International Journal of Pharmaceutics 381 (2009) 205–213

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Melatonin-loaded lecithin/chitosan nanoparticles: Physicochemical characterisation and permeability through Caco-2 cell monolayers

Anita Hafner[a,**], Jasmina Lovrić[a], Dario Voinovich[b], Jelena Filipović-Grčić[a,*]

[a] Department of Pharmaceutics, Faculty of Pharmacy & Biochemistry, University of Zagreb, A. Kovacica 1, 10000 Zagreb, Croatia
[b] Department of Pharmaceutical Sciences, University of Trieste, Trieste, Italy

ARTICLE INFO

Article history:
Received 30 November 2008
Received in revised form 30 April 2009
Accepted 1 July 2009
Available online 9 July 2009

Keywords:
Melatonin
Chitosan
Lecithin
Nanoparticles
Transmucosal permeability

ABSTRACT

In this study, the potential of lecithin/chitosan nanoparticles (NPs) as a mucoadhesive colloidal nanosystem for transmucosal delivery of melatonin was investigated. The size, zeta potential and melatonin loading of the lecithin/chitosan NPs were investigated as a function of lecithin type (Lipoid S45, S75 and S100) and chitosan content in the preparation. The NPs were characterised by mean diameter and zeta potential ranging between 121.6 and 347.5 nm, and 7.5 and 32.7 mV, respectively, and increasing with lecithin-negative charge and chitosan content in the preparation. Melatonin loadings were up to 7.1%. All NPs were characterised by prolonged release profiles with an initial burst (approximately 25%), followed by a slow release phase. Approximately 60–70% of melatonin was released in 4 h. The permeability of melatonin was investigated using Caco-2 cells as an in vitro model of the epithelial barrier. Melatonin permeability from an NP suspension prepared with Lipoid S45 lecithin and a lecithin-to-chitosan weight ratio (L/C) of 20:1 (sample C2) was significantly improved compared to the permeability of melatonin from the solution ($P < 0.001$) and from all other NPs investigated ($P < 0.05$). The results obtained by the cell viability studies (MTT and LDH leakage assays) showed that C2 NP suspension did not induce plasma membrane damage or decrease cell viability and could be safely applied to Caco-2 cells in the concentration range tested (<400 μg/ml).

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Melatonin is an indole amide neurohormone synthesised mainly in the pineal gland and secreted into the blood in a circadian rhythm. It has been shown to play a critical role in the body's internal timekeeping system, which regulates the sleep–wake cycle along with other circadian and seasonal rhythms. Melatonin is also known for its immunostimulative and cytoprotective activity (Mao et al., 2004). Exogenous melatonin has been used in the treatment of various circadian rhythm disorders such as jetlag and insomnia. Due to its antioxidative properties, the potential clinical benefit of melatonin can be expected in the treatment of various cancers and neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Schaffazick et al., 2008). Although melatonin is a compound that is easily absorbed across the mucosa, its sensitivity to oxidation presents a significant problem for achieving a therapeutic dose. Moreover, its low oral bioavailability, due to variable absorption and extensive first-pass metabolism, indicates the need for new routes of administration and an appropriate delivery system to be developed.

Nasal drug delivery, as an alternative route for the administration of systemically acting drugs that have low oral bioavailability, is advantageous due to rapid drug onset and potential for central nervous system delivery (Costantino et al., 2007; van den Berg et al., 2004). This delivery method, however, is generally restricted to very potent drugs or drugs characterised by rather high water solubility (Ekelund et al., 2005). Another drawback for the nasal administration of drugs is mucociliary clearance, which limits the time allowed for drug absorption to occur. Appropriate delivery systems, however, can be developed to overcome the problems related to both poor water solubility and nasal clearance mechanism.

An enhanced bioavailability of melatonin was observed in rabbits after nasal administration of melatonin solution formulated with 40% PEG with or without 1% sodium glycocolate (94% and 55%, respectively) (Bechgaard et al., 1999). Moreover, an 84% absolute melatonin bioavailability from starch microspheres (prepared by an emulsification cross-linking technique) after nasal administration to rabbits was observed (Mao et al., 2004). In addition, nasal clearance studies have shown that more than 80% of the starch microspheres were present in the nasal mucosa 2 h after administration compared to only 30% of starch from the solution. This

* Corresponding author. Tel.: +385 1 63 94 761; fax: +385 1 46 12 691.
** Corresponding author. Tel.: +385 1 63 94 765; fax: +385 1 46 12 691.
E-mail addresses: ahafner@pharma.hr (A. Hafner), jfilipov@pharma.hr (J. Filipović-Grčić).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.07.001

suggests the possibility of making a clinically relevant nasal melatonin formulation.

Polymeric nanoparticles are being widely investigated as drug delivery systems due to their numerous advantages, such as their ability to increase bioavailability, deliver poorly water-soluble compounds and protect mucosa from the toxic effects of drugs. Melatonin-loaded polysorbate 80-coated Eudragit S100-nanoparticles provided an increase in the in vitro effect of melatonin against lipid peroxidation in comparison to the drug in aqueous solution (Schaffazick et al., 2005). Later, it was shown that a single administration of 10 mg/kg melatonin-loaded nanocapsules showed significant antioxidant activity against lipid peroxidation in the mouse brain and liver, while the aqueous solution with the same dose was ineffective (Schaffazick et al., 2008). This supports that polymeric nanoparticles can be a promising technological platform for melatonin delivery.

The primary scope of this study was to develop a colloidal melatonin nanocarrier with sufficient melatonin entrapment and prominent mucoadhesive properties. To achieve these goals, lecithin and chitosan were chosen as constitutive materials.

Chitosan is a biocompatible and biodegradable polycationic polymer. Being a weak base with pK_a of 6.5, it is positively charged at a pH of the nasal mucosa, which is reported to be within the range of 5.5–6.5 (England et al., 1999). Due to the positive charges, chitosan can electrostatically interact with mucus or negatively charged mucosal surfaces. This is to provide a longer contact time for drug transport across the nasal membrane before the substance can be cleared by the mucociliary clearance mechanism (Singla and Chawla, 2001).

Lecithin is a natural lipid mixture of phospholipids that has been frequently used for the preparation of various delivery nanosystems such as micro and nanoemulsions (Kelmann et al., 2007), liposomes (Pavelić et al., 2005), micelles (Ceraulo et al., 2008) and solid lipid nanoparticles (Schubert et al., 2006).

Melatonin encapsulation efficiency, the size and morphology, the zeta potential, bioadhesive and in vitro drug release properties of the NPs were studied as a function of chitosan content and type of lecithin used for their preparation. Lecithin/chitosan NPs were also evaluated in terms of their influence on melatonin mucosal permeability using the Caco-2 cell monolayer.

2. Materials and methods

2.1. Reagents and chemicals

The following materials were used as received: chitosan in the form of hydrochloride salt (Protasan® UP CL 113, deacetylation degree 86%, Novamatrix, Norway); soybean lecithins (commercial mixtures of lipids, phospholipids, and fatty acids; a kind gift from Lipoid GmbH, Germany), Lipoid S45 (fat-free soybean lecithin with 45% phosphatidyl choline), Lipoid S75 (fat-free soybean lecithin with 70% phosphatidyl choline) and Lipoid S100 (phosphatidyl choline from fat-free soybean lecithin); melatonin (Sigma, USA); mucin (type III: partially purified, from porcine stomach, Sigma) and basic fuchsin (Sigma). All other chemicals or solvents used were of analytical grade and purchased from Kemika (Croatia).

2.2. Preparation of lecithin/chitosan NPs

Lecithin/chitosan NPs were prepared according to the method previously reported by Sonvico et al. (2006). Lecithin (S45, S75 or S100) was dissolved in 96% ethanol at a concentration of 2.5% (w/v). Melatonin was dissolved in the ethanolic solution of lecithin at a concentration of 0.5% (w/v), obtaining a lecithin-to-melatonin weight ratio of 5:1. Chitosan was solubilised in distilled water at a concentration of 1% (w/v). Melatonin-loaded NP suspensions were obtained by injection of 4 ml ethanolic lecithin/melatonin solution (syringe inner diameter of 0.75 mm) into 46 ml of water-diluted chitosan solutions that were magnetically stirred (900 rpm). Appropriate volumes of 1% chitosan solution were diluted with distilled water prior to ethanolic lecithin/melatonin solution injection in order to obtain L/C ratios of 20:1, 10:1 and 5:1 in the prepared NP suspensions.

For the comparison, melatonin-loaded S45, S75 and S100 lecithin NPs were prepared by injection of ethanolic melatonin/lecithin solutions into the distilled water.

Melatonin-free (empty) NPs were prepared following the same procedure as for melatonin-loaded NPs.

2.3. Determination of melatonin loading

Encapsulation (entrapment) efficiency (EE%) was measured by determining the amount of non-entrapped melatonin by using the dialysis technique (Maestrelli et al., 2005). For separating non-entrapped melatonin from NPs, an appropriate volume (4 ml) of melatonin-loaded NP suspension was placed into a dialysis bag of cellulose acetate (Spectra/Por® membranes, MW cut off 12–14000), which was immersed into 100 ml of distilled water and magnetically stirred at 30 rpm. Experiments were performed under sink conditions (melatonin water solubility 0.1 mg/ml). At scheduled time intervals, the samples (2 ml) were withdrawn from the receiver solution and replaced with the fresh water. Samples were diluted with acetonitrile to obtain a water-to-acetonitrile volume ratio of 45:55 and then analysed for melatonin content using HPLC. The experiment was stopped when constant drug concentration values were detected in subsequent withdrawals from the receiver phase, taking into account the progressive dilution of the medium.

Entrapment efficiency was then calculated according to the following equation:

$$\mathrm{EE\%} = \frac{[\text{total drug}] - [\text{diffused drug}]}{[\text{total drug}]} \times 100$$

Drug loading (DL%) was calculated according to the following equation:

$$\mathrm{DL\%} = \frac{[\text{total drug}] - [\text{diffused drug}]}{\text{examined quantity of NPs}} \times 100$$

The dialysed NP suspension samples (without non-entrapped melatonin) were used in all further studies.

2.4. Physical characterisation of the particle size, surface charge and shape

The average particle size and size distribution (polydispersity index; PDI) was determined by photon correlation spectroscopy (Zetasizer 3000 HS, Malvern Instruments, Malvern, UK). Measurements were performed at a scattering angle of 90° and at a temperature of 25 °C. Samples were subjected to measurement following the dilution with 0.45 μm filtered distilled water in order to avoid multiple scattering. The hydrodynamic diameter was calculated from the autocorrelation function of the intensity of light scattered from particles with the assumption that the particles had a spherical form. The zeta potential was obtained by laser Doppler anemometry using a Zetasizer 3000 HS. For the measurements, samples were placed in an electrophoretic cell, where a potential of 150 mV was established. The NP suspension samples were subjected to measurement following the dilution with a 10 mM NaCl solution.

The morphology of the NPs was observed by transmission electron microscopy (TEM) using a PHILIPS EM 208 at an accelerating voltage of 100 kV.

2.5. Stability studies

Samples of NP suspensions were stored at 4 °C for a period of two months. Aliquots were withdrawn at scheduled time points (10, 30 and 60 days) and analysed in terms of average diameter and zeta potential of the NPs. For the determination of melatonin retention, aliquots (1.5 ml) of all samples were dialysed against distilled water (30 ml; 30 min) and the concentrations of free melatonin (representing leakage) were determined using HPLC.

2.6. Mucus glycoprotein assay and adsorption of mucin on lecithin/chitosan NPs

To study the adsorption of mucin on the lecithin/chitosan NPs, the procedure described by Filipović-Grčić et al. (2001) was applied. Briefly, NP suspensions were ultracentrifuged at 120,000 × *g* for 90 min in order to separate NPs from chitosan in free-soluble form. The NPs were then resuspended in distilled water. Equal volumes of NP suspensions and aqueous solutions of mucin (0.5 mg/ml), pH 6.0, were vortexed and shaken at 20 °C for 60 min. The suspensions were then centrifuged at 60,000 × *g* for 30 min, and the supernatant was used for the determination of the free mucin content. All assays were performed in triplicate.

A colorimetric assay for glycoproteins based on the periodic acid/Schiff staining (He et al., 1998) was used for the determination of mucin concentration. The mucin adsorbed on the surface of the lecithin/chitosan NPs was calculated from the total and free mucin.

2.7. In vitro release studies

The release of melatonin from lecithin/chitosan NPs was assessed using a dialysis technique under sink conditions over a 24-h period (Mao et al., 2004). The rate of melatonin diffusion to receptor medium is related to its concentration in the receptor medium as well as to its release from NPs, enabling the comparison of the release behaviour of systems investigated. The appropriate volume of melatonin-loaded NP suspension containing 180 μg of melatonin was placed into a dialysis bag of cellulose acetate (Spectra/Por® membranes, MW cut off 12–14000), which was immersed into 30 ml of acetate buffer solution, pH 5.6, at 37 °C and magnetically stirred at 30 rpm. At scheduled time intervals, the agitation was stopped and samples (1 ml) were withdrawn and replaced with the fresh buffer solution. Samples were diluted with acetonitrile to obtain a water-to-acetonitrile volume ratio of 45:55 and analysed for melatonin content using HPLC. All experiments were carried out in triplicate.

2.8. Cell culture conditions

Caco-2 cells (American Type Culture Collection [ATCC], Rockville, MD, USA) were cultured in MEM medium with Hank's Balanced Salt Solution (HBSS), L-glutamine, sodium bicarbonate and neomycin (Institute of Immunology Inc., Zagreb, Croatia) supplemented with 10% foetal bovine serum (GIBCO, Invitrogen, Paisley, UK). Cells were passaged at 80–90% confluence using a 0.25% trypsin/0.20% ethylene diamine tetraacetic acid (EDTA) solution. The medium was changed approximately every 48 h. Cultures were maintained at 95% humidity and 37 °C in an atmosphere of 5% CO_2.

2.9. Cell viability study

To assess the potential cytotoxicity of investigated NP suspensions, Caco-2 cells were seeded onto 24-well plates (Sarstedt, Newton, NC, USA) at a density of 10^5 cells/cm^2 and allowed to reach confluence in 6–8 days. The NP stock suspension was mixed with HBSS (pH 6.0), reaching a final NP concentration of 50, 200 or 400 μg/ml. Prior to the treatment with NP suspensions, the cell culture medium was withdrawn and replaced with HBSS (pH 6.0) with or without NPs. Cells were treated with NPs for 2 h, after which in vitro cell viability was determined by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma) and a lactate dehydrogenase (LDH) leakage assay. Two sets of controls were used in these experiments: cells incubated in the cell culture medium to account for the loss of viability due to incubation in HBSS and untreated cells incubated in HBSS.

2.9.1. LDH leakage assay

Lactate dehydrogenase (LDH) leakage assays were performed to assess the integrity of the plasma membrane following treatment with NPs. Normally found in the cytosol, LDH is a stable enzyme that is rapidly released into the medium upon damage of the plasma membrane. After a 2-h treatment with NPs, the activity of the enzyme present in the medium was determined using an LDH assay kit (Herbos Dijagnostika d.o.o., Croatia). This kit assesses activity based on the enzymatic reaction in which lactate is oxidised in pyruvate and nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH. The increase in NADH concentration was monitored by continuous absorbance measuring at 340 nm using a semi-automated biochemical analyser (Trace Scientific, Ltd.).

The activity of LDH was calculated according to the following equation:

$$A\,(\mathrm{U/l}) = \frac{\Delta A}{\min} \times 9683$$

Cells exposed to 1% Triton X-100 (100% lysis) and non-treated cells incubated in HBSS were used as positive and negative controls, respectively. In preliminary experiments, it was determined that incubation of cells in HBSS in the time period of the experiment did not induce LDH enzyme release. Cell viability was expressed according to the following equation:

$$\text{Cell viability}\,(\%) = \frac{A_{\text{positive control}} - A_{\text{sample}}}{A_{\text{positive control}} - A_{\text{negative control}}} \times 100$$

2.9.2. MTT assay

Colorimetric MTT assays were performed to assess the activity of living cells via mitochondrial dehydrogenases. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals that are insoluble in aqueous solutions. After a 2-h treatment with NPs, HBSS-containing NPs were removed. The cells were then washed twice with PBS and incubated in fresh medium (500 ml/well) for 24 h. A total of 50 ml of MTT stock solution (5 mg/ml) was added to each well. The cells were then incubated for 1 h at 37 °C. The medium was subsequently removed, the cells were lysed and the formazan was dissolved with DMSO. The amount of formazan was quantified spectrophotometrically at 595 nm (Ultrospec Plus, Pharmacia LKB, Cambridge, UK). Mitochondrial activity was expressed relative to a control group treated with distilled water.

2.10. Caco-2 cell monolayer permeability assay

Caco-2 cells were seeded onto the polycarbonate 6-well Transwell® inserts (3.0-μm mean pore size, 4.67 cm^2 surface area, Corning Costar Inc., NY, USA) at a density of 1×10^5 cells/well and the confluent monolayers (20–22 days) were used for permeability studies. Cells were cultured as mentioned earlier with frequent medium changes (every other day for the first 10 days and every day thereafter). The transepithelial electrical resistance (TEER) of the monolayer was measured using an epithelial voltohmmeter (EVOM,

WPI Inc., USA) to determine the formation of the monolayer and its integrity during the experiment. Permeability studies were carried out in HBSS buffered with 30 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 6.0. Test samples were prepared by diluting melatonin solution (500 μM) and melatonin-loaded NP suspension with HBSS up to a 50 μM melatonin concentration. Prior to the experiment, the monolayers were washed with HBSS, after which HBSS was placed into the upper and lower compartments. The cells were then incubated for 1 h. At the start of the experiment, the upper compartment was emptied and 1 ml of the test sample was added. Samples (100 μL) were taken from the lower compartment at regular time intervals over 180 min and replaced with the same volume of fresh buffer. At the end of the experiment, the 100 μL of the solution remaining in the upper compartment was also collected. All experiments were conducted in triplicate at pH 6.0, 37 °C, 5% CO_2 and 95% relative humidity.

Samples were diluted with acetonitrile to obtain a water-to-acetonitrile volume ratio of 45:55 and then analysed for melatonin content using HPLC. Apparent permeability coefficients (P_{app}) were calculated according to the following equation:

$$P_{app} = \frac{\partial Q}{\partial t} \times \frac{1}{AC_0}$$

where ($\partial Q/\partial t$) is the permeability rate, A is the diffusion area of the monolayer and C_0 is the initial concentration of melatonin in the upper compartment (Sadeghi et al., 2008).

2.11. HPLC assay of melatonin

The quantitative determination of melatonin was performed using HPLC. The system consisted of an SCL-10Avp system controller, LC-10Advp pump, DGU-14A degasser, SPD-10AVvp UV–VIS detector, CTO-10Avp column oven (Shimadzu, Japan) and a Pathfinder® C18 AP column (4.6 mm × 150 mm; Shimadzu). The mobile phase consisted of acetonitrile/water (55:45, v/v) (Schaffazick et al., 2005). The flow rate was 1 ml/min and the oven temperature was maintained at 40 °C with an injection volume 20 μL. The column eluent was monitored at 229 nm. Melatonin peaks were separated with a retention time of 2 min.

2.12. Statistical analysis

Statistical data analyses were performed on all data using a one-way ANOVA followed by a multi-parametric Tukey's post hoc test with $P < 0.05$ set as the minimal level of significance. Calculations were performed with the GraphPad Prism program (GraphPad Software, Inc., San Diego, USA; www.graphpad.com).

3. Results and discussion

3.1. Characterisation of NPs

The composition and main characteristics of the NPs prepared are given in Table 1. Nine samples of melatonin-loaded lecithin/chitosan NP suspensions were prepared by injection of ethanolic lecithin/melatonin solution into an aqueous chitosan hydrochloride solution, varying the type of lecithin (S100, S75, and S45) and lecithin-to-chitosan weight ratio employed (20:1, 10:1 and 5:1). Resultant pH values of prepared NP suspensions were between 4.5 and 5.0. In all preparations, the melatonin-to-lecithin ratio was kept constant (5:1, w/w). Three types of lecithin used for the preparation of the NPs differed in the content of lipids that contributed to the negative charge of lecithin, ascending from lecithin S100 to lecithin S45 and giving rise to the interaction with positively charged chitosan. The overall negative charge of Lipoid S45 lecithin

Table 1
Influence of lecithin type (Lipoid S100, S75 and S45) and lecithin-to-chitosan ratio (L/C) on main characteristics of melatonin-loaded lecithin/chitosan NPs.

L/C (w/w)	Type of lecithin														
	Lipoid S100					Lipoid S75					Lipoid S45				
	NP	Drug loading (%)[a]	Size (nm)	PDI	Zeta potential (mV)	NP	Drug loading (%)[a]	Size (nm)	PDI	Zeta potential (mV)	NP	Drug loading (%)[a]	Size (nm)	PDI	Zeta potential (mV)
1/0	A1	3.9 ± 0.1	80.8 ± 1.9	0.25	−12.4 ± 2.8	B1	4.1 ± 0.1	62.7 ± 2.6	0.42	−26.6 ± 3.9	C1	4.9 ± 0.3	97.0 ± 1.6	0.29	−33.3 ± 1.5
20/1	A2	4.9 ± 0.1	125.6 ± 0.6	0.34	7.5 ± 1.2	B2	5.0 ± 0.2	234.5 ± 5.9	0.46	18.1 ± 0.6	C2	6.4 ± 0.3	246.2 ± 8.1	0.20	24.5 ± 0.6
10/1	A3	5.0 ± 0.1	121.6 ± 2.8	0.46	16.4 ± 0.8	B3	5.3 ± 0.2	158.4 ± 10.1	0.48	20.5 ± 0.7	C3	7.1 ± 0.2	329.1 ± 10.5	0.29	28.4 ± 1.4
5/1	A4	5.2 ± 0.2	143.3 ± 3.3	0.45	20 ± 0.9	B4	5.3 ± 0.1	239.7 ± 5.3	0.58	29.9 ± 3.5	C4	7.1 ± 0.1	347.5 ± 3.2	0.49	32.7 ± 0.8

PDI = Polydispersity index.
Values are mean ± SD ($n = 3$). *Do not differ from corresponding lecithin NPs ($P > 0.05$). The shaded portion in the table presented the samples of NPs that were selected (included) in studies of mucoadhesivity, in vitro release, cell viability and permeability.
[a] Drug loading % = ([total drug] − [diffused drug])/examined quantity of NPs × 100.

and the formation of chitosan/lecithin NPs due to electrostatic interaction between the negatively charged fraction of the lipid material and the positively charged chitosan have already been described (Sonvico et al., 2006; Gerelli et al., 2008).

The NPs were separated from unentrapped melatonin using the dialysis method. The suitability of the dialysis technique for EE% determination has been demonstrated. The results obtained did not show significant differences in comparison with those obtained by the ultracentrifugation technique (López-Pinto et al., 2005; Fočo et al., 2005).

The main NP characteristics, size and surface charge, were determined using dialysed systems. Following dilution with water or 10 mM NaCl prior to size and surface charge measurements, pH values in the dialysed systems increased to 5.8–6.0. As pH of the nasal mucosa was reported to be within the range of 5.5–6.5 (England et al., 1999), it may be expected that the size and surface charge of NPs in the dialysed systems would be preserved even after contacting nasal mucosa.

The NP suspensions preserved their macroscopic homogeneous aspects after dialysis. The TEM images indicated that melatonin-loaded lecithin/chitosan NPs were roughly spherical and subspherical in shape, separated from each other, suggesting possible stabilisation of the nanosuspension by NP positive surface charges. Fig. 1 is a representative TEM image from sample C3.

The size and the surface charge of the NPs increased with the increase in both the chitosan content and the negative charge of lecithin used in the preparation (Table 1).

Lecithin/chitosan NPs obtained were characterised by mean diameters ranging between 121.6 ± 2.8 and 347.5 ± 3.2 nm, and were significantly larger ($P < 0.001$) than comparable lecithin NPs (samples A1, B1 and C1).

The negative surface charge of lecithin NPs ascended from lecithin S100 (−12.4 mV; sample A1) to lecithin S45 NPs (−33.3 mV; sample C1). The zeta potential was inverted from negative values for lecithin NPs to positive values for lecithin/chitosan NPs, especially for NPs prepared with lecithins S75 and S45. These were characterised by a zeta potential between 18.1 and 32.7 mV. This observation is of great importance, since positively charged NPs can interact with negatively charged mucins, exhibiting bioadhesive properties.

Compared to lecithin NPs, significantly higher melatonin loadings were obtained within lecithin/chitosan NPs ($P < 0.05$). The EE% and the drug loadings of lecithin/chitosan NPs ranged from 26.0 to 38.2% and from 4.9 to 7.1%, respectively. The best melatonin loadings were obtained with nanosystems composed of lecithin S45 (samples C1–C4).

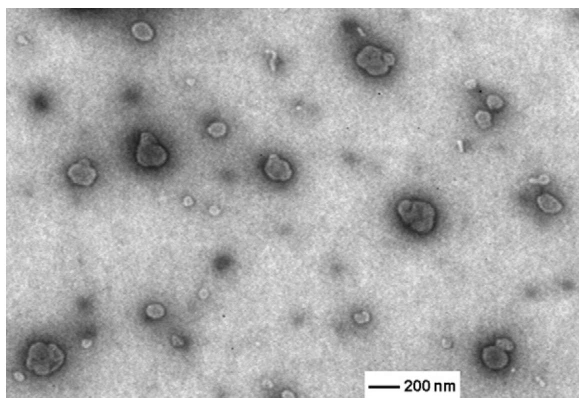

Fig. 1. Representative transmission electron microscope (TEM) image of melatonin-loaded lecithin/chitosan NPs prepared with S45 lecithin and an L/C weight ratio of 20:1 (C3). Size bar = 200 nm.

Nanoparticles prepared with lecithin S45 (samples C1–C4) and NPs prepared with lecithin S100 and S75, with a lecithin-to-chitosan weight ratio of 20:1 (samples A2 and B2, respectively), were included in further studies.

3.2. Mucoadhesive properties of lecithin/chitosan NPs

Mucaodhesive properties of lecithin/chitosan NPs were determined by the content of mucin adsorbed onto NPs suspended in mucin aqueous solution at pH 6.0 (Fig. 2). The amount of mucin adsorbed ranged between 0.02 and 0.13 mg. The results showed that mucin adsorption actually depended on the surface charge of the NPs. Mucin adsorption, like the zeta potential of the NPs, increased with the increase of the chitosan content in the preparation and with the negative charge of lecithin used for the NP preparation. The clear correlation between mucoadhesion, zeta potential and the synergistic effects of the size and zeta potential on the mucoadhesive properties of the chitosan-based delivery systems has already been reported (He et al., 1998; Martinac et al., 2005; Zhang et al., 2008). The influence of size on mucoadhesion behaviour is evident in the cases of the C2 and C3 NPs. Although being characterised by a lower zeta potential, C2 NPs showed similar mucoadhesive behaviour to C3 NPs (Fig. 2), probably due to their smaller mean diameter and higher total surface area available for the interaction with the mucin.

The amount of mucin adsorbed on NPs allows for the prediction of the relative amount of chitosan in its free-soluble form in the NP suspensions. The adsorbed mucin is related to the total surface area and surface charge of the NPs. The surface charge of the NPs is related to the amount of NP-constituent chitosan. Thus, taking into account the amount of mucin adsorbed (Fig. 2) and the total chitosan content in the suspensions, it may be concluded that the amount of chitosan in free-soluble form increased in the following order: C2 < B2 < A2 < C3 < C4.

3.3. Stability studies

The stability of the lecithin/chitosan NPs was determined by monitoring the changes of drug loading, size, polydispersity and zeta potential of NPs over a two-month period at 4 °C. All NPs were characterised by good melatonin retention properties, as melatonin leakage in two months was, in all cases, between 5 and 10% of the melatonin entrapped (Fig. 3). Results obtained from size and zeta potential measurements indicated that all nanosystems were stable over a one-month period. In two months, however, significant changes in either size, zeta potential or polydispersity were observed in the case of all NPs with the exception of C2 and C3 NPs (data not shown). These results indicate that the most stabile melatonin-loaded NPs were obtained when lecithin S45 with

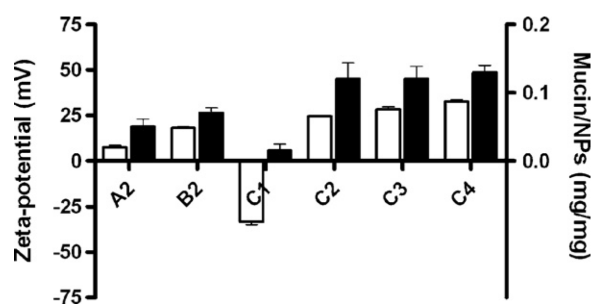

Fig. 2. Influence of lecithin type and chitosan content on zeta potential (□) and mucoadhesive properties (■) of lecithin/chitosan NPs at pH 6.0. Mucoadhesive properties are presented by the amount of mucin (mg) adsorbed per mg NPs. C1 lecithin NPs served as control. Data are expressed as mean ± SD ($n = 3$).

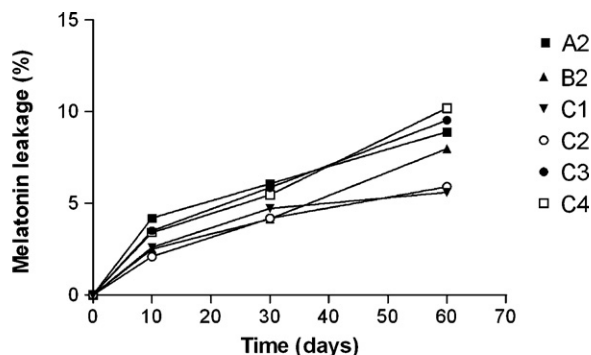

Fig. 3. Melatonin leakage from lecithin NPs (C1) and lecithin/chitosan NPs (A2, B2, C2, C3 and C4) over a two-month period. Indicated values are the means of at least three experiments ± SD.

weight ratios to chitosan of 20:1 and 10:1 were employed for the nanosystem preparation.

3.4. In vitro release studies

All NPs were characterised by prolonged release profiles, which is a desirable property for formulations treating various circadian rhythm disorders (Bénès et al., 1997). Melatonin release rate decreased with both, the presence of chitosan in the system and the increase in negative charge of lecithin used for NP preparation. Melatonin release from S45 lecithin/chitosan NPs (C2, C3 and C4) was slower ($t_{50\%}$ 1.7, 2.3 and 2.3 h, respectively) than melatonin release from S45 lecithin NPs (C1; $t_{50\%}$ = 1.0 h). Among the NPs with equal chitosan content (A2, B2 and C2; Fig. 4.), the fastest drug release was observed in the case of S100 lecithin/chitosan NPs (A2) and the slowest in the case of S45 lecithin/chitosan NPs (C2), confirming the strongest interaction between chitosan and lecithin S45.

3.5. Evaluation of cell viability

First contact between nanoparticles and cells occurs at the plasma membrane. Nanoparticles with positively charged groups at their surface, such as cationic polymer polyethyleneimine (PEI) or polyamidoamine (PAMAM) dendrimers, were shown to induce physical disruption of the plasma membrane (Unfried et al., 2007). This disruption can result in enhanced membrane permeability and release of cytosolic enzymes to the culture medium (Hong et al., 2004). In addition, NPs are candidates for cellular uptake by endocytosis (Jones et al., 2003), a process in which macromolecules or particles are carried into the cell in membrane bound vesicles derived by invagination and pinching off of the pieces of the plasma membrane (Conner and Schmid, 2003). One of the major requirements for the cationic polymer nanoparticles for drug delivery is low cytotoxicity. Chitosan and its derivatives have been reported to be less toxic than other cationic polymers such as polyethyleneimine (Amidi et al., 2006). Various chitosans and chitosan derivatives have been reported for NP preparation. The toxicity of those chitosans, however, was different depending on the type of cells and derivatives studied.

A cytotoxicity study of S45 lecithin (C1) and lecithin/chitosan NPs (A2, B2, C2, C3 and C4) was then performed on Caco-2 cells at NP concentrations ranging from 50 to 400 μg/ml and 2-h incubation times. Cells incubated in complete cell culture media were used to account for the loss of viability due to incubation of cells in HBSS. The incubation in HBSS for the time interval of the experiment did not induce a significant negative effect on cell viability. Thus, in these experiments, untreated cells incubated in HBSS were used as a negative control to which the viabilities of all NP-treated cells were compared.

The effect of nanosystems investigated on the metabolic activity of Caco-2 cells is shown in Fig. 5. This cell viability assay did not show significant toxic effects of S45 lecithin NPs and C2 NPs consisting of lecithin S45-to-chitosan ratio of 20:1 at any concentration ($P > 0.05$). A decrease in the cell viability was observed when the cells were incubated with NPs at 200 and 400 μg/ml ($P < 0.001$). A substantial decrease in the cell viability was observed after incubation with C4 NPs at concentrations as low as 50 μg/ml compared to untreated cells incubated in HBSS ($P < 0.001$). The differences between the cytotoxicity of NPs may be ascribed to differences in the charge density of NPs and the chitosan content in its free-soluble form in NP suspension, both of which may affect the interaction of the systems with the cell membranes and, thus, their toxicity. These results are consistent with the results of the effect of S45 lecithin NPs (C1) and lecithin/chitosan NPs (A2, B2, C2, C3 and C4) on LDH leakage (Fig. 6). Moreover, the cell viabilities obtained with these two cytotoxicity tests were found to be highly correlated (r^2 = 0.9964). The LDH assay, however, showed that membrane integrity was mainly preserved after a 2-h incubation with the lecithin/chitosan NPs (A2, B2, C3 and C4) at a concentration of 200 μg/ml, whereas the cell viability evaluated by MTT assay was found to be lower under the same conditions. This suggests an intracellular origin for the cytotoxicity.

3.6. Caco-2 cell monolayer permeability assay

The influence of lecithin/chitosan NPs on melatonin permeability was investigated on the Caco-2 cell monolayer. The Caco-2 cell line has shown to be a relatively simple and well-characterised in vitro model that is useful for drug transport screening studies. Although the Caco-2 cell line has an intestinal origin, it has already been used to study more general membrane effects of drug delivery systems intended for nasal administration (Ekelund et al., 2005; Mao et al., 2005; Singh et al., 2006; Furubayashi et al., 2007). Test samples were prepared by diluting melatonin stock solution (M) and the selected melatonin-loaded NP suspensions (C1, C2, C3, A2 and B2) with HBSS buffer (pH 6.0) up to the equal melatonin concentration (50 μM). As NPs differed in drug loading (Table 1), dilution of suspensions to be equal to the melatonin concentration resulted in test samples with NP concentrations ranging from 163 to 237 μg/ml. Melatonin solution and S45 lecithin NPs (C1) served as negative controls. Formulation C4 appeared to be unstable in HBSS buffer. The permeability study was therefore not performed with this sample.

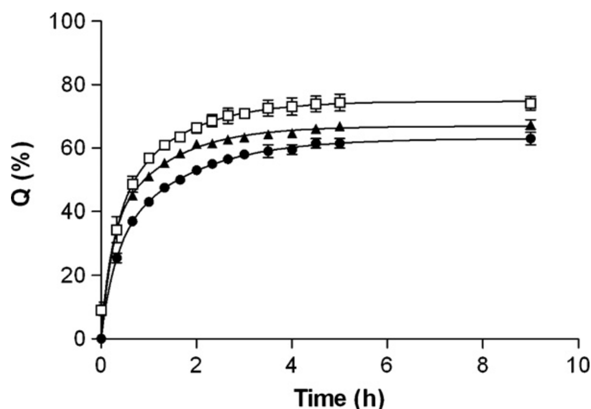

Fig. 4. Release profiles of melatonin from the A2 (□), B2 (▲) and C2 (●) NPs (L/C 20:1, w/w) in acetate buffer at pH 5.6 and 37 °C. Data are expressed as mean ± SD (n = 3).

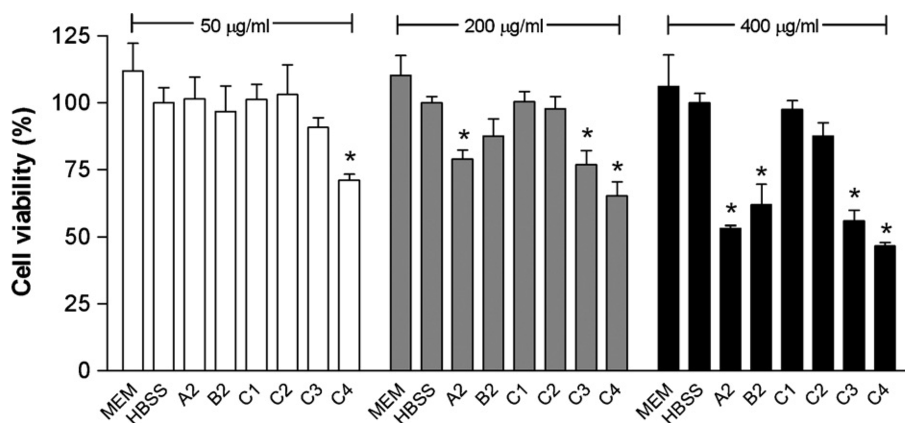

Fig. 5. Influence of S45 lecithin NPs (C1) and lecithin/chitosan NPs (A2, B2, C2, C3 and C4) on the viability of Caco-2 cells (MTT assay). Incubation time was 2 h and NP concentration ranged between 50 and 400 μg/ml. Data are expressed as mean ± SD ($n = 3$). *Differs from untreated cells incubated in HBSS ($P < 0.001$).

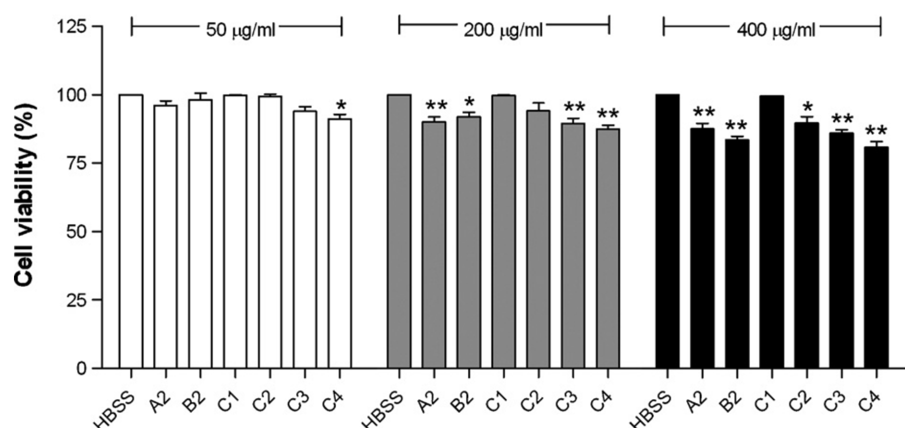

Fig. 6. Influence of S45 lecithin NPs (C1) and lecithin/chitosan NPs (A2, B2, C2, C3 and C4) on the viability of Caco-2 cells (LDH leakage assay). Incubation time was 2 h and NP concentration ranged between 50 and 400 μg/ml. Data are expressed as mean ± SD ($n = 3$). *Differs from untreated cells incubated in HBSS ($P < 0.05$), **Differs from untreated cells incubated in HBSS ($P < 0.001$).

The effect of NP suspensions on tight junction opening was investigated by monitoring the TEER values across the Caco-2 cell monolayer for 24 h starting from the application of the test samples to the cells. Measured TEER values are presented as the percentage of the initial TEER values (at $t = 0$; Fig. 7). The largest decrease in TEER was observed within the first hour of the experiment. Although slower, TEER continued to decrease within the second hour of the experiment. As expected, negative controls (M and C1) had the least effect on tight junction openings, reducing the TEER values by up to 90 and 80% of the initial value, respectively. All lecithin/chitosan NP suspensions caused more pronounced decrease in TEER (up to 50–65% of the initial value).

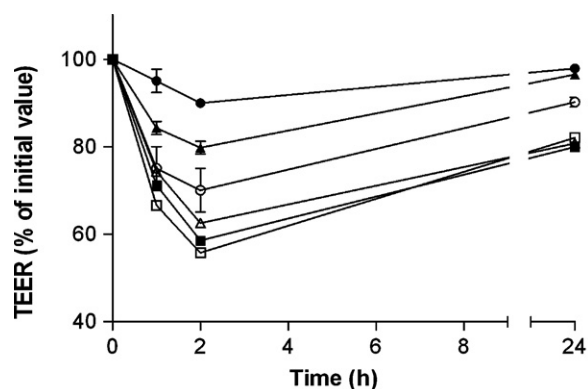

Fig. 7. Influence of melatonin solution (M; ●), S45 lecithin NPs (C1; ▲) and lecithin/chitosan NPs (A2; ■, B2; ▽, C2; ○ and C3; □) on the TEER of the Caco-2 cell monolayer. After 2 h, the monolayer was rinsed with HBSS and the cells were placed in culture medium. Data are expressed as mean ± SD ($n = 3$). *Differs from negative controls (M and C1; $P < 0.01$).

The effect of chitosan in a form of solution on TEER and paracellular transport was extensively studied. Chitosan is known to decrease TEER and promote absorption in different mucosal cell lines, such as bronchial Calu-3 (Florea et al., 2006), 16HBE14o (Kudsiova and Lawrence, 2008) and intestinal Caco-2 (Smith et al., 2004). The ability of chitosan to decrease the TEER is due to its positive charge in the systems with pH values below the pK_a of chitosan (<6.5) and its interaction with negatively charged glycoproteins on the cell surface or in the interior of the tight junctions (Smith et al., 2004). Recently, chitosan particles have been evaluated in terms of their effect on TEER and permeability across various cell monolayers, but mostly across the Caco-2 cell monolayer. Sadeghi et al. (2008) observed that, in comparison to the free-soluble polymers, the NPs prepared by ionic gelation of the chitosan and its quaternized derivatives had a much lower effect on decreasing the TEER by the opening of tight junctions due to reduced available amount of positive charge at the surface of the nanoparticles in comparison to the chitosan in free-soluble form.

The decrease in TEER primarily depended on the chitosan content in the NP suspension applied to the cells (Fig. 7). The largest decrease in TEER values (50% of the initial value) was observed in the case of the C3 NP suspension, characterised by the highest chi-

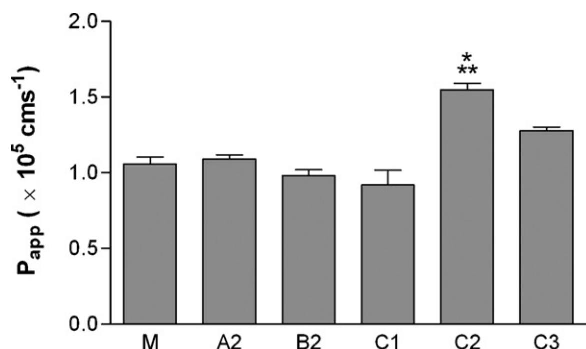

Fig. 8. Permeation of melatonin in Caco-2 cells from aqueous solution (M), lecithin NP (C1) and lecithin/chitosan NP suspensions (A2, B2, C2, and C3) presented by apparent permeability coefficients (P_{app}). Indicated values are the means of at least three experiments ± SD (n = 3). *Differs from other NP suspensions (P < 0.05), **Differs from melatonin aqueous solution (P < 0.001).

tosan content (L/C 10:1, w/w) with the highest NP surface charge (28.4 mV). The C2 NP suspensions had the least effect on the TEER (65% of initial value). The A2 and B2 NP suspensions, which had the same chitosan content as the C2 NP suspension (L/C 20:1, w/w) and a lower surface charge (7.5 and 18.1 mV, respectively), exhibited a greater effect on TEER values (around 55% of the initial value). Since this effect was more similar to that of the C3 NP suspension it can be concluded that the free-soluble chitosan in the systems is more effective in reducing the TEER than the NPs themselves. Because NPs were prepared by ionic interaction between negatively charged lecithin and positively charged chitosan, the amount of positive chitosan charge available for the interaction with tight junctions was reduced compared to the chitosan in free-soluble form. This is consistent with previous findings (Sadeghi et al., 2008). Due to the most intensive interaction of chitosan with S45 lecithin, the C2 NP suspension was characterised by the lowest free chitosan content. It has been found that a similar S45 lecithin/chitosan nanosystem containing the same lecithin/chitosan ratio (20:1) as in this study contained only 3% of chitosan in the free-soluble form (Gerelli et al., 2008).

For all samples tested, an increase in TEER was observed 22 h after removing the NPs from the cells. This increase was related to the reversibility of the tight junctions opening. Following negative controls (M and C1), the largest increase in TEER (up to 90% of the initial value) was observed for cells incubated with the C2 NP suspension. The TEER values of the cells incubated with the A2 and B2 NP suspensions containing the same chitosan content as the C2 NP suspensions, but consisting of different types of lecithin (S100 and S75, respectively), increased to only 75% of the initial value. This difference in TEER reversibility was consistent with the difference in toxicity of the NP suspensions compared under the same conditions (pH 6.0, NP concentration approximately 200 μg/ml).

It can be concluded that the effect of C2 NP suspension on TEER across the Caco-2 cell monolayer was almost completely reversible, and could be attributed to a temporary opening of the tight junctions between adjacent Caco-2 cells. These results are very promising, since the TEER appears to be a very sensitive marker for epithelial toxicity in Caco-2 cell experiments (Ekelund et al., 2005). These results are consistent with the results obtained by cell viability studies that showed that the C2 NP suspension could be safely applied to Caco-2 cells in the concentration range tested.

The permeation of melatonin from aqueous solution and NP formulations across the Caco-2 cell monolayer is demonstrated by the apparent permeability coefficients (Fig. 8). Results obtained indicated that, in most cases, incorporation of melatonin into lecithin/chitosan NPs did not significantly affect its permeability. For lecithin/chitosan NPs prepared with lecithins S100 and S75 (A2 and B2), apparent permeability coefficients were slightly higher than the apparent permeability coefficient for lecithin S45 NPs (C1). This can be explained by the higher influence of lecithin/chitosan NPs on the TEER values of the Caco-2 cell monolayer and consequently improved paracellular transport of released melatonin through the opened tight junctions.

The permeability of melatonin from C2 NPs was significantly improved compared to the permeability of melatonin from the solution (M; P < 0.001) and from all other NPs investigated (A2, B2 and C3; P < 0.05). The C2 NPs were characterised by a drug content of 6.4%, a mean diameter of 246.2 ± 8.1 nm and a zeta potential of 24.5 mV. Despite the higher surface charge (28.4 mV) and the higher drug content (7.1%), C3 NPs demonstrated a lower effect on melatonin permeability compared to C2 NPs. This can be explained by the significant difference in size of the C2 and C3 NPs (P < 0.001). The mean diameter of C3 NPs was 329.1 ± 10.5 nm. Since the C2 NPs were smaller in size, they had larger total surface area available for the interaction with the cellular membrane. Chen et al. (2008) have shown transcytosis across Caco-2 cell monolayers of N-trimethyl chitosan NPs that were similar in size to the NPs used in our study (∼300 nm). Moreover, Sadeghi et al. (2008) have shown that TMC, DMEC and DEMC nanoparticles that were approximately 200 nm in size are primarily transported through the Caco-2 monolayer by the intracellular pathway. The interdependent effect of key particle variables such as size, shape, chemical composition and surface charge on cellular entry is not yet understood, and it is possible that slight changes in these variables could grant entry to certain types of NPs while not permitting the entrance of others. Although in our study, we have no direct evidence that the NPs were endocytosed or transcytosed, our results show enhanced permeation of melatonin through the cell monolayer in the case of C2 NPs. According to recent literature, we speculate that the effect observed could be due to the ability of these NPs to more easily cross the epithelial barrier via the intracellular pathway.

4. Conclusions

We have shown that lecithin/chitosan NPs are a promising colloidal nanocarrier for the transmucosal delivery of melatonin. The matrix formed by ionic interactions between lecithin and chitosan provided both sufficient entrapment efficiency- and prominent surface charge-dependent mucoadhesive properties. The size and surface charge of the NPs prepared depended on both lecithin type and the lecithin-to-chitosan weight ratio. Nanoparticle suspension prepared with S45 lecithin and an L/C ratio of 20:1 (w/w) enhanced melatonin permeability compared to its permeability from the solution without inducing plasma membrane damage or a decrease in cell viability at the concentration tested. Further studies, however, are required to elucidate the exact mechanism(s) by which lecithin/chitosan NPs enhance melatonin transport across the epithelial barrier.

Acknowledgement

This work was supported by Grant 006-0061117-1244 of the Ministry of Science, Education and Sports of the Republic of Croatia.

References

Amidi, M., Romeijn, S.G., Borchard, G., Junginger, H.E., Hennink, W.E., Jiskoot, W., 2006. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J. Control. Release 111, 107–116.

Bechgaard, E., Lindhardt, K., Martinsen, L., 1999. Intranasal absorption of melatonin in vivo bioavailability study. Int. J. Pharm. 182, 1–5.

Bénès, L., Claustrat, B., Horrière, F., Geoffriau, M., Konsil, J., Parrott, K.A., de Grande, G., McQuinn, R.L., Ayres, J.W., 1997. Transmucosal, oral, controlled-release, and

transdermal drug administration in human subjects: a crossover study with melatonin. J. Pharm. Sci. 86, 1115–1119.
Ceraulo, L., Fanara, S., Turco Liveri, V., Ruggirello, A., Panzeri, W., Mele, A., 2008. Orientation and molecular contacts of melatonin confined into AOT and lecithin reversed micellar systems. Colloids Surf. A: Physicochem. Eng. Aspects 316, 307–312.
Chen, F., Zhang, Z.R., Yuan, F., Qin, X., Wang, M., Huang, Y., 2008. In vitro and in vivo study of N-trimethyl chitosan nanoparticles for oral protein delivery. Int. J. Pharm. 349, 226–233.
Conner, S.D., Schmid, S.L., 2003. Regulated portals of entry into the cell. Nature 422, 37–44.
Costantino, H.R., Illum, L., Brandt, G., Johnson, P.H., Quay, S.C., 2007. Intranasal delivery: physicochemical and therapeutic aspects. Int. J. Pharm. 337, 1–24.
Ekelund, K., Östh, K., Påhlstorp, C., Björk, E., Ulvenlund, S., Johansson, F., 2005. Correlation between epithelial toxicity and surfactant structure as derived from the effects of polyethyleneoxide surfactants on Caco-2 cell monolayers and pig nasal mucosa. J. Pharm. Sci. 94, 730–744.
England, R.J.A., Homer, J.J., Knight, L.C., Ell, S.R., 1999. Nasal pH measurement: a reliable and repeatable parameter. Clin. Otolaryngol. 24, 67–68.
Filipović-Grčić, J., Škalko-Basnet, N., Jalšenjak, I., 2001. Mucoadhesive chitosan-coated liposomes: characteristics and stability. J. Microencapsulation 18, 3–12.
Florea, B.I., Thanou, M., Junginger, H.E., Borchard, G., 2006. Enhancement of bronchial octreotide absorption by chitosan and N-trimethyl chitosan shows linear in vitro/in vivo correlation. J. Control. Release 10, 353–361.
Fočo, A., Gašperlin, M., Kristl, J., 2005. Investigation of liposomes as carriers of sodium ascorbyl phosphate for cutaneous photoprotection. Int. J. Pharm. 291, 21–29.
Furubayashi, T., Kamaguchi, A., Kawaharada, K., Masaoka, Y., Kataoka, M., Yamashita, S., Higashi, Y., Sakane, T., 2007. Kinetic model to predict the absorption of nasally applied drugs from in vitro transcellular permeability of drugs. Biol. Pharm. Bull. 30, 1007–1010.
Gerelli, Y., Barbieri, S., Di Bari, M.T., Deriu, A., Cantù, L., Brocca, P., Sonvico, F., Colombo, P., May, R., Motta, S., 2008. Structure of self-organized multilayer nanoparticles for drug delivery. Langmuir 24, 11378–11384.
He, P., Davis, S.S., Illum, L., 1998. In vitro evaluation of the mucoadhesive properties of chitosan microspheres. Int. J. Pharm. 166, 75–88.
Hong, S., Bielinska, A.U., Mecke, A., Keszler, B., Beals, J.L., Shi, X., Balogh, L., Orr, B.G., Baker Jr., J.R., Banaszak Holl, M.M., 2004. Interaction of poly(amidoamine) dendrimers with supported lipid bilayers and cells: hole formation and the relation to transport. Bioconjugate Chem. 15, 774–782.
Jones, A.T., Gumbleton, M., Duncan, R., 2003. Understanding endocytic pathways and intracellular trafficking: a prerequisite for effective design of advanced drug delivery systems. Adv. Drug Deliv. Rev. 55, 1353–1357.
Kelmann, R.G., Kuminek, G., Teixeira, H.F., Koester, L.S., 2007. Carbamazepine parenteral nanoemulsions prepared by spontaneous emulsification process. Int. J. Pharm. 342, 231–239.
Kudsiova, L., Lawrence, M.J., 2008. A comparison of the effect of chitosan and chitosan-coated vesicles on monolayer integrity and permeability across Caco-2 and 16HBE14o-cells. J. Pharm. Sci. 97, 3998–4010.
López-Pinto, J.M., González-Rodríguez, M.L., Rabasco, A.M., 2005. Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. Int. J. Pharm. 298, 1–12.
Maestrelli, F., González-Rodriguez, M.L., Rabasco, A.M., Mura, P., 2005. Preparation and characterisation of liposomes encapsulating ketoprofen-cyclodextrin complexes for transdermal drug delivery. Int. J. Pharm. 298, 55–67.
Mao, S., Chen, J., Wei, Z., Liu, H., Bi, D., 2004. Intranasal administration of melatonin starch microspheres. Int. J. Pharm. 272, 37–43.
Mao, S., Germershaus, O., Fischer, D., Linn, T., Schnepf, R., Kissel, T., 2005. Uptake and transport of PEG-graft-trimethyl-chitosan copolymer-insulin nanocomplexes by epithelial cells. Pharm. Res. 22, 2058–2068.
Martinac, A., Filipović-Grčić, J., Voinovich, D., Perissutti, B., Franceschinis, E., 2005. Development and bioadhesive properties of chitosan-ethylcellulose microspheres for nasal delivery. Int. J. Pharm. 291, 69–77.
Pavelić, Z., Škalko-Basnet, N., Filipović-Grčić, J., Martinac, A., Jalšenjak, I., 2005. Development and in vitro evaluation of a liposomal vaginal delivery system for acyclovir. J. Control. Release 106, 34–43.
Sadeghi, A.M.M., Dorkoosh, F.A., Avadi, M.R., Weinhold, M., Bayat, A., Delie, F., Gurny, R., Larijani, B., Rafiee-Tehrani, M., Junginger, H.E., 2008. Permeation enhancer effect of chitosan and chitosan derivatives: comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells. Eur. J. Pharm. Biopharm. 70, 270–278.
Schaffazick, S.R., Pohlmann, A.R., de Cordova, C.A.S., Creczynski-Pasa, T.B., Guterres, S.S., 2005. Protective properties of melatonin-loaded nanoparticles against lipid peroxidation. Int. J. Pharm. 289, 209–213.
Schaffazick, S.R., Siqueira, I.R., Badejo, A.S., Jornada, D.S., Pohlmann, A.R., Netto, C.A., Guterres, S.S., 2008. Incorporation in polymeric nanocapsules improves the antioxidant effect of melatonin against lipid peroxidation in mice brain and liver. Eur. J. Pharm. Biopharm. 69, 64–71.
Schubert, M.A., Harms, M., Müller-Goymann, C.C., 2006. Structural investigations on lipid nanoparticles containing high amounts of lecithin. Eur. J. Pharm. Sci. 27, 226–236.
Singh, J., Pandit, S., Bramwell, V.W., Alpar, H.O., 2006. Diphtheria toxoid loaded poly-(ε-caprolactone) nanoparticles as mucosal vaccine delivery systems. Methods 38, 96–105.
Singla, A.K., Chawla, M., 2001. Chitosan: some pharmaceutical and biological aspects—an update. J. Pharm. Pharmacol. 53, 1047–1067.
Smith, J., Wood, E., Dornish, M., 2004. Effect of chitosan on epithelial cell tight junctions. Pharm. Res. 21, 43–49.
Sonvico, F., Cagnani, A., Rossi, A., Motta, S., Di Bari, M.T., Cavatorta, F., Alonso, M.J., Deriu, A., Colombo, P., 2006. Formation of self-organized nanoparticles by lecithin/chitosan ionic interaction. Int. J. Pharm. 324, 67–73.
Unfried, K., Albrecht, C., Klotz, L.O., Von Mikecz, A., Grether-Beck, S., Schins, R.P.F., 2007. Cellular responses to nanoparticles: target structures and mechanisms. Nanotoxicology 1, 52–71.
van den Berg, M.P., Merkus, P., Romeijn, S.G., Verhoef, J.C., Merkus, F.W., 2004. Uptake of melatonin into the cerebrospinal fluid after nasal and intravenous delivery: studies in rats and comparison with a human study. Pharm. Res. 21, 799–802.
Zhang, X., Zhang, H., Wu, Z., Wang, Z., Niu, H., Li, C., 2008. Nasal absorption enhancement of insulin using PEG-grafted chitosan nanoparticles. Eur. J. Pharm. Biopharm. 68, 526–534.