Department of Pharmaceutical Technology[1], Faculty of Pharmacy, Hacettepe University, Ankara; Department of Pharmaceutical Technology[2], Faculty of Pharmacy, Ataturk University, Erzurum; Department of Neurology[3], Institute of Neurological Sciences and Psychiatry, Faculty of Medicine, Hacettepe University, Ankara, Turkey; Laboratory of Pharmaceutical Technology and Biopharmacy[4], UMR CNRS 8612, Faculty of Pharmacy, University of Paris-XI, Chatenay-Malabry, France; Department of Pharmaceutical Technology[5], Faculty of Pharmacy, University of Santiago de Compostela; Departamento de Química Orgánica[6], Facultad de Química, and Unidad de RMN de Biomoleculas asociada al CSIC, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

Preparation and evaluation of alpha-phenyl-*n*-tert-butyl nitrone (PBN)-encapsulated chitosan and PEGylated chitosan nanoparticles

O. Pinarbasli[1], Y. Aktas[2], T. Dalkara[3], K. Andrieux[4], M. J. Alonso[5], E. Fernandez-Megia[6], R. Novoa-Carballal[6], R. Riguera[6], P. Couvreur[4], Y. Capan[1]

Received December 19, 2008, accepted December 29, 2008

Yilmaz Capan, Ph. D., Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey
ycapan@hacettepe.edu.tr

Pharmazie 64: 436–439 (2009) *doi: 10.1691/ph.2009.8374*

Alpha-phenyl-*n*-tert-butyl nitrone (PBN) shows its major effect by scavenging free radicals formed in the ischemia and it has the ability to penetrate through the blood brain barrier easily. The *in vivo* stability of PBN is very low and when administered systemically, it has a mean plasma half life of about three hours. Therefore, formulations which are able to prolong the plasma residence time of PBN are of major interest, because oxygen radicals are usually continuously formed under pathological conditions. In this study, PBN, a nitrone compound having neuroprotective properties, was encapsulated in chitosan (CS) and chitosan-poly(ethylene glycol) (CS-PEG) nanoparticles for treatment of diseases such as stroke, in which sustained free radical production is reported. The nanoparticles were characterized through particle size determination, zeta potential, encapsulation efficiency, surface morphology determinations and *in vitro* release studies. The surface morphologies were evaluated by transmission electron microscopy (TEM) and nanoparticles having spherical shapes were characterized. The particle size distribution was between ~97 nm and ~322 nm; and the zeta potentials varied between ~9 mV and ~33 mV. Size of the nanoparticle formulations was important for the release of PBN from nanoparticles. The quantitative determination of PBN has been evaluated by a validated analytical HPLC method. The presented chitosan-based nanotechnology opens new perspectives for testing antioxidant activity *in vivo*.

1. Introduction

There has been considerable interest in the potential therapeutic benefit of nitrone-derived free radical trapping agents as neuroprotective agents (Green et al. 2003). One of the nitrone compounds having neuroprotective properties is the alpha-phenyl-*n*-tert-butyl nitrone (PBN). PBN is a free radical spin-trap, which has been widely used as a pharmacological tool in studies assessing the role of free radical formation in ischemic brain damage. The compound has been shown to reduce infarct size in several studies of transient occlusion of the middle cerebral artery (MCAO) in rats (Nakashima et al. 1999; Schmid-Elsaesser et al. 2000). PBN blocks the production of free radicals and increases the decomposition of free radicals. This property of PBN may be named as radical scavenger effect (Yang et al. 2000). PBN is a molecule that can easily pass the blood brain barrier (BBB) (Dehouck et al. 2002). The bioavailability of nitron compunds is generally high and their half live is of about 3 h. However, the stability of PBN in blood is relatively low and the residence time in blood is also too short (Chan et al. 1997).

On the other hand, 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 (Hirano et al. 1991). Chitosan nanoparticles are prepared by ionotropic gelation due to the simplicity and the lack of toxic solvents in this technique (Xu and Du, 2003). For crosslinking nanoparticles, pentasodium tripolyphosate (TPP) is used and nanoparticles are formed through complexation of the negatively charged groups of TPP and positively charged amino groups of chitosan (Shu and Zhu 2002). However, following intravenous injection, these nanoparticles are cleared rapidly from the blood (within minutes) by elements of the reticuloendothelial system (RES), particularly the hepatic Kupffer cells. On the contrary, chitosan nanoparticles sterically stabilized by poly(ethylene glycol) (PEG) are expected to circulate for a prolonged period of

time into the blood circulation due to reduced recognition by the macrophages of the RES.
Thus, in order to stabilize PBN in the systemic circulation and to increase its blood residence time after intravenous administration, we describe here the encapsulation of this molecule in chitosan (CS) and PEG-coated chitosan nanoparticles (CS-PEG). Release characteristics of PBN from these nanoparticles is also described.

2. Investigations, results and discussion

CS and CS-PEG nanoparticles formed spontaneously after addition of the TPP solution to the CS or CS-PEG solution as observed by TEM (Fig. 1 and Fig. 2).
The physicochemical properties of blank and PBN-containing nanoparticulate formulations were investigated. The CS and CS-PEG nanoparticles had a particle diameter (Z-average) ranging from approximately 280–322 nm and 97–116 nm with a polydispersity index values of 0.221 and 0.123 respectively as seen in Table 1. This low polydispersity index implies a relatively homogeneous distribution of nanoparticles with similar diameters. It is noteworthy that the hydrodynamic diameter of the particles measured by light scattering (PCS) is higher than the size estimated from microscopy (TEM). This may be attributed to the high swelling capacity of the CS nanospheres in water. The nanoparticles studied by TEM were dried samples hence, were shrunken whereas nanoparticles studied by light scattering in aqueous suspension were swollen, which may have led to the observed difference in particle size as determined by light scattering and by TEM. We observed that the size of CS-PEG nanoparticles was smaller than that of CS nanoparticles. This may be explained by the colloid stabilization exerted by PEG.
The zeta potential commonly used to characterize the surface charge of nanoparticles. As shown in Table 1, CS and CS-PEG nanoparticles had a positive zeta potential ranging approximately from 25 to 33 mV and from 9 to 19 mV, respectively. All of the nanoparticles are positively charged, but the charge value of CS nanoparticles are higher than that of the CS-PEG nanoparticles because of the positive charges of the ammonium groups was masked by the presence of PEG groups. The addition of the PBN, a positively charged molecule, resulted in an increase of the zeta potential values compared with blank nanoparticles.
The formulation with the PBN concentration of 400 ng/mL provided the highest loading capacity in both CS and CS-PEG nanoparticles whereas PBN loading did not dramatically modify the size of the obtained particles (Table 2). The results indicate that the association efficiency values decrease as a function of initial concentration of PBN. However the total amount of the PBN encapsulated per weight of the nanoparticles (PBN) loading was related to the PBN concentration. There are several factors which could affect the encapsulation of the PBN in the CS and CS-PEG nanoparticles, such as CS/TPP or CS-PEG/TPP ratio, auxiliary molecules, and interaction between the PBN and CS or TPP (Janes et al. 2001).
Figure 3 shows the release profiles of PBN from the nanoparticles in PBS (pH 7.4). An initial, fast release (15–20% in 60 min) suggests that some PBN was localized on the surface of the nanoparticle. For each nanoparticle type (either CS or CS-PEG), quite similar profiles were obtained, whereas a higher amount was released from the 400 ng/mL formulations (40–50% at 24 h) as compared to the 200 ng/mL formulations (35–44% at 24 h). PBN release was relatively higher from CS-PEG nanoparticles than from CS nanoparticles, as a result of the larger surface area and the more hydrophilic character which accounted for an improved penetration of the aqueous release medium into the polymer matrix of the CS-PEG nanoparticles. The release of PBN from CS and CS-PEG nanoparticles in PBS was likely due to a dissociation mechanism. The residual drug remaining at 24 h may, therefore, account for stable complexes between chitosan and PBN. Thus, the drug was likely associated to the nanoparticles in three different states: at the nanoparticle surface, in the core as a reversible complex with chitosan, or in the core as an irreversible complex with chitosan (Agnihotri and Aminabhavi 2004). In a nut shell, the release rate was determined by the size and by the repartition of the drug inside of the nanoparticles.

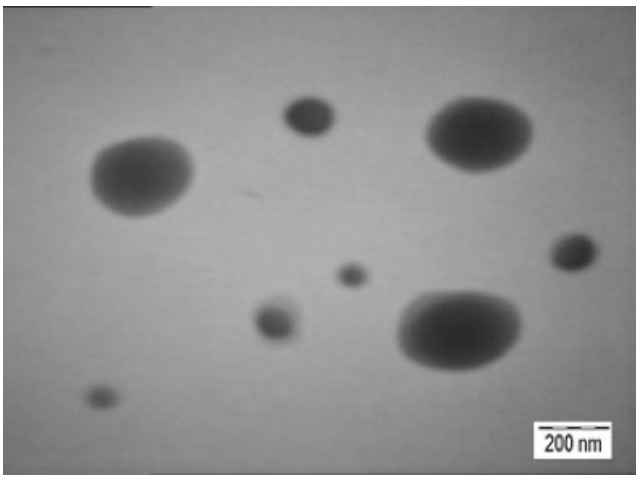

Fig. 1: TEM images of blank CS nanoparticles

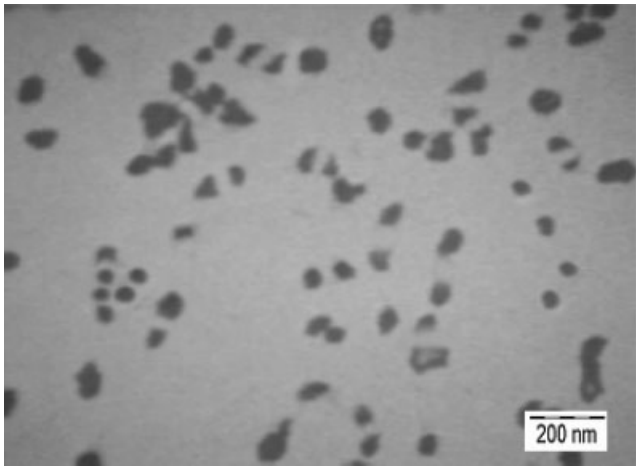

Fig. 2: TEM images of blank CS-PEG nanoparticles

Table 1: Particle diameter, polydispersity index (PDI) and zeta potential values of CS and CS-PEG nanoparticles containing different concentrations of PBN

Formulations-PBN concentration	Particle diameter (nm)	PDI	Zeta potential (mV)
CS (Blank)	322 ± 5.5	0.2 ± 0.019	25.6 ± 1.3
CS-PEG (Blank)	115.9 ± 3.8	0.121 ± 0.047	9.3 ± 0.5
CS-200 ng/mL	300.6 ± 3.5	0.217 ± 0.019	30.3 ± 1.4
CS-400 ng/mL	280.2 ± 3.6	0.218 ± 0.017	32.9 ± 1.0
CS-PEG-200 ng/mL	99.6 ± 5.6	0.184 ± 0.022	14.9 ± 0.7
CS-PEG-400 ng/mL	97.6 ± 1.8	0.223 ± 0.016	18.9 ± 0.6

Table 2: Association efficiency (AE%), loading capacity (LC%) and encapsulated amount of CS and CS-PEG nanoparticles containing different concentrations of PBN

Formulations-PBN concentration	AE%	LC%	Encapsulated amount (ng)
CS (Blank)	–	–	–
CS-PEG (Blank)	–	–	–
CS-200 ng/mL	16.63 ± 3.41	$0.003 \pm 0.68 \times 10^{-3}$	33.26
CS-400 ng/mL	14.22 ± 3.29	$0.006 \pm 1.31 \times 10^{-3}$	56.88
CS-PEG-200 ng/mL	18.8 ± 4.36	$0.004 \pm 0.87 \times 10^{-3}$	37.6
CS-PEG-400 ng/mL	13.46 ± 2.65	$0.005 \pm 1.06 \times 10^{-3}$	53.85

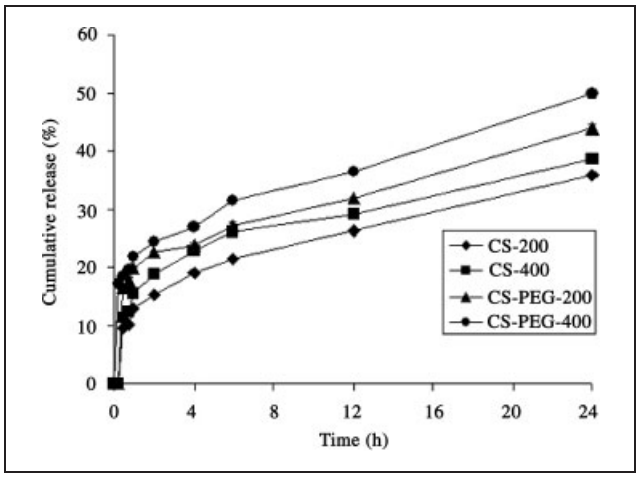

Fig. 3: Release profiles of PBN from the CS and CS-PEG nanoparticles: (◆) CS-200 ng/mL, (■) CS-400 ng/mL, (▲) CS-PEG-200 ng/mL, (●) CS-PEG-400 ng/mL

This study shows the feasability to encapsulate PBN in nanoparticulate formulation, which has never been done before. It opens interesting prospects to increase plasma residence time of PBN by using loaded-chitosan and chitosan-poly(ethylene glycol) nanoparticles.

3. Experimental

3.1. 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. (2005a). 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.

3.2. Preparation of chitosan and chitosan-poly(ethylene glycol) (PEG) nanoparticles

Chitosan (CS) and chitosan-poly(ethylene glycol) (CS-PEG) nanoparticles were prepared according to the ionotropic gelation process (Calvo et al. 1997; Vila et al. 2004; Aktas et al. 2005b). Blank CS nanoparticles were obtained upon the dropwise addition of a TPP aqueous solution (0.4 mg/mL) to a equal volume of CS solution (1.75 mg/mL) stirred at room temperature. Nanoparticle formation resulted from the interaction between the negative groups of TPP and the positively charged amino groups of chitosan. Likewise blank CS-PEG nanoparticles were obtained upon the addition of a 0.4 mL TPP aqueous solution (0.84 mg/mL) to a 1 mL CS-PEG solution (1 mg/mL) stirred at room temperature. In the blank formulations the ratios of the CS/TPP and CS-PEG/TPP were 4.375 and 2.976 respectively. The ratio of CS/TPP and CS-PEG/TPP were established according to previous studies (Aktas et al. 2005a). PBN loaded nanoparticles were obtained according to the same procedure, and the ratio of polymer/TPP remaining unchanged. Increasing amounts of PBN (200 ng/mL, 400 ng/mL) were dissolved in the polymer solution before the addition of the TPP in order to investigate the effect of the PBN concentration on the nanoparticle properties. Nanoparticles were collected by centrifugation at 13.500 rpm on a 10 μL glycerol bed, for 1 h and supernatants were discarded.

3.3. Characterization of the nanoparticles

Morphological examination of the CS and CS-PEG nanoparticles was performed using Transmission electron microscopy (TEM) (TEM; LEO 906E, Sony, Japan). Practically, the samples were resuspended in water, stained with 2% (w/v) uranyl acetate and placed on copper grids to dry for TEM analysis.

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).

3.4. Evaluation of PBN encapsulation

PBN loaded nanoparticles were separated from the aqueous suspension medium by ultracentrifugation at 13.500 rpm, 4 °C for 1 h. The amount of free PBN was measured in the clear supernatant by validated HPLC method (Pinarbasli et al. 2007). A C18 reversed-phase column was used as the stationary phase and methanol : water (55 : 45) as the mobile phase. PBN was detected at a wavelength of 286 nm.

PBN loading capacity (LC) of the nanoparticles and their association efficiency (AE) were calculated according to the following equations:

$$LC\% = 100X \frac{\text{Total PBN amount} - \text{Free PBN amount}}{\text{Nanoparticle weight}} \quad (1)$$

$$AE\% = 100X \frac{\text{Total PBN amount} - \text{Free PBN amount}}{\text{Total PBN amount}} \quad (2)$$

3.5. Determination of PBN release from nanoparticles

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 1600 ng/mL ($y = 0.1840x - 0.1038$, $R^2 = 0.9998$). The limit of detection was 5 ng/mL.

Acknowledgement: We would like to acknowledge Prof. Esra Atabenli for the TEM investigations of the nanoparticle formulations in Ankara University, Faculty of Medicine, Department of Histology and Embryology. This research was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK).

References

Agnihotri SA, Aminabhavi TM (2004) Controlled release of clozapine through chitosan microparticles prepared by a novel method. J Control Release 96: 245–259.

Aktas Y, Yemisci M, Andrieux K, Gursoy RN, Alonso MJ, Fernandez-Megia E, Novoa-Carballal R, Quinoa E, Riguera R, Sargon MF, Celik HH, Demir AS, Hıncal AA, Dalkara T, Capan Y, Couvreur P (2005a) Development and brain delivery of chitosan-PEG nanoparticles functionalized with the monoclonal antibody OX26. Bioconjugate Chem 16: 1503–1511.

Aktas Y, Andrieux K, Alonso MJ, Calvo P, Gursoy RN, Couvreur P, Capan Y (2005b) Preparation and in vitro evaluation of chitosan nanoparticles containing a caspase inhibitor. Int J Pharm 298: 378–383.

Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ (1997) Novel hydrophilic chitosan-poly(ethylene oxide) nanoparticles as protein carriers. J Appl Pol Sci 63: 125–132.

Chan TYY, Privat SJ, Narducy KW, Chaplin MD, Waterbury LD (1997) Pharmacokinetics and Bioavailability of *N*-tert-butyl-alpha-phenyl nitrone in rats and dogs. Proc West Pharmacol Soc 40: 57–59.

Dehouck M, Cecchelli R, Green AR, Renftel M, Lundquist S (2002) In vitro blood-brain barrier permeability and cerebral endothelial cell uptake of the neuroprotective nitrone compound NXY 059 in normoxic, hypoxic and ischemic conditions. Brain Res 955: 229–235.

Green RA, Ashwood T, Odergren T, Jackson, MD (2003) Nitrones as neuroprotective agents in cerebral ischemia, with particular reference to NXY-059. Pharmacol Therap 100: 195–214.

Hirano S, Hirochi K, Hayashi K, Mikami T, Tachibana H (1991) Cosmetic and pharmaceutical use of chitin and chitosan. In: Gebelein CG, Dunn RL (Eds.), Cosmetics and Pharmaceutical Applications of Polymers. Plenum Press, New York, pp. 95–104.

Janes KA, Calvo P, Alonso MJ (2001) Polysaccharide colloidal nanoparticles as delivery systems for macromolecules. Adv Drug Delivery Rev 47: 83–97.

Nakashima M, Niwa M, Iwai T, Uematsu T (1999) Involvement of free radicals in cerebral vascular reperfusion injury evaluated in a transient focal cerebral ischemia model of rat. Free Radic Biol Med 26: 722–729.

Pinarbasli O, Aktas Y, Capan Y (2007) Development and validation of an analytical method for the determination of PBN from chitosan nanoparticles. In: Senel S, Varum KM, Sumnu MM, Hincal AA (Eds.), Advances in Chitin Science, Alp Ofset, Ankara, Vol X, pp. 370–375.

Schmid-Elsaesser R, Hungerhuber E, Zausinger S, Baethmann A, Reulen HJ (2000) Neuroprotective effects of the novel brain-penetrating antioxidant U-101033E and the spin-trapping agent alpha-phenyl-*n*-tert-butyl nitrone (PBN). Exp Brain Res 130: 60–66.

Shu XZ, Zhu KJ (2002) Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure. Int J Pharm 233: 217–225.

Xu Y, Du Y (2003) Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. Int J Pharm 250: 215–226.

Vila A, Sanchez A, Janes K, Behrens I, Kissel T, Vila-Jato JL, Alonso MJ (2004) Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. Eur J Pharm 57: 123–131.

Yang Y, Li Q, Shuaib A (2000) Neuroprotection by 2-h postischemia administration of two free radical scavengers, alpha-phenyl-*n*-tert-butyl-nitrone (PBN) and *N*-tert-butyl-(2-sulfophenyl)-nitrone (S-PBN), in rats subjected to focal embolic cerebral ischemia. Experim Neurol 163: 39–45.