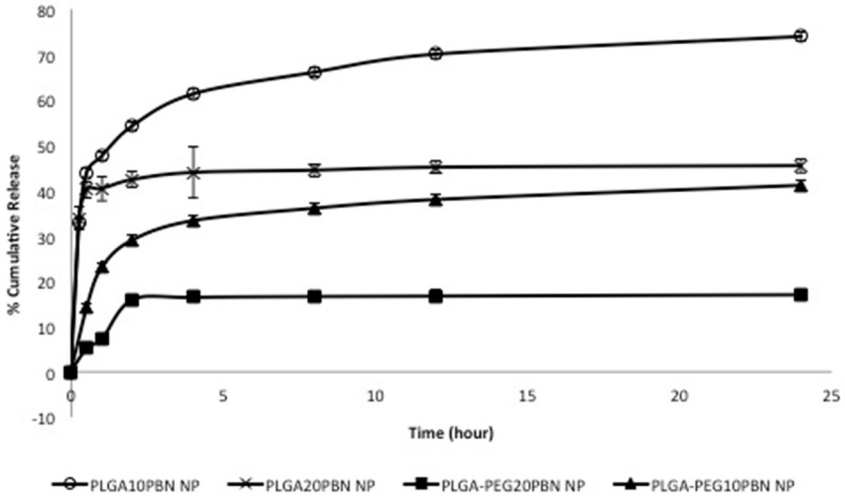

Figure 8. In vitro release profiles of PBN-loaded PLGA and PLGA-PEG nanoparticles (n=6).