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Preparation and in vitro evaluation of chitosan nanoparticles containing a caspase inhibitor

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Abstract

The aim of this work was to develop a formulation for Z-DEVD-FMK, a peptide which is a caspase inhibitor and has been used in experimental animal studies for a decade. Peptide loaded chitosan nanoparticles were obtained by ionotropic gelation process and Z-DEVD-FMK was quantified by an HPLC method. The influence of the initial peptide concentration on the nanoparticle characteristics and release behavior was evaluated. The CS nanoparticles have a particle diameter (Z-average) ranging from approximately 313–412 nm and a positive zeta potential (20–28 mV). The formulation with the initial peptide concentration of 400 ng/ml provided the highest loading capacity (0.46%) and the highest extent of release (65% at 24 h) suggesting the possibility to achieve a therapeutic dose. According to the data obtained, this chitosan-based nanotechnology opens new and interesting perspectives for anticaspase activity.

Keywords: Chitosan; Nanoparticles; Z-DEVD-FMK; HPLC

1. Introduction

Apoptotic cell death is a prominent phenomenon in neurodegenerative disorder, such as cerebral ischemia. The interleukin converting enzyme (ICE) family of cysteine proteases, now referred to as caspases is a group of apoptosis-regulatory genes that may play a role in ischemic brain injury. Caspase inhibitors attenuate apoptotic cell death during development of ischemia and reduce infarct volume (Thornberrry, 1997; Chen et al., 1998). Z-DEVD-FMK is one of the caspase inhibitors used in the preven-

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tion of apoptotic cell death (Kondratyev and Gale, 2000).

The delivery of hydrophilic drugs to the brain is still a great challenge for the treatment of many brain-related diseases, since hydrophilic drugs cannot cross the blood–brain barrier (BBB). The blood–brain barrier is a very dense biological barrier, and it possesses a unique morphological and physiological characteristic with cerebrovascular endothelial cells, which are tightly connected to each other and supported by glial cells (Ballabh et al., 2004). The BBB represents an insurmountable obstacle for a large number of drugs, including antibiotics, antineoplastic agents, and a variety of central nervous system (CNS)-active drugs, especially neuropeptides. One of the possibilities to overcome this barrier is targeted drug delivery to the brain using nanoparticles (Calvo et al., 2001, 2002; Brigger et al., 2002). Drugs that have successfully been transported into the brain using nanoparticulate drug carrier systems include the hexapeptide dalargin, the dipeptide kytorphin, loperamide, tubocurarine, the NMDA receptor antagonist MRZ 2/576, and doxorubicin (Kreuter, 2001).

Biodegradable nanoparticulate systems have received considerable attention as potential drug delivery vehicles. Chitosan (CS), a polysaccharide known to be a favorable pharmaceutical material because of its biocompatibility and biodegradability, forms an ideal hydrophilic carrier system (Calvo et al., 1997; Mitra et al., 2001; Alonso and Sanchez, 2003). Moreover, chitosan has been shown to be non-toxic and tissue compatible in a range of tests (Aspden et al., 1997). Chitosan nanoparticles are attractive non-viral and cationic carriers for the delivery of peptides, proteins, oligonucleotides, and plasmids (Mao et al., 2001; Janes et al., 2001; Alonso and Sanchez, 2003). They have the capacity to protect sensitive bioactive materials from enzymatic and chemical degradation in vivo and during storage, and to facilitate the transport of charged molecules across the absorptive epithelial cells (Mao et al., 2001).

Chitosan nanoparticles are obtained by the process of ionotropic gelation based on the interaction between the negative groups of the pentasodium tripolyphosphate (TPP) and the positively charged amino groups of CS. This process has been used to prepare CS nanoparticles for the delivery of peptides and proteins (Vila et al., 2004) including insulin (Fernàndez-Urrusuno et al., 1999) and cyclosporine (De Campos et al., 2001).

The aim of this work was to develop a new formulation of the anticaspase peptide, Z-DEVD-FMK based on chitosan nanoparticles for possible targeted delivery to the CNS.

2. Experimental Section

2.1. Materials

The polymer Chitosan Protasan Cl 113 (MW: <150 kD, deacetylation degree: 75–90%) was purchased from FMC Biopolymers (Norway). TPP was supplied by Sigma Chemical Co. (USA). The caspase inhibitor peptide Z-DEVD-FMK, Z-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-FMK (molecular weight is 668 g/mol) was purchased from Enzyme Systems (USA). Ultrapure water was obtained with MilliQ equipment (Waters, USA).

2.2. Preparation of chitosan nanoparticles

CS nanoparticles were prepared according to the ionotropic gelation process (Calvo et al., 1997; Vila et al., 2004). Blank nanoparticles were obtained upon the addition of a TPP aqueous solution (0.4 mg/ml) to a CS solution (1.75 mg/ml) stirred at room temperature. The formation of nanoparticles was a result of the interaction between the negative groups of TPP and the positively charged amino groups of chitosan. The ratio of chitosan/TPP was established according to the preliminary studies. Z-DEVD-FMK loaded nanoparticles were obtained according to the same procedure and the ratio of chitosan/TPP remained unchanged. Variable amounts of peptide were incorporated to the chitosan solution prior to the formation of nanoparticles in order to investigate the effect of the initial peptide concentration on the nanoparticle characteristics and in vitro release profiles. Nanoparticles were collected by centrifugation at 10,000 rpm on a 10 μl glycerol bed, for 1 h and supernatants were discarded.

2.3. Characterization of the nanoparticles

The morphological examination of the chitosan nanoparticles was performed using a transmission electron microscope (TEM); (CM12 Philips, USA). The samples were resuspended in water and stained with

1% (w/v) phosphotungstic acid and placed on copper grids to dry for TEM analysis.

Scanning electron microscopy (SEM) was performed using a LEO 1530 (LEO Electron Microscopy Inc., Thornwood, NY) operating between 1 and 3 kV with a filament current of about 0.5 mA. Liquid samples were deposited on vitreous carbon stubs and dried at room temperature. They were coated with a palladium–platinum layer of about 4 nm using a Cressington sputter-coater 208HR with a rotary-planetary-tilt stage, equipped with a MTM-20 thickness controller.

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 3000HS (Malvern Instruments, UK).

2.4. Evaluation of Z-DEVD-FMK encapsulation

The peptide loaded nanoparticles were separated from the aqueous suspension medium by ultracentrifugation at 10,000 rpm and 4 °C for 1 h. The amount of free peptide was measured in the clear supernatant by a reversed-phase (RP) HPLC method. A C18 RP column was used as the stationary phase and water: acetonitrile (80:20) containing 0.1% trifluoracetic acid (TFA) as the mobile phase. The peptide was detected at a wavelength of 215 nm.

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

$$\%\mathrm{LC} = 100 \times \frac{\text{Total peptide} - \text{Free peptide}}{\text{Nanoparticle weight}}$$

$$\%\mathrm{AE} = 100 \times \frac{\text{Total peptide} - \text{Free peptide}}{\text{Total peptide}}$$

2.5. Evaluation of in vitro Z-DEVD-FMK release

Precipitated nanoparticles (1 mg) were resuspended in 1 ml of phosphate buffered saline solution (PBS) and incubated at 37 °C under light agitation. At appropriate time intervals, individual samples were centrifugated and the amount of the peptide in the release medium was determined by HPLC. The calibration curve obtained from the HPLC method was linear between 25 and 150 ng/ml ($y = 2.6309x - 20.542$, $R^2 = 0.9975$). The limit of detection was 10 ng/ml.

3. Results

3.1. Physicochemical characterization of nanoparticles and peptide association

Spherical nanoparticles were formed spontaneously upon the incorporation of TPP solution to the chitosan solution under magnetic stirring as observed by TEM (Fig. 1a) and SEM (Fig. 1b). The particle diameter (Z-average) ranged from approximately 313–412 nm as seen in Table 1. It is noteworthy that the hydrodynamic

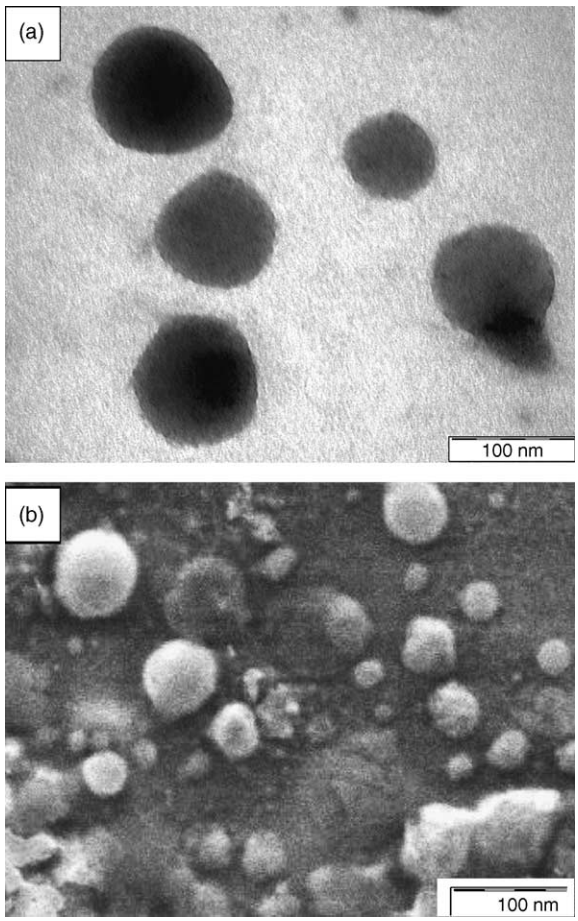

Fig. 1. TEM (a) and SEM (b) photographs of blank chitosan nanoparticles.

Table 1
Particle diameter and zeta potential values of chitosan nanoparticles containing different concentrations of peptide

Initial peptide concentration (ng/ml)	Particle diameter (nm)	Zeta potential (mV)	%AE	%LC
0	339.0 ± 5.7	28.4 ± 0.2	–	–
200	411.9 ± 5.2	23.0 ± 0.6	12.60 ± 0.49	$0.25 \pm 6.38 \times 10^{-5}$
400	312.9 ± 8.7	21.0 ± 0.5	9.26 ± 0.92	$0.46 \pm 26 \times 10^{-5}$
600	329.3 ± 0.3	20.0 ± 0.3	11.33 ± 0.69	$0.35 \pm 27.3 \times 10^{-5}$
800	383.4 ± 4.6	20.3 ± 4.6	7.87 ± 0.77	$0.39 \pm 14 \times 10^{-5}$

diameter of the particles measured by light scattering is higher than the size estimated from microscopy particularly because of the high swelling capacity of chitosan nanoparticles. It seems that there is no linear correlation between the peptide concentration and the size of the nanoparticles. Similar results were observed in the previous studies on chitosan nanoparticles (Fernàndez-Urrusuno et al., 1999). The nanoparticles observed by TEM display lower values of diameter due to drying of the sample suspension (Fig. 1a).

CS nanoparticles have a positive zeta potential ranging from 20 to 28 mV, approximately, depending on the peptide content (Table 1). Being a negatively charged molecule, the addition of the Z-DEVD-FMK resulted in lowering of the zeta potential values in comparison with the blank nanoparticles. For a parenteral administration, a neutralization of the residual positive charge of the CS nanoparticles encapsulating Z-DEVD-FMK should be investigated.

The %AE and %LC could not be correlated with the initial peptide concentration. The formulation with the initial peptide concentration of 400 ng/ml provided the highest loading capacity (0.46%). However, regarding the particle size and %AE, larger particles were observed in higher association cases except with the 800 ng/ml initial peptide concentration where a saturation of the association seems to occur. This may be because of the interaction of the negatively charged peptide and the cationic chitosan.

3.2. In vitro Z-DEVD-FMK release

Fig. 2 displays the release profiles of Z-DEVD-FMK from the nanoparticles in PBS. An initial, fast release (25–45% in 60 min) suggests that some peptide is localized on the surface of the nanoparticles. The highest extent of release (65% at 24 h) was observed for the formulation prepared with the 400 ng/ml initial concentration in comparison with the 30–35% at

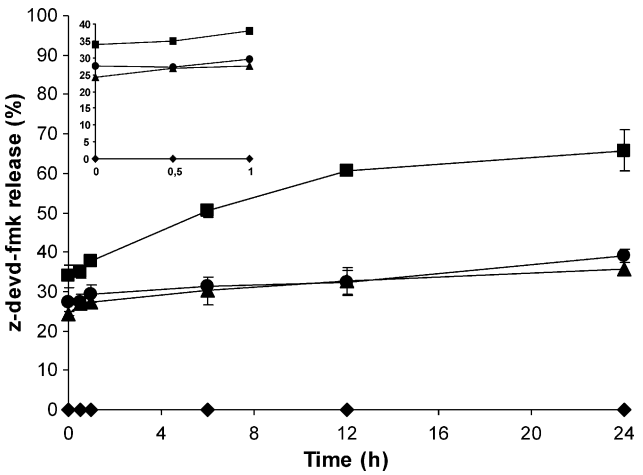

Fig. 2. Release profiles of chitosan nanoparticles containing different concentrations of peptide: (♦) 200 ng/ml, (■) 400 ng/ml, (▲) 600 ng/ml, and (●) 800 ng/ml.

24 h observed for formulations at 600 and 800 ng/ml, which show quite similar profiles. The release of the peptide from CS nanoparticles in PBS may be caused by a dissociation mechanism. The amount of peptide which was not released at 24 h may be attributed to stable complexes formed between chitosan and the peptide. There was no release with the 200 ng/ml sample. This may be explained again by a possible complex formation between chitosan and the entire amount of the peptide loaded. The peptide could be associated to the nanoparticles in three different states: (a) at the nanoparticle surface, (b) in the core as a reversible complex with chitosan, or (c) in the core as an irreversible complex with chitosan. The repartition of the peptide between these three states depends on the initial peptide concentration. Size and repartition of the peptide inside nanoparticles influence the release rate.

It is shown in the literature that the therapeutic dose of the peptide is in the range of 80–480 ng (Ma et al., 2001). Thus, it appears that with 400 ng/ml initial concentration, the therapeutic dose could be achieved by adjusting the quantity of the peptide-loaded nanoparticles administered.

4. Conclusion

Previous studies on chitosan nanoparticles have reported encapsulation of several compounds, their in vitro release profiles and in vivo applications. In this study, we have encapsulated Z-DEVD-FMK which has not been formulated in a drug delivery system yet. Moreover, we have developed an HPLC method for the quantification of the Z-DEVD-FMK. This work can be considered as the first step for further studies on the application of Z-DEVD-FMK loaded-chitosan nanoparticles and a promising step for the possible targeted delivery to the CNS.

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