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Research paper

Chitosan microspheres for intrapulmonary administration of moxifloxacin: Interaction with biomembrane models and *in vitro* permeation studies

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Abstract

Chitosan microspheres loaded moxifloxacin were prepared to obtain sustained release of the drug after intrapulmonary administration. The microspheres were produced by the spray-drying method using glutaraldehyde as the crosslinking agent. The particles were spherical with a smooth but distorted surface morphology and were of small size, ranging from 2.5 to 6.0 μm, thus suitable for inhalation. *In vitro* release studies showed a significant burst effect for all crosslinked systems, followed by a prolonged moxifloxacin release, particularly in the presence of the highest glutaraldehyde concentration. Lipid vesicles made of dipalmitoylphosphatidylcholine (DPPC) were used as an *in vitro* biomembrane model to evaluate the influence of chitosan microspheres on the interaction of moxifloxacin with biological membranes. Differential scanning calorimetry was used as a simple and non-invasive technique of analysis. Moxifloxacin freely permeates through DPPC liposomes, interacting with the hydrophobic zone of the bilayers (lowering of the ΔH value and loss of the cooperativity of the main transition peak). Uncrosslinked microspheres rapidly swelled and dissolved releasing free chitosan that was able to interact with liposomes (increase of ΔH value), probably altering the biomembrane permeability to the drug. Crosslinked microspheres did not show this property. Pulmonary absorption of moxifloxacin-loaded chitosan microspheres was evaluated compared to the free drug. A monolayer of Calu-3 human bronchial epithelial cells mounted on Franz diffusion cells was used as an *in vitro* bronchial epithelium model. Microspheres retard the absorption of moxifloxacin and within 6 h the cumulative amount of permeated drug was about 18%, 11% and 7% (w/w) for free moxifloxacin, moxifloxacin-loaded crosslinked and moxifloxacin-loaded uncrosslinked microspheres, respectively.

Keywords: Chitosan microparticles; Moxifloxacin; Biomembrane interaction; *In vitro* permeation; Calu-3 cell monolayer

1. Introduction

Pulmonary administration of drugs to treat localized disease states within the bronchi has been employed for years. In this way the drugs can be delivered into the diseased regions, thus reducing side effects due to drug distribution to other organs. Inhalation of aerosolized drugs can also represent an ideal method for drug delivery to the systemic circulation [1]. However, drug disposition in the lung following inhalation still depends on simultaneous processes, these are: deposition, absorption, metabolism and mucociliary clearance [2], and generally there is the need to frequently administer therapeutic dose (3–4 times daily), thus limiting patient compliance. Formulation strategies to prolong the drug's retention time in the lung can be

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useful to improve or retard absorption, minimizing the biodistribution throughout the systemic circulation, thus influencing therapeutic effects and reducing side effects. Chitosan microparticles can be used for this purpose. Chitosan [(1 → 4)-2-amino-2-deoxy-β-D-glucan] is a hydrophilic linear polysaccharide characterized by low toxicity and high biodegradability and biocompatibility [3]. Chitosan has been shown to be degraded mainly by lysozyme (EC 3.2.1.17) [4], which commonly exists in various human body fluids and tissues and represents one of the most abundant enzymes in the lung, in which it is synthesized and secreted by glandular serous cells, surface epithelial cells, and macrophages in the human airways [5]. Chitosan is able to form microparticulate carriers with good bioadhesive properties [6] related to attractive electrostatic forces between the negative charged glycoprotein of mucin and the positive charged amino groups of the polymer. Moreover, by means of a reduction of mucociliary clearance and the opening of tight intercellular junctions [7], chitosan is able to increase drug bioavailability after nasal administration [8].

In this study we used chitosan to create a microparticulate system for pulmonary administration of moxifloxacin (MXF), an 8-methoxy-quinolone (Fig. 1) with a broad spectrum of antimicrobial activity against common respiratory pathogens, including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, as well as atypicals such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*. MXF is orally or i.v. administered at a dose of 400 mg taken once daily for 5 or 10 days, depending on the specific infection and shows a very high lung distribution. Its intrapulmonary concentration is superior to that obtained in plasma. The most common adverse events associated with MXF administration are gastrointestinal disturbances, such as nausea and diarrhea, due to the impact on the human intestinal microflora, mainly on the enterobacteria [9]. These side effects can be avoided by loading the drug into a biodegradable microparticulate system suitable for inhalation. A bioadesive polymer used as carrier, such as chitosan, can reduce the number of MXF administrations, increasing patient compliance.

MXF-loaded chitosan microspheres were prepared by the spray-drying technique using different amounts of glutaraldehyde as crosslinking agent. The systems were characterized for surface morphology and sizes. Technological studies were performed to evaluate the drug encapsulation efficiency and *in vitro* drug release. To assay the influence of the polymeric carrier on MXF bioavailability, an *in vitro* MXF–biomembrane interaction study was performed by using differential scanning calorimety (DSC) and dipalmitoylphosphatidylcholine (DPPC) vesicles as a model of biological membranes. The suitability of the microparticles as sustained intrapulmonary release systems for MXF was assayed by an *in vitro* model of human bronchial epithelium made of a Calu-3 cell monolayer mounted on Franz diffusion cells.

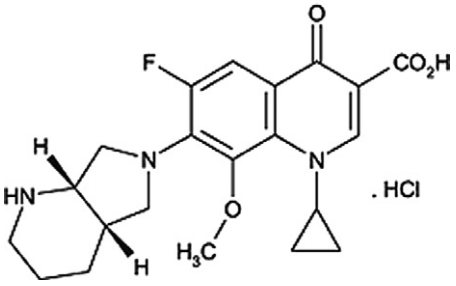

Fig. 1. Chemical structure of moxifloxacin.

2. Materials and methods

2.1. Materials

Moxifloxacin hydrochloride (MXF) was kindly provided by Bayer S.P.A. (BAY 12-8039) (Leverkusen, Germany). Chitosan (copolymer of β(1 → 4) linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose; degree of deacetylation 85%) and glutaraldehyde (GL, 50% aqueous solution) were supplied by Sigma–Aldrich (Milan, Italy). Dipalmitoyl-rac-glycero-3-phosphatidylcholine (DPPC) was purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). All other chemicals and solvents were of analytical reagent grade. De-ionized double-distilled water was used throughout the study.

2.2. Preparation of chitosan microspheres

Microspheres were obtained by the spray-drying technique. Chitosan (0.1%, 0.2% and 0.5% w/v) was dissolved at 50 °C in 200 ml of 1% acetic acid solution by mechanical stirring. MXF (12.5%, 25% and 50%, w/w based on polymer) and different amounts of 50% GL aqueous solution (0, 0.25, 0.5, 1, 2 and 4 ml) were added to the previously filtered chitosan solutions (0.45 μm Nylon Millipore filter). The solutions were stirred mechanically for 15 min, they were then spray-dried in a Bùchi Minispray Model B190 (Bùchi Laboratoriums-Tecknik AG, Flawil, Switzerland) with a standard 0.7 mm nozzle. The manufacturing parameters were: inlet temperature 160 °C; pump rate 10 ml/min; compressed air flow 700 l/h.

2.3. Characterization of MXF-loaded chitosan microspheres

The morphological examination of the microspheres was performed by scanning electron microscopy (SEM) using a Philips mod 500 SEM at 10 kV. Microsphere samples were dried for 24 h at 40 °C before analysis (Buchi TO-51 desiccator). A small amount of each sample was stuck on double-sided adhesive tape attached to a metallic sample stand, then coated, under argon atmosphere, with a thin layer of Au, using a POLARON E5100 SEM Coating Unit.

The size of the microspheres was determined using a Zeiss Axiovert S100 inverted optical microscope (Carl Zeiss, Inc., Thornwood, NY) ($n = 600$ particles). Powders

were placed on microscope slides and images were captured with an EPIX digital camera.

Physical–chemical characterization of microspheres was performed by differential scanning calorimetry (DSC) using a Mettler DSC 12 E (Mettler Toledo Italia, Milan, Italy); equipped with a Haake thermocryostat mod. D8-G (Haake, Karlsruhe, Germany). Mettler TA89E and FP89 system software package was used for data acquisition. Indium was used to calibrate the instrument. Samples of MXF-loaded uncrosslinked (chitosan, 0.2%, w/v; theoretical drug content, 50%, w/w) and MXF-loaded crosslinked microspheres (chitosan, 0.2%, w/v; theoretical drug content, 50%, w/w; GL, 0.5 ml) (15 mg), blank microspheres (13 mg), free drug (2 mg) and polymer (13 mg) were analyzed at a speed of 10 °C min^{-1} in the 30–300 °C temperature range.

Loading capacity of microspheres was determined by dissolving 10 mg of each sample in 10 ml of 0.1 N HCl. The obtained solutions were analyzed by HPLC for quantitative determination of MXF. HPLC analyses were performed at room temperature (25 °C) using a 1050 Hewlett Packard apparatus (Hewlett Packard, Cernusco S/N, Milan, Italy) on a 5 μm end-capped Lichrospher® 100 RP-18e cartridge (250 × 4 mm ID) (Hewlett Packard) equipped with a 5 μm end-capped Lichrospher® 100 RP-18e guard cartridge (4 × 4 mm ID) (Hewlett Packard) and eluted isocratically with acetonitrile/H_3PO_4 (0.4%, w/v) (65:35 v/v). The flow rate was fixed at 1 ml/min and UV light at 296 nm was used for detection. The linear regression coefficient determined in the range 0.148–29.7 μg/ml was 0.99998 ($n = 6$). The method sensitivity was 0.148 μg/ml (signal to noise ratio 3:1).

2.4. In vitro dissolution of MXF-loaded microspheres

In vitro release tests were carried out in phosphate buffer solution (PBS, pH 7.4) at 37 ± 0.5 °C. A suitable amount of microspheres (50 mg) was added to 200 ml of the releasing medium in stoppered bottles and shaken at 100 strokes min^{-1} for 4 days. Aliquots of 0.5 ml were withdrawn at fixed time intervals (5, 15, 30, 45, 60, 120, 180, 240, 360 min and 24, 48, 72, 96 h.) and filtered through 0.45 μm Millipore filters. The sample volumes were replaced with the same amounts of fresh PBS (pH 7.4). All samples were analyzed by HPLC to determine the concentration of MXF. The obtained values were corrected for the dilution used during the sampling. The experiments were carried out in triplicate.

2.5. Liposome preparation

DPPC multilamellar liposomes were prepared in the presence and absence of the free drug or MXF-loaded microspheres, at a temperature above the gel–liquid crystalline phase transition. Lipids (10 mg) were solubilized in chloroform/methanol solution (50/50, v/v) in a round-bottomed flask and the solvent was removed under nitrogen in a rotoevaporator. The resulting film was kept for 4 h at 40 °C under high vacuum (Buchi TO-51 dryer) to remove the residual solvent. DPPC vesicles were prepared by adding 400 μl of PBS (pH 7.4) to the film, then alternatively vortexed (3 min) and warmed in a water bath at 55 °C for 5 min. This procedure was repeated 3 times. MXF was added during the preparation of the film, then it was dissolved together with the lipids at different molar fractions (0, 0.01, 0.02, 0.03, 0.06, and 0.09). MXF-loaded uncrosslinked microspheres (chitosan, 0.2%, w/v; theoretical drug content, 50%, w/w) and MXF-loaded crosslinked microspheres (chitosan, 0.2%, w/v; theoretical drug content, 50%, w/w; GL, 0.5 ml) were added during liposome preparation by suspending an amount of each sample in PBS (pH 7.4) so as to obtain molar fractions equal to those of the free drug. The biomembrane suspension was left at room temperature for 3 h to anneal the bilayer structures. Aliquots of 40 μl (1 mg of the lipid) were then transferred to 40 μl DSC aluminum pans and submitted to DSC analysis. All samples were submitted to four heating/cooling cycles in the temperature range 20–60 °C at a scanning rate of 1 °C/min. Data from the first scan were always discarded to avoid mixing artifacts. Due to periodic recalibration, a control sample consisting of a drug-free DPPC biomembrane suspension accompanied each set of experiments. The endothermal peak coming from the second scan of the control sample was used as a reference template. All samples, after calorimetric scans, were removed from the pans and aliquots were used to determine the amount of the phospholipids using Bartlett's [10] phosphorus assay. Three replicates were performed for each DSC experiment.

2.6. Kinetic experiments

MXF-loaded uncrosslinked and crosslinked microspheres (chitosan, 0.2%, w/v; theoretical drug content, 50%, w/w; GL, 0.5 ml) were added to the DPPC liposomes in amounts to obtain a 0.09 molar fraction of the drug with respect to the lipid (10 mg). Forty microlitres of each sample was placed in 40 μl DSC aluminum pans and incubated in a Haake thermocryostat mod. D8-G at 37 °C. The samples were analyzed immediately after preparation and after 12, 24, 36 and 48 h of incubation. All samples were submitted to DSC scans after four heating/cooling cycles in the temperature range 20–60 °C at a scanning rate of 1 °C/min.

2.7. Cell culture

Calu-3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), penicillin (100 U/ml), 1% L-glutamine and 1% non-essential amino acids (NEAA). For the experiments the cells were trypsinized, counted in a haemocytometer and plated onto Snapwell™ polyester inserts (1.2 cm^2, 0.4 μm pore size; Costar Corporation) at

a density of 5×10^5 cells/cm^2. Cells were maintained in a humidified 5% CO_2 air atmosphere at 37 °C. The growth media were replaced every two days. Confluent cell monolayers were obtained 12 days after seeding.

2.8. Permeation experiments through Calu-3 bronchial epithelial cell monolayer

Permeation experiments were performed by means of Franz type diffusion cells, placing the Snapwell™ containing the Calu-3 cells monolayer in the donor compartment. An aliquot of 200 μl of each sample, containing 200 μg/ml (PBS, pH 7.4) of free MXF or the equivalent amount of the MXF-loaded uncrosslinked and crosslinked microspheres (chitosan, 0.2%, w/v; theoretical drug content, 50%, w/w; GL, 0.5 ml), was applied to the monolayer. The acceptor phase was PBS (pH 7.4). Samples of the receiving solution were withdrawn at different times during the experimental period (6 h); the sample volumes were replaced with the same amounts of fresh PBS (pH 7.4). All samples were analyzed by HPLC to determine the concentration of MXF. The obtained values were corrected for the dilution used during the sampling. Cell-free Snapwells were used as control. Each experiment was performed 6 times.

2.9. Evaluation of monolayer integrity

Before starting permeation experiments through the Calu-3 cell monolayer, the confluence of the endothelial cells was controlled by measuring sodium fluorescein transport and transepithelial electrical resistance (TEER).

2.9.1. Sodium fluorescein transport

An amount (200 μl) of 1 mg/ml in PBS (pH 7.4) of sodium fluorescein was applied into Snapwells™ mounted on Franz diffusion cells. PBS (pH 7.4) was used as acceptor phase. Samples of the receiving solution were withdrawn after 1 h and at the end of the experimental period (6 h). The amount of sodium fluorescein in the acceptor compartment was determined spectrophotometrically at 486 nm. A monolayer was considered to be a ''tight monolayer'' when the amount of sodium fluorescein in the acceptor compartment was less than 1% of the initial amount in the donor compartment [11]. Cell-free Snapwells™ were used as control.

2.9.2. Transepithelial electrical resistance measurement

Transepithelial electrical resistance (TEER) across the monolayer was measured using Millicell-ERS (Millipore, Bedford, MA). The measurements were made before the application of the different samples on the monolayer, during permeation and at the end of the experiments (0, 2, 4 and 6 h). The assayed samples then were removed and replaced with fresh culture medium. After 2 h (8 h from the beginning of the experiments) the TEER was remeasured. Each monolayer was measured three time and the mean value was calculated. The mean resistance of a cell-free Snapwells™ was subtracted from the resistance measured across each layer to yield the TEER value of the cell monolayer.

3. Results and discussion

3.1. Microsphere characterization

MXF-loaded microspheres were produced by the spray-drying technique using different GL amounts as crosslinking agent. All microparticles showed a normal distribution of sizes, ranging from about 2.5 to 6 μm (Table 1) and thus suitable for inhalation. Crosslinked microspheres had a yellow-brown color, which was more intense with the increasing crosslinking density. SEM analysis showed a spherical surface, smooth but distorted, for all samples, probably due to the low viscosity grade of the chitosan solutions

Table 1
The percentage yield, mean particle size and encapsulation parameters of spray-dried chitosan microspheres loading MXF

Chitosan concentration (%, w/v)	Theoretical MXF content (%, w/w)	GL solution (ml)	Yield[a] (%)	DC[a] (%, w/w)	EE[a] (%)	Mean particle size[b] (μm)
0.1	12.5	–	79.2 ± 1.1	7.4 ± 0.4	59.2 ± 3.2	2.5 ± 0.5
0.1	25	–	80.23 ± 2.8	15.3 ± 0.6	61.2 ± 2.3	2.7 ± 0.6
0.1	50	–	78.9 ± 1.9	27.9 ± 0.8	55.8 ± 1.6	3.0 ± 0.8
0.2	12.5	–	85.6 ± 1.5	11.5 ± 0.6	92.0 ± 4.8	2.8 ± 0.7
0.2	25	–	84.3 ± 3.1	23.6 ± 1.0	94.4 ± 4.0	3.2 ± 1.0
0.2	50	–	86.9 ± 2.5	39.9 ± 1.9	79.8 ± 3.9	3.4 ± 0.9
0.2	50	0.25	87.6 ± 1.2	33.1 ± 0.8	66.2 ± 1.5	3.4 ± 1.2
0.2	50	0.5	88.1 ± 1.4	32.5 ± 1.5	65.0 ± 3.0	3.2 ± 0.8
0.2	50	1	86.1 ± 2.5	29.5 ± 1.0	59.0 ±2.1	4.0 ± 1.1
0.2	50	2	81 ± 1	27.8 ± 1.2	55.6 ± 2.3	3.9 ± 1.2
0.2	50	4	85.8 ± 3.4	26.2 ± 1.2	52.4 ± 2.4	3.8 ± 1.5
0.5	12.5	–	88.4 ± 2.8	11.7 ± 0.6	93.6 ± 4.5	5.1 ± 0.9
0.5	25	–	88.5 ± 1.1	23.8 ± 1.1	95.2 ± 4.5	5.8 ± 2.0
0.5	50	–	86.9 ± 1.2	40 ± 1.3	80. ± 2.2	4.5 ± 1.4

[a] The values are means of three determinations (±SD).
[b] The values are means of 600 determinations (±SD).

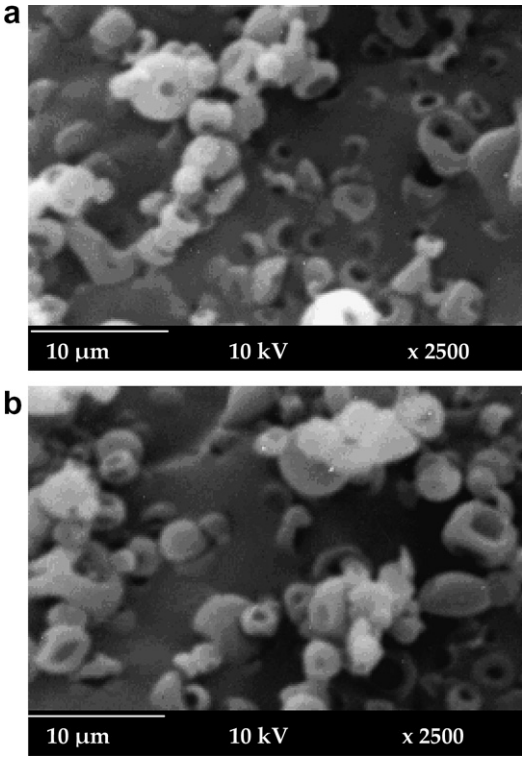

Fig. 2. Scanning electron microscopy pictures of MXF-loaded uncrosslinked (a) and crosslinked chitosan microphores (b) (chitosan concentration 0.2%, w/v; theoretical MXF content, 50%; w/w, GL, 0.5 ml).

used [12] or to the fast evaporation of water or high shear generated during the spray-drying process [13] (Fig. 2).

The variation of the formulative parameters (different concentrations of chitosan, MXF and GL) differently influenced the microparticle sizes. Higher chitosan concentrations produced larger sized microspheres, due to the presence of a greater polymer amount in the same volume of a liquid droplet [12]. No significant variations in terms of particle sizes were observed for MXF and GL concentrations. It is probable that the influence of the drug on droplet concentration is not significant with respect to the chitosan concentration and is not able to modify microsphere size and GL, only modify the nature of the polymer (crosslinked rather than uncrosslinked) not the concentration. The percentage yield of all microparticles produced was between 80% and 88.5%. The drug content (DC) and the encapsulation efficiency (EE) of the spray-dried chitosan microspheres are reported in Table 1. Higher EE was observed at the higher chitosan concentrations. A greater amount of polymer is obviously able to incorporate a greater amount of the drug. No influence on EE was observed varying the MXF percentage from 12.5% to 25% but a decrease from about 90% to 80% was observed increasing the MXF concentration to 50% (w/w). The DC% of this system was higher with respect to the microspheres prepared with the lesser MXF amount. A reduction of EE was produced by reticulation, particularly significant at the highest GL concentration; GL is probably able to interact with the amine groups of MXF, forming a complex that could be spray dried separately from the microspheres [12].

Physico-chemical characterization of microspheres was performed by DSC analysis. The DSC thermograms of pure MXF and microspheres are shown in Fig. 3. Pure MXF had a single endothermic peak due to the drug melting at 248 °C (Fig. 3a), which broadened and shifted a little to a lower temperature (203 °C) in the trace of MXF blank microsphere physical mixture (Fig. 3d), showing the presence of the drug in a crystalline form. MXF melting peak totally disappeared in the calorimetric curve of MXF-loaded uncrosslinked and crosslinked chitosan microspheres, evidencing the presence of a solid dispersion of the amorphous drug into the chitosan matrix (Figs. 3e and f). The main events of all calorimetric curves of the samples containing chitosan were two peaks, the first was endothermic at about 100 °C and the second was exothermic at about 270 °C. As reported by Neto et al. [14] these peaks were due to the loss of water linked to amine or hydroxyl groups of chitosan and to degradation of the main chain of the polymer, respectively.

No endothermic peak related to glass transition (T_g) of the pure chitosan and microspheres was seen in the experimental conditions.

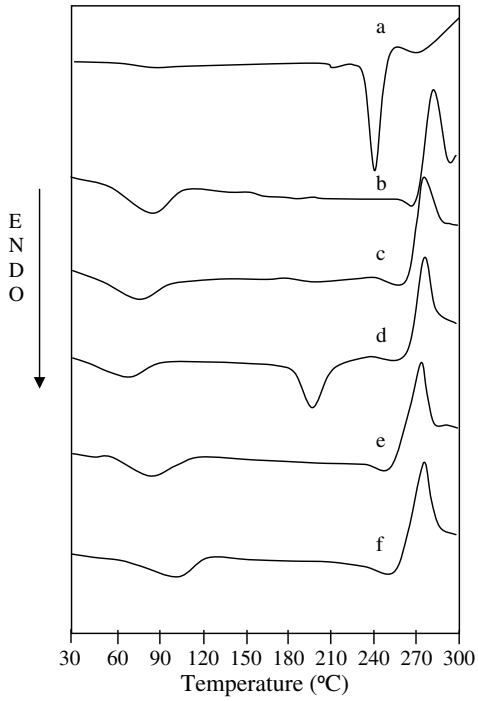

Fig. 3. DSC scans of chitosan microspheres. (a) Free MXF; (b) pure chitosan; (c) unloaded microspheres; (d) MXF: unloaded microsphere physical mixture; (e) MXF-loaded uncrosslinked microspheres (chitosan, 0.2%, w/v; theoretical drug content, 50%, w/w); (f) MXF-loaded crosslinked microspheres (0.5 ml of GL aqueous solution).

3.2. In vitro drug release

The release of MXF from crosslinked and uncrosslinked chitosan microspheres (theoretical drug content 25% w/w) prepared from 0.2% w/v chitosan concentration is shown in Fig. 4. A significant burst effect was observed for all crosslinked samples, particularly at the highest GL concentration. About 60%, 40% and 25% (w/w) of MXF was released within the first hour for the systems prepared in the presence of 4, 2 and 0.5 ml of GL, respectively. Reticulation probably produces a superficial distribution of MXF in the microspheres [15]. The drug was then rapidly released. The MXF that remained inside the systems was localized more internally and, as expected, it was released slowly, particularly in the presence of the highest GL concentration. As reported [12,16], reticulation produces microspheres with a rigid structure that limits drug diffusion; within 4 days only a further 32% (w/w), 22% (w/w) and 10% (w/w) of loaded MXF was released from the systems prepared with 0.5, 2 and 4 ml of crosslinking agent, respectively. The uncrosslinked microspheres did not maintain the form of spheres in water, they swelled and rapidly dissolved. For this reason a very fast release of the loaded drug was expected. However, all the drug loaded into the uncrosslinked microspheres was released within 24 h. An electrostatic interaction between the carboxylic group of MXF and the amine groups of chitosan probably occurred, thus reducing the release of the free drug from the chitosan network.

3.3. MXF–biomembrane interaction

An *in vitro* drug–biomembrane interaction study may provide important and useful information to understand the drug pharmacology as well as to optimize the development of a sustained release system of the drug. Pure lecithins, such as DPPC, have well-defined thermotropic properties [17,18] and hence any changes in these properties can be easily related to the type of drug-membrane interaction and to the localization in the ordered structure of the bilayer [19]. To evaluate the influence of MXF on the thermotropic properties of lecithin-based vesicles, DSC analysis was carried out.

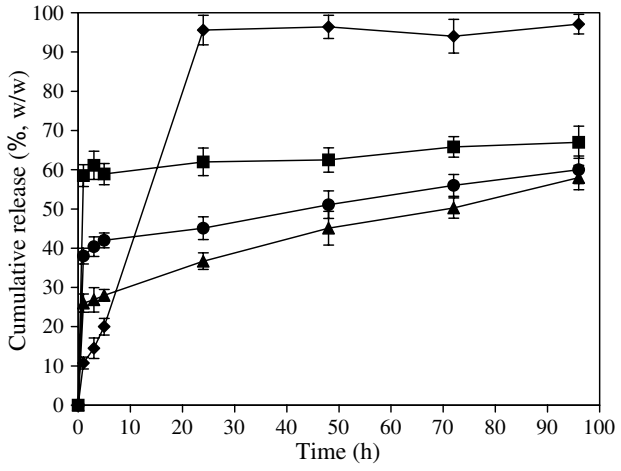

Fig. 4. *In vitro* release profiles of MXF from chitosan microspheres in PBS (pH 7.4) under constant stirring. ♦, Uncrosslinked microspheres; ■, crosslinked with 4 ml of GL; ●, crosslinked with 2 ml of GL; ▲, crosslinked with 0.5 ml of GL.

When DPPC liposomes were submitted to DSC scan, two thermal transitions were observed [17,18]. A sharp acyl chain melting transition ($P_\beta \rightarrow L_\alpha$) at about 41 °C, which is associated to the passage from a highly ordered gel state, in which phospholipid acyl chains are in *all-trans* conformation, to a less packed and more fluid liquid crystal phase due to an increased *trans–gauche* isomerization of acyl chains. A small broad pretransition, so-called $L_\beta \rightarrow P_\beta$, was observed at about 36 °C. This seems to be due to a conformational change and/or packing order rearrangement at the level of phosphor-diester groups and glycerol backbone of DPPC (data not shown).

The incorporation of MXF in DPPC liposomes produces a slight shift of temperature and a progressive depression of the pretransition peak (lowering ΔH) at increasing drug molar fractions (data not shown). Because the pretransition is highly sensitive to the presence of other molecules in the polar region of the phospholipids, the influence on pretransition cannot be ascribed to a specific molecular change. No influence on the main phase transition temperature of the DPPC bilayer was exerted by different MXF molar fractions (Table 2). A reduction of ΔH value of the main transition was observed at MXF molar ratios up to 0.03. A slight broadening of the peak was also observed, evidencing a moderate loss of cooperativity between the phospholipid alkyl chain. This trend can be related to the hydrophobic characteristics of the drug at the experimental pH value (7.4), which permits the drug to freely permeate through the biomembranes. During this passage MXF is able to interact with the hydrophobic zone of the bilayers, lowering the ΔH value. Similar results were obtained by Fresta et al. [20] for ofloxacin.

Table 3 shows the effect of different fractions of MXF-loaded microspheres on the DPPC vesicles' main phase transition. No influence was observed on temperature, which remained unchanged in the presence of both crosslinked and uncrosslinked microspheres. However, the two systems influenced the ΔH value of transition peak of

Table 2
Thermotropic parameters of mesophase transition from gel (P_β) to liquid crystalline phase (L_α) of multilamellar vesicle dispersions made up of DPPC at different molar fractions of MXF

MXF molar fraction	Transition temperature (K)	ΔH (kJ mol^{-1})	$\Delta T_{1/2}$ (K)
0	314.5	−37.90	2.1
0.01	314.1	−37.10	2.0
0.03	314.3	−37.70	2.2
0.06	314.3	−35.00	2.5
0.09	314.3	−32.90	2.6

Table 3
Thermotropic parameters of mesophase transition from gel (P_β) to liquid crystalline phase (L_α) of multilamellar vesicle dispersions made up of DPPC at different molar fractions of MXF-loaded uncrosslinked[a] and crosslinked microspheres[b]

MXF molar fraction	Transition temperature (K)		ΔH (kJ mol^{-1})		$\Delta T_{1/2}$ (K)	
	Uncrosslinked microspheres	Crosslinked microspheres	Uncrosslinked microspheres	Crosslinked microspheres	Uncrosslinked microspheres	Crosslinked microspheres
0	314.5		−37.90		2.1	
0.01	314.2	314.1	−39.90	−37.40	2.0	2.1
0.03	314.1	314.1	−39.55	−37.60	2.1	2.1
0.06	314.2	314.1	−37.10	−36.60	2.2	2.0
0.09	314.2	314.2	−37.90	−35.25	2.4	2.6

[a] Microspheres were made of 0.2% (w/v) chitosan concentration and theoretical drug content of 50 mg.
[b] Microspheres were made of 0.2% (w/v) chitosan concentration, theoretical drug content of 50 mg and 0.5 ml of GL solution.

DPPC liposomes in a different way. The influence on ΔH derives from the superimposition of different actions on biomembrane models exerted by microspheres and/or by dissolved chitosan from microspheres and by free MXF released from the systems. At lower drug molar fractions (below 0.06) no influence of released MXF was predictable, in fact, free MXF is able to reduce the ΔH value of the main phase transition peak only at higher molar fractions (Table 2). We must consider that in the experimental conditions (DSC scans were performed after 3 h from the liposome preparation) the drug was partially released from the microspheres (see Fig. 4), thus free MXF was able to interact with the DPPC bilayer less than the theoretical drug molar fraction (about 50% and 10% for crosslinked and uncrosslinked microspheres, respectively).

The increase of ΔH value observed for the uncrosslinked system up to 0.03 theoretical drug molar fraction could be due to an action exerted by chitosan on biomembranes. Fang et al. [21] reported that chitosan is able, at pH values below 6.0, to perturb the DPPC membranes, lowering the ΔH value of the main phase transition by a long-range electrostatic repulsion between the amines of chitosan and the cationic choline headgroup of DPPC. This perturbation is obviously higher at lower pH values due to the protonation of amine groups.

Our DSC scans were performed in PBS at a pH value of 7.4; in these conditions the amine groups of the polymer were present principally as free base, thus no repulsion was observed but an electrostatic interaction with the phosphate group of the polar heads of lipids could be present. In this way, the electrostatic repulsion forces among choline groups were reduced ensuring a tight packing of the phospholipid alkyl chains, which enhances van der Waals reticular energy of the hydrophobic domain, and leads to the enhancement in the ΔH value. Increasing the theoretical MXF molar fraction, we observed, for uncrosslinked microspheres, a reduction of ΔH value until to value of pure DPPC. This trend was probably the result of the opposite action of chitosan and MXF on the bilayer. In fact, it is conceivable that the increase of the ΔH value produced by chitosan was counterbalanced by the ΔH lowering produced by the penetration of MXF into the biomembrane model.

No influence was exerted until 0.03 drug molar fraction by crosslinked microspheres on DPPC liposomes (no variation of the ΔH value was observed), this is probably a result of the reticulation that not only reduces the number of free amine groups of chitosan able to interact with polar lipid heads, but, in the mean time, reduces the rate of microspheres swelled and consequently the rate of chitosan dissolution. A significant decrease of the ΔH value was observed at theoretical drug molar fractions up to 0.03. Because no effect was exerted by reticulate chitosan on the DPPC bilayer (data not shown), this effect could be due exclusively to the MXF penetration into the hydrophobic domain of liposomes. The absence of correspondence between these ΔH values and those detected for free MXF was the result of the sustained release of the drug from the system that reduces the amount of free MXF interacting with the biomembrane model.

Fig. 5 shows the release kinetics of MXF from crosslinked and uncrosslinked microspheres to DPPC liposomes. A progressive decrease of the ΔH value of the

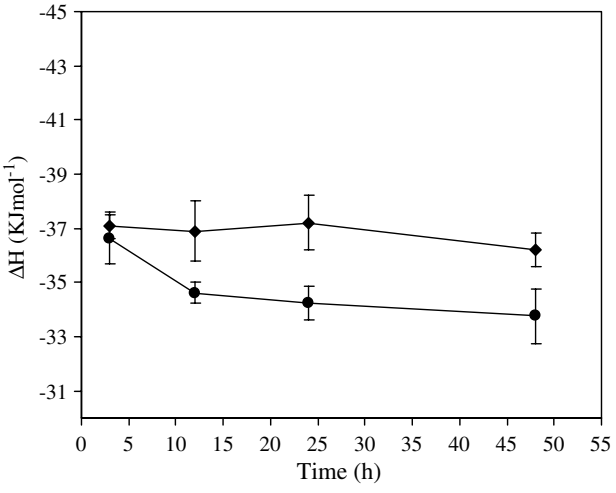

Fig. 5. Enthalpy variation of $P_\beta \rightarrow L_\alpha$ transition of DPPC multilamellar vesicles as a function of MXF released from microspheres crosslinked with 0.5 ml of GL (●) and uncrosslinked microspheres (♦) to void liposomes after incubating at 37 °C for 12, 24, 36 and 48 h. All samples were submitted to DSC scans using four heating/cooling cycles in the temperature range 20–60 °C at a scanning rate of 1 °C/min.

main transition peak over time was observed for MXF-loaded crosslinked microspheres, evidencing a progressive transfer of MXF from the microspheres to liposomes. MXF-loaded uncrosslinked microspheres first produce (until 24 h) no effect on the ΔH value, thereafter there is only a slight decrease. Probably, within the first 24 h of incubation the effects of free MXF and dissolved chitosan on the biomembrane were counterbalanced, but, increasing the time of incubation the predominant effect was due to free MXF that diffused from swollen chitosan microspheres to lipid biomembranes.

3.4. In vitro permeation studies

To evaluate the potentiality of chitosan microspheres as a sustained release system for intrapulmonary administration of MXF, an *in vitro* study was performed using a monolayer of Calu-3 cells. The Calu-3 cells are a human bronchial epithelial cell line isolated from an adeno-carcinoma of the lung [22]. They form confluent monolayers of mixed phenotype, including ciliated and secreting cells [23]. Calu-3 cells were recently used as a model to study the uptake of microparticulate carriers into the pulmonary epithelium [24].

For permeability studies, the monolayer was mounted on Franz diffusion cells and the receptor phase was sampled within 6 h to assay the amount of permeated drug. The monolayer integrity and cell vitality in the experimental conditions were assayed by evaluating sodium fluorescein transport through the monolayer and TEER measurements. Less than 1% of fluorescein was detected within 6 h in the receptor compartment, demonstrating confluence and cell vitality for all the experimental time. The corresponding TEER values were between 400 and 500 $\Omega\,cm^2$ all the time. The monolayer treated with fluorescein could not be used for other experiments. However, because Geys et al. [25] demonstrated a linear correlation between fluorescein transport through the monolayer and TEER value; these latter can be used as a control of cell vitality before application of the microspheres and during experiments. Only the monolayers with a TEER value equal to the control were used for the permeation studies.

No cytotoxicity was observed in the presence of the free drug or both crosslinked and uncrosslinked microspheres during the experiments, this was also demonstrated by Huang et al. [26]. In fact, no significant variation on TEER measurements was observed for the monolayers treated with free MXF and MXF-loaded crosslinked microspheres (chitosan concentration, 0.2% (w/v); theoretical drug content, 50% (w/w), GL, 0.5 ml) (see Fig. 6). Only in the presence of MXF-loaded uncrosslinked microspheres did we observe a slight reduction of the initial TEER value (about 20%). However, cell vitality was not compromised and after removing the sample and incubation with the culture medium, the TEER value of the monolayer increased again. According to Artursson et al. [27] chitosan can open the tight junctions reducing the TEER value and probably

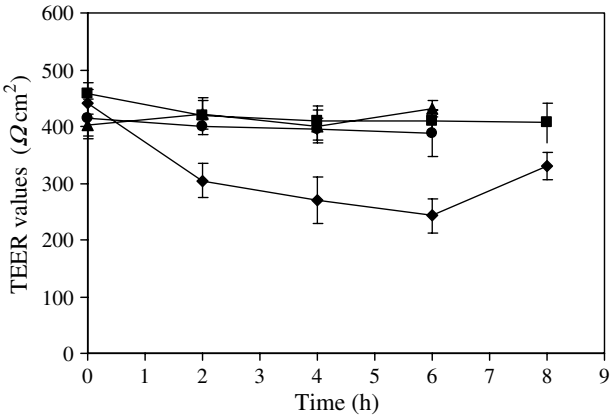

Fig. 6. Effect of chitosan microspheres on the TEER of a Calu-3 cell monolayer at pH 7.4. Each point represents the mean ± SD of 3 experiments. Keys: ■, control; ●, free MXF; ♦, MXF-loaded uncrosslinked microspheres; ▲, MXF-loaded crosslinked microspheres.

increasing drug permeation. This action was reversible and after removing the samples the junctions were restored.

The influence of the carrier on the absorption of MXF through the monolayers is shown in Fig. 7. A rapid absorption of the free drug with respect to MXF-loaded crosslinked or uncrosslinked microspheres was observed. Within 6 h the cumulative amount of permeated MXF was about 18%, 11% and 7% (w/w) for free MXF, MXF-loaded crosslinked and MXF-loaded uncrosslinked microspheres, respectively. The trend observed was in part expected. In fact, considering that only the free drug would be able to cross the monolayer, the first factor that plays an important role in the absorption of MXF through the Calu-3 cells would be the amount of free MXF available for permeation. Obviously, in the sample containing not loaded MXF, the drug was immediately available as a function of its dissolution rate into PBS and a rapid permeation was expected. As concerns MXF loaded into chitosan microspheres, only the drug superficially

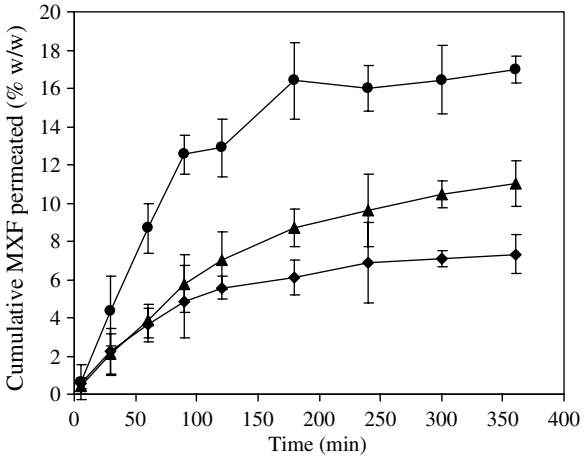

Fig. 7. Permeation profiles through a Calu-3 cell monolayer of free MXF or loaded into crosslinked and uncrosslinked chitosan microspheres. ●, Free MXF; ♦, MXF-loaded uncrosslinked microspheres; ▲, MXF-loaded crosslinked microspheres.

absorbed on the systems was immediately released (see burst effect in the drug release profiles), the other one was released from the polymeric matrix with different rates as a result of different degrees of crosslinking. This fact produced a retardation in the MXF permeation with respect to the free drug.

Given the aim of this study, that is, to realize a system able to progressively release the drug, after an initial immediate release, thus reducing the number of administrations, the crosslinking of chitosan with GL would be a very important step to obtain a rigid structure. In this way, the MXF release was prolonged as well as its absorption. However, *in vitro* permeation profiles showed, after an initial comparable absorption (within the first hours of the experiment), a more rapid MXF absorption from crosslinked rather than uncrosslinked microspheres. This trend could, in part, be justified by the high burst effect observed for crosslinked systems (about 30%, w/w, of loaded MXF), and not for the uncrosslinked microspheres (about 10%, w/w, of loaded MXF). The immediate release of consistent amounts of MXF for the crosslinked system can counterbalance the sustained drug release observed in the second phase of its release profile, thus obtaining a higher MXF permeation through the monolayer with respect to uncrosslinked microspheres. It is also conceivable that Calu-3 cells are able to internalize chitosan microspheres, permitting the penetration of MXF not only as free drug but also loaded in the microspheres. Because only crosslinked chitosan maintained the structure of microspheres in water, cellular uptake was expressed only for these systems. Other studies are in progress to confirm this hypothesis. Concerning the first portion of release profiles, in which, although there was the presence of higher amounts of free MXF for crosslinked systems, overlapped curves were obtained for both systems, another factor can be considered, the ability of free chitosan to interact with biological membranes. As demonstrated by the measured TEER value, dissolved chitosan from uncrosslinked microspheres can produce leakage in the monolayer, enhancing the amount of permeated MXF. On the other hand, the results obtained in the permeation experiments correlated well with our MXF–biomembrane interaction studies which demonstrated a rapid penetration of free MXF through the phospholipid bilayer and the ability of uncrosslinked microspheres only to alter the packing of the liposome bilayer, so allowing higher penetration of the drug through the biomembrane.

4. Conclusions

Spray drying produces spherical particles with suitable sizes for inhalation and high MXF encapsulation efficiency. *In vitro* DSC studies demonstrated that uncrosslinked chitosan microspheres are able to alter the packing of a biomembrane model due to an interaction of free chitosan dissolved from the microspheres with the phospholipid polar heads. As a result of reticulation with glutaraldehyde, chitosan microspheres lose this ability and do not produce any alteration on MXF permeation. These results correlate well with *in vitro* biological studies performed on Calu-3 cell monolayers that showed an influence of uncrosslinked microspheres on the monolayer, thus increasing the amount of permeated MXF in the first hour of the experiment with respect to the crosslinked system. Successively, the high burst effect observed in the release studies for crosslinked microspheres produced a higher permeation of MXF for this system with respect to uncrosslinked microspheres. These *in vitro* results would seem to demonstrate that the reticulation with GL is not needed to retard absorption of MXF through pulmonary epithelium, however, we must consider that, due to the loss of cellular vitality, *in vitro* biological studies were performed for a limited time with respect to the release studies. These latter demonstrated a prolonged MXF release from crosslinked microspheres up to 4 days. It is conceivable that such an *in vivo* system is able to immediately release the necessary dose after inhalation due to the appearance of this effect and successively maintain it for some days. *In vivo* studies are necessary to clarify this concept.

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