Spray-dried Microspheres Based on Chitosan and Lecithin Cyclosporin A Delivery System

Ž. Davosir Klarić,[a] A. Hafner,[b] S. Zubčić,[a] M. Dürrigl,[c] and J. Filipović-Grčić[b,*]

[1]Croatian Agency for Medicinal Products and Medical Devices, Ksaverska cesta 4, 10 000 Zagreb, Croatia

[2]Department of Pharmaceutics, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, 10 000 Zagreb, Croatia

[3]PharmaS Ltd., Radnička cesta 47, 10 000 Zagreb, Croatia

Original scientific paper
Received: May 7, 2012
Accepted: July 24, 2012

Conventional and composed cyclosporin A (CsA)-loaded polymeric microspheres (MS) were prepared by spray-drying of CsA/chitosan one-phase system (solutions) and CsA/lecithin/chitosan two-phase system (suspensions). Microspheres were characterised in terms of production yield, entrapment efficiency, size distribution, zeta-potential, thermal properties, swelling ability and drug release profile.

Conventional MS were characterised by mean diameter ranging from 1.15 ± 0.91 to 1.27 ± 0.84 μm and CsA entrapment efficiency varying from 72.6 to 87.3%. Composed MS were characterised by larger mean diameter (1.32 ± 1.08 to 1.53 ± 1.15 μm) and higher CsA entrapment efficiency (86.6–94.3%) compared to the corresponding conventional MS. Only composed MS showed swelling ability, which was proportional to chitosan base content in the preparation. In vitro CsA release profile depended on both, the type of the spray-dried system and the chitosan used, as these factors were crucial in determining CsA entrapment pattern and swelling/dissolution ability of MS.

Key words:
Chitosan, lecithin, microspheres, nanoparticles, cyclosporin, spray-drying, ocular delivery

Introduction

Cyclosporin A (CsA) is highly lipophilic neutral cyclic peptide consisting of 11 amino acids of which 7 N-methylated. Its molecular formula is $C_{62}H_{111}N_{11}O_{12}$, has a molecular weight of 1202.64 Daltons, and contains four intermolecular hydrogen bonds that affect the strength of pronounced cyclical structure. Such an unusual structural properties affect the extremely low solubility in water (6.6 mg/ml), or extreme hydrophobicity (logP = 3.0).[1] Pursued by poor and erratic bioavailability from oral, transdermal and ocular routes, the delivery of CsA is a challenging task and explains the constant efforts by the pharmaceutical scientists to design effective delivery systems.[1] Although progress has been made, there is still possibility for improvement in the application of CsA, as none of these formulations is ideal.[2]

At concentrations of 50–300 ng/g of ocular tissue, CsA effectively suppresses the immune response and inflammation in most ocular disorders.[3] Local immunosuppression caused by CsA is effective for the management of extra ocular disorders. The target sites for the treatment of these diseases are the cornea and conjunctiva, and a number of drug delivery systems for topical CsA delivery in the eye were investigated so far.[4] The local ocular drug delivery is restricted by the dynamics of the lachrymal drainage system, which is the natural defense mechanism of the eye. This system introduces tear fluid to the eye and rapidly drains the fluid together with any instilled formulation from the precorneal area to the nasal cavity and throat. The high elimination rate results in short duration of contact of the drug with its absorption sites and consequently in a low local bioavailability. Increased ocular bioavailability can be achieved by the use of viscosity enhanced aqueous eye drops, suspensions, oily drops and unguents, mucoadhesive ocular delivery systems such as solutions and microparticle suspensions, in-situ gelling systems triggered by pH, temperature, or ions change, colloidal delivery systems such as liposomes and nanoparticles, and ocular inserts.[5–11]

The aim of this study was the development and characterisation of ophthalmic drug delivery system – MS, which depending on size, charge and bioadhesive properties, can improve absorption and bioavailability of CsA. MS as drug delivery system with large surface to volume ratio can make firm contact and allow for longer retention of the drug at the site of application/absorption.

*Corresponding author: Jelena Filipović-Grčić, E-mail: jfilipov@pharma.hr

Chitosan (CS), a polycationic biopolymer is well known for its several favourable biological properties such as biodegradability, nontoxicity and biocompatibility.[12] Chitosan increases transepithelial permeability due to its ability to reversibly disrupt the epithelial tight junctional complexes up to a pH of around 6.[13,14] These exceptional biological features of CS make it an ideal polymer in drug delivery systems such as micro- and nanoparticles intended for topical and ocular drug delivery.[15] Chitosan-based colloidal systems were found to work as transmucosal drug carriers, either facilitating the transport of drugs to the inner eye (chitosan-coated colloidal systems containing indomethacin) or their accumulation into the corneal/conjunctival epithelia (chitosan nanoparticles containing CsA).[15]

Nanocarriers, such as nanoparticles, have the capacity to deliver ocular drugs to specific target sites and hold promise to revolutionize the therapy of many eye diseases. The surface characteristics of the nanocarriers, among other properties, have an influence on the interaction with the ocular surface structures. Chitosan nanoparticles loaded with CsA can contact intimately with the corneal and conjunctiva surfaces, thereby increasing delivery to external ocular tissues without compromising inner ocular structures and systemic drug exposure. This helps to provide these target tissues with long-term drug levels.[4] CsA was incorporated successfully into cationic Eudragit RS 100 nanoparticles (EPNs). In vitro release studies showed the extended release, and in vivo results demonstrated the prolonged residence time of CsA in the deeper layers (vitreous humour) of the eye with positively charged EPNs.[16] Topical ophthalmic efficacy of CsA was enhanced via administration of solid lipid nanoparticles (SLNs). [17] In this study, conventional and composed CsA-loaded MS were prepared by spray-drying of CsA/chitosan one-phase feeds (solutions) and CsA/lecithin/chitosan two-phase feeds (suspensions). Microspheres obtained were characterised in terms of production yield, entrapment efficiency, size distribution, zeta-potential, physical state of the drug incorporated in the polymer matrix, swelling ability and drug release profile.

Materials and methods

Materials

CsA was obtained from Galena (Czech Republic). Chitosan base (CB, Protasan UP Base, deacetylation degree 84%) and chitosan in the salt form as chitosan chloride (CS, Protasan UP CL 213, deacetylation degree 86%) were purchased from NovaMatrix (Norway). Fat-free soybean lecithin with 45% phosphatidyl choline (Lipoid S45; LS45) and phosphatidyl choline from fat-free soybean lecithin (Lipoid S100; LS100) were a kind gift from Lipoid GmbH (Germany).

Simulated tear fluid (STF) was prepared as an aqueous solution containing 6.7 g NaCl, 2.0 g $NaHCO_3$ and 0.08 g $CaCl_2 \times 2H_2O$ per litre. All other chemicals or solvents used were of analytical grade and purchased from Kemika (Croatia).

Preparation of CsA loaded MS

MS were prepared by spray-drying method, using a Mini Spray Dryer Büchi 190 (Flawil, Switzerland) with a standard 0.5 mm nozzle. The drying conditions were as follows: dry air rate of 700 NL/h; inlet air temperature of 157 °C, outlet air temperature of 81 °C and feed pump rate of 0.25 L/h. The resulting dried products were collected and kept away from rehydration until further tests.

Lecithin (LS45 or LS100) was dissolved in 96% ethanol at a concentration of 0.4% (w/v). Chitosan chloride (CS) was solubilised in distilled water at a concentration of 2% (w/v). Chitosan base (CB) was solubilised in 0,5% acetic acid at a concentration of 2% (w/v). CB solution and CS solution were mixed (CB/CS), obtaining a CB-to-CS weight ratio of 3:2. For the preparation of CsA-loaded chitosan MS, CsA was dissolved in 96% ethanol at a concentration of 0.13% (w/v). For the preparation of CsA-loaded chitosan/lecithin MS, CsA was dissolved in the ethanolic solution of lecithin at a concentration of 0.13% (w/v), obtaining a lecithin-to-CsA weight ratio of 3:1.

Feed dispersions were obtained as follows: 22.5 ml of ethanolic CsA or lecithin/CsA solution was injected (syringe inner diameter of 0.75 mm) into 35 ml of distilled water that was magnetically stirred (900 rpm). Appropriate volume of CB, CS or CB/CS solution was then injected to CsA or lecithin/CsA suspension magnetically stirred in order to obtain CsA/chitosan ratio of 1:9. Prepared dispersions were subjected to spray-drying under process conditions described above. CsA-free (empty) MS were prepared following the same procedure as for CsA-loaded MS.

In vitro characterization of MS

Evaluation of entrapment efficiency and yield of MS

The CsA content in MS was determined directly by measuring the encapsulated CsA amount in MS. A known weight of spray-dried powder was dissolved in 96% ethanol, magnetically stirred (300 rpm) for 24 h, and put for 1 h in an ultrasonic bath. After centrifugation for 30 min at 3,000 rpm, supernatant was filtered and evaporated to dryness

and subsequently dissolved in 30 ml of acetonitrile:water (1:1 v/v) mixture. The CsA content of the samples was determined by HPLC (Shimadzu LC-10 Series). A Nucleosil 100-5-C-18 column (5 μm, 4x125 mm, Agilent, USA) was used. The injection volume was 20 μl. The mobile phase consisted of phosphoric acid/1,1-dimethylethyl methyl ether/acetonitrile/water (1/50/430/20) mixture. The detector wavelength, flow rate, and column temperature were 210 nm, 1.5 mL/min and 80 °C respectively. This method is described in CsA Monograph in European Pharmacopoeia.

Entrapment efficiency of the CsA (1) and the yield of MS (2) were calculated according to the following equations:

$$EE = (\text{Weight of drug determined (mg)} / \text{Weight of drug added (mg)}) \times 100 \quad (1)$$

$$Yield = (\text{The total weight of obtained MS (mg)} / \text{drug+polymer weight (mg)}) \times 100 \quad (2)$$

MS size and zeta potential analyses

A microscopic imaging analysis technique for determination of MS size distribution was applied. MS morphology and size distribution were determined with an Olympus BH-2 microscope equipped with a camera (CCD Camera ICD-42E, Ikegami Tsushinki Co., Japan) and a computer-controlled image analysis system (Optomax V, Cambridge). The MS were dispersed on a microscope slide. A microscopic field is scanned by video camera. The images of the scanned fields are digitalised and analysed by the software to determine MS equivalent spherical diameter (ESD) from area and particle size distribution. In all measurements at least 1500 particles were examined.

The zeta potential of the particles was determined using Photon Correlation Spectroscopy with a Zetasizer 3000 (Malvern Instruments, Malvern, UK) in 10 mM NaCl solution (pH 6.7) at 25 °C. The analyses were performed in triplicate.

MS swelling analyses

The water-absorbing capacity of MS was determined by a volumetric method using a Franz diffusion cells at room temperature. A water-permeable polyamide membrane with 0.45 μm pore size was placed between the donor compartment with MS (3 mg) and receptor compartment, which was filled with STF. The level of STF in graduated part of Franz diffusion cell lowered due to liquid uptake of the MS. The amount of STF, equal to the amount of STF absorbed by the MS was then added to the receptor compartment. The liquid uptake of each MS sample was expressed as volume of STF added per milligram of the MS in 15 min swelling process.

Modulated differential scanning calorimetry (MDSC)

Modulated differential scanning calorimetry (MDSC) analyses were performed on a TA Instrument Q1000 Modulated DSC (TA Instruments, New Castle, DE, USA) using aluminium hermetic pans with pierced lids to allow for removal of residual solvent, with approximately 5 ± 2 mg sample, under a dynamic nitrogen atmosphere (50 ml/min). Samples were heated at 5 °C/min from 20 °C to 180 °C using a modulation temperature amplitude of ± 0.8 °C and period of 60 s. The glass transition temperature (T_g) was determined using the TA Universal Analyses 2000 Software by extrapolating the linear portion of the DSC curve above and below the glass transition point and determining the midpoint temperature in the reverse heat flow curve. The analyses were performed in triplicate.

In vitro release studies

Drug release was determined as follows: CsA loaded MS (3 mg) were placed in glass beaker filled with STF (20 mL) maintained in gentle agitation by means of a magnetic stirrer (600 rpm). At scheduled time intervals, the filtered (pore size 0.45 μm) samples (0.5 ml) were withdrawn from the beaker and replaced with the fresh medium. The drug content in the samples was quantified by the HPLC method described previously. The analyses were performed in triplicate.

Statistical analysis

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

Results and discussion

Preparation of CsA loaded MS

Chitosan MS were prepared by spray-drying from CsA/chitosan solution or CsA/lecithin/chitosan suspension.

Thermal efficiency of the spray-drying method depends on the input of heat energy (inlet temperature) and the amount of heat used in the process of evaporation. The optimal efficiency of spray-drying method can be achieved by balancing the amount of input energy and the amount of energy needed, depending on the sample volume to be sprayed. When the solvent is water inlet air temperature must be higher than 100 °C. It was found that the inlet air temperature to produce chitosan MS from chitosan

aqueous solution should be around 160 °C. If the inlet air temperature is lower than 140 °C, or the sample flow rate is greater than 10 ml/min, the solvent from the droplets cannot completely evaporate and droplets adopt the inner wall of the main chamber of the spray-drier[18].

In this study, the preliminary investigations have determined conditions under which dry, non-agglomerated MS were obtained, and at which the highest production yields were achieved (standardized conditions).

Chitosan and lecithin were selected as drug carriers in the preparation of CsA-loaded MS because of their natural origin, biocompatibility and biodegradability. Two types of chitosan (chitosan base (CB) and chitosan chloride (CS)) and their mixtures (CB/CS 3:2, w/w) were used.

Used chitosans differ in solubility, degree of deacetylation and viscosity (Table 2). Chitosan as a base (pKa $\approx$ 6.5) is insoluble in neutral and alkaline medium, while the organic and inorganic chitosan salts, like chitosan chloride used in this study, are well soluble in water. Two types of soybean lecithin, LS45 and LS100 were used for the preparation of MS, differing in components that contribute to a negative charge. LS45 contains more components that contribute to the negative charge than LS100 and is therefore more negatively charged. The intensity of interaction of lecithin with the positively charged chitosan affects the degree of compaction of MS. All prepared MS have the same weight ratio of CsA, lecithin and chitosan, which is 1:3:9 respectively.

The single-phase systems (solutions) were prepared by dissolving CsA in ethanolic chitosan solution and two-phase systems (suspensions) were prepared by dispersing ethanolic CsA/lecithin solution in aqueous chitosan solution, and were subjected to spray-drying to obtain MS. Specifically, injection of ethanol solution of CsA/lecithin and viscous solution of chitosan in the water resulted in the formation of CsA-loaded lecithin vesicles suspended in the aqueous solution of chitosan. The interaction between the negatively charged components on the lecithin vesicles surface and positively charged amino groups of chitosan is assumed. Electrostatic interactions between lecithin and chitosan have been extensively described in the literature.[19,20] Chitosan/lecithin nanoparticles can occur due to the self-assembly of lecithin molecules and chitosan due to ionic interactions.[19] The structure of nanoparticles was studied by combining various analytical methods such as cryo-TEM, dynamic light scattering (DLS) and small angle neutron scattering (SANS).[20] Chitosan/lecithin nanoparticles are positively charged. The charge depends on the type of lecithin and the mass ratio of chitosan and lecithin in the formulation.[21] Because of the positive charge chitosan/lecithin nanoparticles electrostatically react with negatively charged mucin and mucosal components, which reduces mucociliary clearance of nanoparticles.[21]

In vitro characterization of MS

The entrapment efficiency and yield of MS

The preparation yields obtained are relatively high with respect to the applied method and ranged from 46.7 ± 5.2 to 57.3 ± 5.5%. Losses in the process of spray-drying are the result of sample adhesion to the cylinder wall. Also the lightest and smallest particles together with the air leave the spray-drier because there is no filter that would keep them in the system.

MS preparation yields of single-phase sprayed systems (CsA-loaded chitosan MS) were not significantly different from the MS yields obtained by spray-drying of two-phase systems (CsA-loaded chitosan/lecithin MS). Two-phase systems, as opposed to single-phase systems, in their composition contain both chitosan and lecithin. It can be concluded that the introduction of lecithin in the feed composition of the MS did not affect the utilization of their preparation.

Two types of chitosan solutions used for the preparation of feed dispersions were of equal concentration but different viscosities (Table 1), which may affect the utilization of the preparation. The feeds of higher concentration and/or viscosity are of the slower flow at the same drying conditions, causing the drying air is less saturated with the evaporated solvent, enhanced particles drying, and less adhesion of particles on cylinder wall of the spry-drier.[22] The results however show that type of chitosan used in the preparation of sprayed systems did not affect the MS preparation yields. It can be concluded that the feeds were not significantly different in viscosity due to multiple dilution of stock chitosan solutions and the presence of ethanol.

The CsA entrapment efficiency of MS was determined after destruction of the known weight of MS in absolute ethanol. EE ranged between 72.6 and 94.3% (Table 1).

The CsA entrapment of MS spray-dried from two-phase feeds is significantly higher compared to the corresponding MS obtained by spray-drying of single-phase feeds. The single-phase spray-dried feeds resulted with CsA-loaded chitosan MS and lipophilic drug being dispersed in a hydrophilic polymer matrix. On the other hand, by spray-drying of the two-phase systems chitosan MS with CsA-loaded lecithin nanoparticles were formed.

Table 1 – *The composition and main characteristics of the MS prepared; yield, encapsulation efficiency, mean diameter and swelling properties*

Sample	Chitosan type								
	CB			CS			CB+CS		
	B	BLS100	BLS45	S	SLS100	SLS45	BS	BSLS100	BSLS45
Lecithin type	–	LS100	LS45	–	LS100	LS45	–	LS100	LS45
Yield (%)	57.3 ± 5.5	54.3 ± 7.3	52.0 ± 5.1	46.7 ± 5.2	48.5 ± 3.2	48.7 ± 3.5	47.5 ± 2.4	53.9 ± 0.5	49.1 ± 4.2
Drug content (%)	7.2 ± 0.06	6.6 ± 0.02	6.6 ± 0.02	8.7 ± 0.03	7.2 ± 0.14	6.9 ± 0.04	7.3 ± 0.02	7.0 ± 0.01	6.5 ± 0.02
EE (%)	72.6 ± 0.7	87.8 ± 0.8	86.6 ± 0.3	87.3 ± 1.3	94.3 ± 1.3	91.5 ± 2.0	73.3 ± 1.0	91.3 ± 0.8	86.7 ± 0.3
Mean diameter (μm)	1.21 ± 0.93	1.32 ± 1.08	1.53 ± 1.15	1.27 ± 0.84	1.38 ± 1.13	1.44 ± 1.17	1.15 ± 0.91	1.15 ± 0.91	1.51 ± 1.08
V_{STF} (μl/mg)	0	51.7 ± 1.7	48.3 ± 1.7	0	0	0	0	23.3 ± 0	20.0 ± 0

Lecithin introduction into the system advanced the encapsulation of lipophilic CsA in chitosan MS.

Given the entrapment efficiency of CsA, MS prepared with LS45 did not significantly differ from the corresponding MS prepared with LS100. For example, EE of CsA in BLS100 and BLS45 MS was 87.8 ± 0.8 and 86.6 ± 0.3% respectively. Also, the SLS100 and SLS45 MS were observed with 94.3 ± 1.3 and 91.5 ± 2.0% CsA EE, respectively.

The size and zeta-potential of MS

The MS were of satisfactory size distribution with about 95% particles with diameter smaller than 5 μm and with mean diameter in the range of 1.15 ± 0.91 to 1.53 ± 1.15 μm (Table 1). The mean diameter of MS prepared by spray-drying of single-phase feeds (CsA-loaded chitosan MS), is in the range of 1.15 ± 0.91 to 1.27 ± 0.84 μm, with 85% particles smaller than 2 μm, and is smaller than the mean diameter of corresponding MS prepared by spray-drying of two-phase feeds (CsA-loaded chitosan/lecithin MS) ranged between 1.32 ± 1.08 and 1.53 ± 1.15 μm, with only 65–70% particles smaller than 2 μm.

The CsA-loaded chitosan/lechitin MS prepared with LS45 were bigger than the corresponding MS prepared with LS100 (Table 1). It is assumed that the size of the MS depended on the strength of interaction between chitosan and lecithin. Since the LS45 has more negative charge than LS100, its interaction with the positively charged amino groups of chitosan is stronger, resulting in the formation of slightly larger particles.

Two types of chitosan solution used for the MS preparation were of equal concentration but of different viscosities (Table 1) that could affect the size of the prepared MS. However, given the multiple dilution of chitosan stock solutions and the presence of ethanol in sprayed feeds, they did not significantly differ in viscosity and thus corresponding MS prepared with different types of chitosan solution were not significantly different in size. For example, mean diameters of conventional CsA-loaded chitosan MS, prepared from the solution of chitosan base, chitosan chloride, or their mixture (CB:CS 3:2, m/m), were 1.21 ± 0.93, 1.27 ± 0.84 and 1.15 ± 0.91 μm, respectively.

Zeta-potential of MS was measured after the dispersion of MS in 10 mM NaCl solution (pH 6.7, temp. 25 °C). The results are shown in Figure 1. The zeta-potential of LS45 and LS100 nanoparticles was –11.0 ± 0.8 and –39.5 ± 1.2 mV, respectively. The negative charge is a consequence of dissociation of functional groups of the components of lecithin.

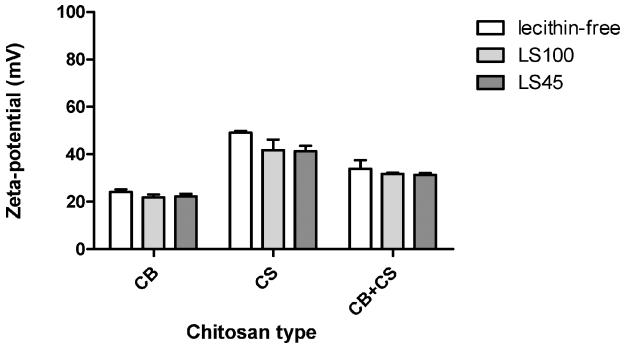


Fig. 1 – *The zeta-potential of chitosan and chitosan/lecithin microspheres prepared with chitosan base (CB), chitosan chloride (CS) and their mixture (CB+CS) in weight ratio of 3:2, and lecithins LS100 and LS45. Values are mean ± SD (n=3).*

All chitosan MS were characterized by a positive zeta-potential in the range of 21.9 ± 1.1 to 49.1 ± 0.8 mV. The positive charge on the surface of the MS derived from the positively charged amino groups of chitosan. Zeta-potential of conven-

tional MS increased, depending on the type of chitosan used in their preparation, in the following sequence: CB < CB+CS < CS. Higher values of the zeta-potential of chitosan chloride microspheres (24.9 mV) than the zeta potential of chitosan base microspheres (9.7 mV) were observed earlier.[18]

Effect of lecithin on the zeta-potential of composed MS was in line with observations for conventional MS (CB < CB+CS < CS). Thus, the zeta-potential of composed MS prepared with CS (SLS100 and SLS45) was about 8 mV lower than the zeta-potential of corresponding conventional MS (S), while the difference in zeta-potential between composed and conventional MS prepared with CB was within the standard deviation. This is probably due to different solubility of CB and CS.

Swelling of MS

Swelling of MS was expressed as the volume of artificial tear fluid absorbed by the 1 mg of MS over time (Table 1). In contact with the artificial tear fluid MS, depending on their composition, swelled (composed MS prepared with CB or CB+CS), dissolved (composed MS prepared with CS) or remained unchanged (conventional MS). The CS dissolves in contact with aqueous media while CB swells. In the absence of contact of polymer with the aqueous media MS remain unchanged. Conventional MS behaved like that. The less EE of lipophilic CsA into conventional MS, and its presence at the MS surface prevented contact of MS with aqueous medium and chitosan from swelling. Swelling and dissolution was observed for composed MS. Lecithin improved integration of lipophilic CsA in composed MS and thereby reduced its presence on the surface of MS, as well as it reduced contact angle and improved MS wetting.

Swelling of MS, except the presence of lecithin, depended on the proportion of CB in their composition. Thus, the MS with 69% CB (BLS100 and BLS45) absorbed more STF (51.7 ± 1.7 and 48.3 ± 1.7 μl/mg, respectively) than the MS with 41.5% CB (BSLS100 and BSLS45, 23.3 ± 0 and 20.0 ± 0 μl/mg, respectively). From these data it is possible to conclude that the type of lecithin did not significantly affect the swelling of MS. If we take into consideration the swelling of BLS100 empty MS in which the proportion of CB is 75% and the volume of fluid absorbed 68.3 ± 1.7 μl/mg, it is evident that neither incorporated CsA did not affect the swelling of MS. Swelling has been proportional to the CB content (Figure 2).

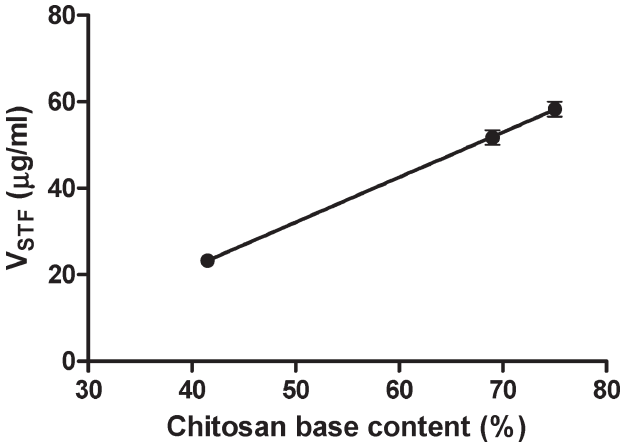


Fig. 2 – *The correlation between swelling properties of microspheres (expressed as volume of STF absorbed per milligram of microspheres in 15 min swelling process) and chitosan base content in preparation. Values are mean ± SD (n=3).*

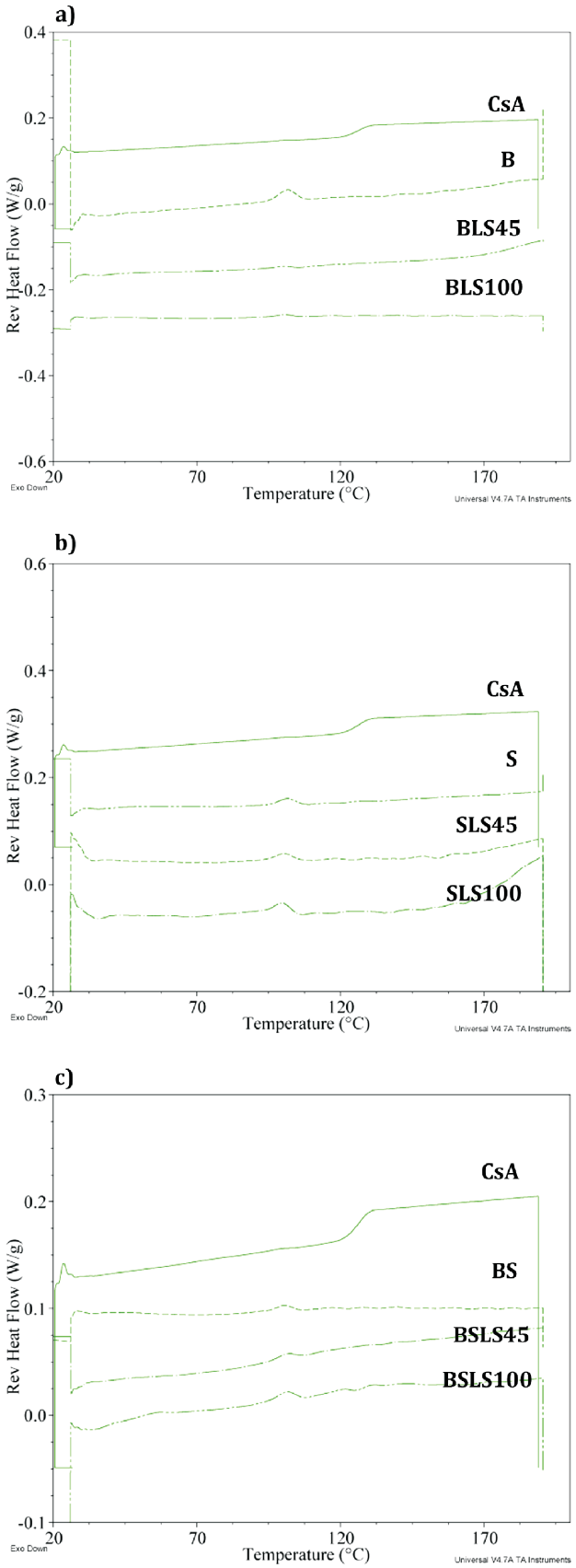
Thermal properties of MS

Thermal properties of drug and MS were assessed using MDSC. It is well known that reversing heat flow curves obtained by MDSC are used to detect overlapping thermal events not easily recognised in total heat flow curves (e.g. glass transitions). CsA was originally in an amorphous state as presented in reversing heat flow curves in Figure 3 that revealed lack of melting peak of crystalline form. Actually, heat capacity change corresponding to T_g was evident at ≈126 °C and correlated well with already reported data.[23,24]

The reversing heat flow curves obtained for the spray-dried MS containing chitosan base, chitosan chloride or combination thereof, including lecithin containing MS are presented in Fig 3a-c). All CsA-loaded chitosan and chitosan/lecithin MS displayed fully amorphous nature after spray-drying process. This preparation process usually produces amorphous structures as crystallisation process is prevented due to extremely fast solvent evaporation.

Reversing heat flow curves obtained for chitosan MS (B, S or BS) have shown no thermal event attributed to CsA itself, confirming that spray-drying of single-phase systems resulted in chitosan MS with CsA dispersed in a polymer matrix.

Moreover, reversing heat flow curves obtained for chitosan/lecithin MS prepared with chitosan base and chitosan chloride (Fig 3a-b) also have shown no thermal event attributed to CsA. This observation suggests that CsA was evenly dispersed within lecithin/chitosan ionic complex. In contrary to that, weak glass transition (being ascribed to CsA) is observed when combination of chitosan base and chloride was used regardless of lecithin type. It is assumed that having four different components in a mixture, makes uniform mixing less feasible resulting in apparent phase separation.



F i g . 3 – *The reversing heat flow curves of CsA itself and CsA-loaded chitosan/lecithin MS where chitosan type is a) chitosan base (CB), b) chitosan chloride (CS) and c) combination of chitosan base and chitosan chloride (CB+CS) in the weight ratio of 3:2.*

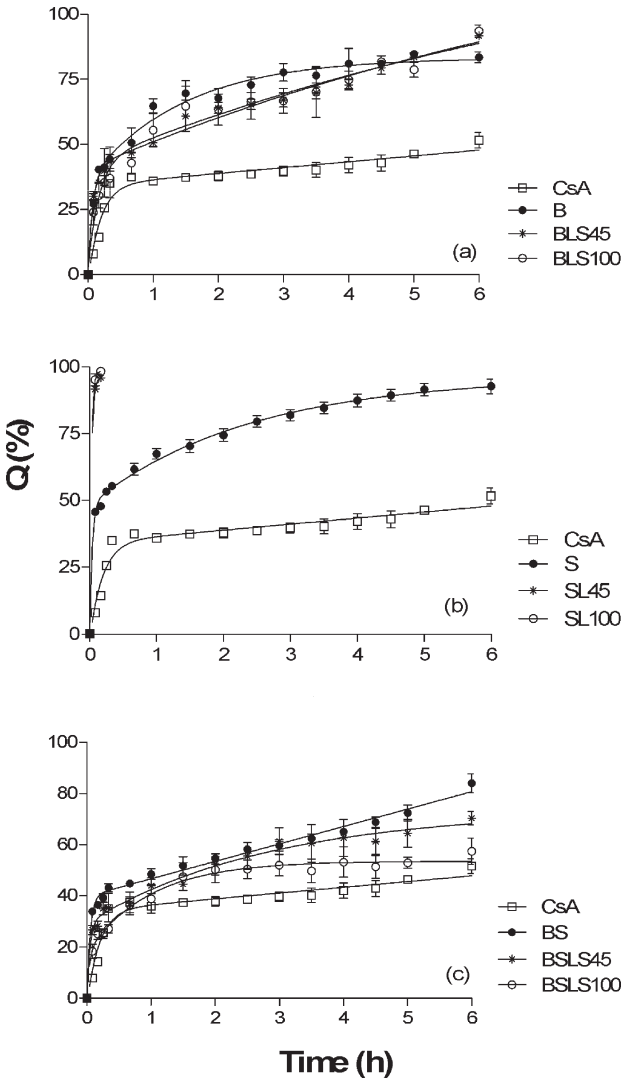
Physical mixtures of exact weight proportions of chitosan, lecithin and CsA were used to confirm capability of applied methodology to detect CsA related glass transition (data not shown). Thermal event appearing at temperature between 95–100 °C was characteristic for all chitosan samples and was unaffected by MS composition. Additional study is needed to understand origin of this thermal event as published data suggest that glass transition temperature of chitosan is expected in the region between 130–220 °C (being dependant on deacetylation, origin, molecular weight).[25]

The release of CsA from MS

In vitro release of active substances from the drug delivery systems is carried out under conditions similar to in vivo conditions, to predict the release behaviour in vivo. In vitro dissolution can be carried out in conditions independent of the in vivo conditions if aimed to compare the formulations or find out the release mechanism.[26] In this work, the release study of CsA from MS was conducted in 20 ml of STF (pH = 6.8), at 37 °C while stirring with a magnetic stirrer (600 rpm). The selected release media corresponded to ophthalmic conditions, while the other terms of the in vitro release model used were independent of the in vivo ocular conditions. The aim of such tests was to compare the prepared MS and to determine the effect of lecithin and chitosan type on the release of CsA from the prepared MS.

The release profiles of CsA from conventional MS prepared with chitosan base, chitosan chloride or their mixture are shown in Figure 4. For all three types of chitosan MS a biphasic release profile of the drug was observed, the initial rapid release followed by a slower phase of release. During the six-hour release test between 83.4 and 92.7% of entrapped CsA was released from MS. The observed CsA release profiles could be attributed to the CsA location within MS. The first phase of the release might be driven by dissolution and passive diffusion of CsA located at or near the MS surface. The second phase may present the release of CsA incorporated into the core of the MS or lecithin nanoparticles.

Thus, for example, the MS prepared with chitosan base, chitosan chloride or their mixture in the first 5 minutes released 27.3 ± 6.8%, 45.6 ± 1.5% and 34.0 ± 1.1% incorporated CsA, respectively (Figure 3). These results are consistent with results obtained by swelling studies of chitosan MS. Specifically, it was assumed that the absence of swelling and/or dissolution of chitosan MS resulted from the presence of lipophilic CsA on the surface of the MS, which prevented chitosan



F i g . 4 – *The release profiles of CsA from chitosan (●), chitosan/LS100 (○) and chitosan/LS45 microspheres (✳) prepared with chitosan base (a), chitosan chloride (b) and their mixture in the weight ratio of 3:2 (c). The dissolution profile of CsA (□) obtained under the conditions applied in the release studies (STF, pH 6,8; 37°C) has also been included. Values are mean ± SD (n=3).*

contact with the aqueous media. Though, incorporation of CsA in chitosan MS favoured its dissolution. Specifically, under conditions that were used for in vitro release, within the six-hour examination of the dissolution of CsA (equal amounts as entrapped in MS) only 51.7 ± 3.0 % of CsA dissolved, which is significantly less than the amount of CsA released from the MS in the same study period (83.4 to 92.7%).

Compared with the release of CsA from conventional chitosan MS prepared with chitosan base (B) or chitosan chloride (S), the release of CsA from MS prepared with the mixture of these two types of chitosan (BS) was better controlled (Figure 3c). After a rapid initial release of CsA from BS MS, the phase of a uniform release of CsA was observed that corresponded to the zero-order kinetics.

The same pattern was observed for CsA release from composed chitosan/lecithin MS prepared with chitosan base (Figure 4a). These observations may be explained by the better CsA encapsulation due to the introduction of lecithin in the composition of MS. There was no significant difference in the release of CsA with respect to the type of lecithin used in the preparation of MS . The uniform release of CsA from the MS can be attributed to emerging chitosan gel in the process of swelling, which is a diffusion barrier in the release of CsA and/or CsA-loaded lecithin nanoparticles.[27,28]

The literature describes the preparation of similar drug delivery microsystems in which the drug-loaded nanoparticles were incorporated into the polymer matrix, and release of nanoparticles and/or drug depended on the swelling and/or degradation of the polymer carrier. Thus, for instance, liposomes were embedded into dextran MS. The release of liposomes was controlled by dextran matrix degradation.[29] Liposomes were encapsulated into microspheres of biodegradable polymers by the solvent extraction/evaporation process to form LIMs.[30] Before incorporation into the MS, liposomes were coated with chitosan to improve their stability. The release of liposomes depended on the size of the pores of the polymer matrix and the size of the liposomes. El-Sherbiny and Smyth[31] incorporated polyethylene glycol modified chitosan nanoparticles into alginate MS. The developed nano-/microspheres carrier systems were formed via spray drying followed by ionotropic crosslinking in mild aqueous medium. The release of drug embedded (bovine serum albumin, BSA) from the MS was controlled by the swelling of alginate.

The release profiles of CsA from MS obtained by spray-drying of the single and two-phase systems composed with chitosan chloride are shown in Figure 4b. Unlike CsA-loaded conventional chitosan MS, CsA-loaded composed chitosan/lecithin MS dissolved within 5 minutes and released CsA-loaded nanoparticles immediately. The results of swelling test showed that conventional MS prepared with chitosan chloride in contact with aqueous media remained unchanged while composed MS prepared with chitosan chloride dissolved at the same conditions, which is the cause of the immediate release of CsA-loaded nanoparticles in aqueous medium.

Complete and high CsA release was obtained from the conventional MS prepared with chitosan chloride, although they have not swelled in water. Up to 50% embedded CsA released in the first

5 minutes, the rest released slowly over 6 hours (Figure 4b).

The literature describes the preparation of similar nanoparticle microcarrier systems that can easily dissolve or disperse in contact with water and release the nanoparticle complexes. Carriers in such systems can be sugars, e.g. mannitol and lactose, as well as Aerosil and silica. Thus, for example, Grenha[32] and colleagues used mannitol as microcarrier of chitosan/TPP and phospholipids nanoparticles (L/CS-NP) with insulin intended for pulmonary application. After resuspension and dissolution of mannitol microparticles, nanoparticles were liberated unchanged in size and release profile of insulin. Sham[33] and colleagues examined the systems in which the carrier of PBCA- gelatin nanoparticles was lactose. In contact with moist of lung epithelium lactose carrier dissolved, and liberated nanoparticles. Amorim[34] and colleagues used spray-drying method for incorporation of chitosan and N-carboxymethyl chitosan nanoparticles with idebenone in a silica matrix, and thus prepared drug delivery system suitable for topical or nasal idebenone aplication. The nanoparticles showed a 10-fold increase of drug stability in comparison with free drug and preserved antioxidant activity in vitro.

Release profiles of CsA from MS prepared with the mixture of chitosan base and chitosan chloride (3:2, w/w) are shown in Fig 4c. Compared with the release of CsA from conventional chitosan MS, the release of CsA from composed chitosan/lecithin MS was significantly slower and less consistent. Rapid initial release of CsA from chitosan/lecithin MS prepared with LS100 and LS45 (18% and 27% respectively) was followed by the slow release phase, and the amount of CsA released soon reached a plateau with maximum of 60% and 70%, respectively. Since chitosan chloride is freely soluble in water, one would expect that CB+CS MS would swell well in aqueous medium, enhancing CsA release like chitosan salt MS with reduced burst release. It seems however that denser chitosan matrix/gel was formed that sustained CsA release from CB+CS MS.

This is corroborated by the fact that the composed chitosan/lecithin MS prepared with the mixture of chitosan base and chitosan chloride (3:2, w/w) showed a slower release of CsA than the corresponding composed MS prepared with chitosan base only (Figure 4). It can be concluded that the release rate depended on the MS swelling ability. The MS, which consisted of chitosan base only , swelled better than the MS prepared with CB+CS mixture.

Conclusions

Spray-dried dispersion formulations of CsA using chitosan/lecithin as matrix were developed and characterized. Developed MS revealed great enhancement in dissolution rate of CsA especially those prepared with chitosan chloride. The dissolution improvement was due to hydrophylisation of cyclosporine. Developed MS formulations revealed dissolution profiles that implied that CsA was embedded in a solid dispersion as confirmed by physicochemical characterization studies and hence might increase its bioavailability.

ACKNOWLEDGEMENTS

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

References

1. *Italia, J.L., Bhardwaj, V., Kumar, M.N.*, Drug Discov Today **11** (2006) 846.
2. *Lallemand, F., Felt-Baeyens, O., Besseghir, K., Behar-Cohen, F., Gurny, R.*, Eur J Pharm Biopharm **56** (2003) 307.
3. *Kaswan, R.L.*, Transplant Proc **20** (1988) 650.
4. *De Campos, A.M., Sanchez, A., Alonso, M.J.*, Int J Pharm **224** (2001) 159.
5. *Aksungur, P., Demirbilek, M., Denkbas, E.B., Vandervoort, J., Ludwig, A., Unlu, N.*, J Control Release **151** (2011) 286.
6. *Davies, N.M.*, Clin Exp Pharmacol Physiol **27** (2000) 558.
7. *Urtti, A.*, Adv Drug Deliv Rev **58** (2006) 1131.
8. *Ludwig, A.*, Adv Drug Deliv Rev **57** (2005) 1595.
9. *Diebold, Y., Jarrin, M., Saez, V., Carvalho, E.L., Orea, M., Calonge, M., Seijo, B., Alonso, M.J.*, Biomaterials **28** (2007) 1553.
10. *Shen, J., Wang, Y., Ping, Q., Xiao, Y., Huang, X.*, J Control Release **137** (2009) 217.
11. *Pepic, I., Hafner, A., Lovric, J., Pirkic, B., Filipovic-Grcic, J.*, J Pharm Sci **99** (2010) 4317.
12. *Illum, L.*, Pharm Res **15** (1998) 1326.
13. *Smith, J., Wood, E., Dornish, M.*, Pharm Res **21** (2004) 43.
14. *Kotze, A.F., Luessen, H.L., de Leeuw, B.J., de Boer, B.G., Verhoef, J.C., Junginger, H.E.*, Pharm Res **14** (1997) 1197.
15. *Alonso, M.J., Sanchez, A.*, J Pharm Pharmacol **55** (2003) 1451.
16. *Basaran, E., Demirel, M., Sirmagul, B., Yazan, Y.*, J Biomed Nanotechnol **7** (2011) 714.
17. *Gokce, E.H., Sandri, G., Egrilmez, S., Bonferoni, M.C., Guneri, T., Caramella, C.*, Curr Eye Res **34** (2009) 996.
18. *He, P., Davis, S.S., Illum, L.*, Int J Pharm **187** (1999) 53.
19. *Sonvico, F., Cagnani, A., Rossi, A., Motta, S., Di Bari, M.T., Cavatorta, F., Alonso, M.J., Deriu, A., Colombo, P.*, Int J Pharm **324** (2006) 67.
20. *Gerelli, Y., Barbieri, S., Di Bari, M.T., Deriu, A., Cantu, L., Brocca, P., Sonvico, F., Colombo, P., May, R., Motta, S.*, Langmuir **24** (2008) 11378.

21. *Hafner, A., Lovric, J., Voinovich, D., Filipovic-Grcic, J.*, Int J Pharm **381** (2009) 205.
22. *Billon, A., Bataille, B., Cassanas, G., Jacob, M.*, Int J Pharm **203** (2000) 159.
23. *Lechuga-Ballesteros, D., Abdul-Fattah, A., Stevenson, C.L., Bennett, D.B.*, J Pharm Sci **92** (2003) 1821.
24. *Tam, J.M., McConville, J.T., Williams, R.O., 3rd, Johnston, K.P.*, J Pharm Sci **97** (2008) 4915.
25. *Alhalaweh, A., Andersson, S., Velaga, S.P.*, Eur J Pharm Sci **38** (2009) 206.
26. *Janes, K.A., Fresneau, M.P., Marazuela, A., Fabra, A., Alonso, M.J.*, J Control Release **73** (2001) 255.
27. *Chan, A.W., Neufeld, R.J.*, Biomaterials **30** (2009) 6119.
28. *Khalid, M.N., Agnely, F., Yagoubi, N., Grossiord, J.L., Couarraze, G.*, Eur J Pharm Sci **15** (2002) 425.
29. *Stenekes, R.J., Loebis, A.E., Fernandes, C.M., Crommelin, D.J., Hennink, W.E.*, Pharm Res **17** (2000) 690.
30. *Feng, S.S., Ruan, G., Li, Q.T.*, Biomaterials **25** (2004) 5181.
31. *El-Sherbiny, I.M., Smyth, H.D.*, Int J Pharm **395** (2010) 132.
32. *Grenha, A., Seijo, B., Serra, C., Remunan-Lopez, C.*, Biomacromolecules **8** (2007) 2072.
33. *Sham, J.O., Zhang, Y., Finlay, W.H., Roa, W.H., Lobenberg, R.*, Int J Pharm **269** (2004) 457.
34. *Amorim Cde, M., Couto, A.G., Netz, D.J., de Freitas, R.A., Bresolin, T.M.*, Nanomedicine **6** (2010) 745.