



## Release of rifampicin from chitosan, PLGA and chitosan-coated PLGA microparticles

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### ABSTRACT

Recently three groups of rifampicin (RIF)-loaded microparticles (MPs), consisting of chitosan (CHT), PLGA and PLGA/CHT mixtures, were assessed in terms of RIF-loading and retention during nebulisation. The CHT-coated PLGA MPs were found to exhibit high RIF-loading ability together with nebulisation ability, stability, and mucoadhesive properties. All MP types had comparable toxicity towards alveolar cells which was significantly lower than that of the free drug. Herein, we study the release of RIF from all MP-types, during incubation in buffer with pH values: 4.40 and 7.40. Results show that CHT particles exhibit a higher burst release compared to PLGA MPs; at pH 4.40, which is explained by the higher solubility of CHT in acidic media. At pH 7.40 burst release from CHT MP's is significantly lower when CHT is crosslinked with glutaraldehyde, which is consistent with their – previously observed – increased stability during nebulization. From PLGA MPs, RIF release was pH independent under the conditions applied, while the amount of PVA (stabilizer) considerably affected drug release. When PLGA MP's were coated with CHT, at pH 7.40 the retention of RIF increased further (compared to non-coated MPs), while at pH 4.40 the release was faster from the CHT-coated particles.

Concluding, it is proven that when PLGA MPs are coated with CHT, in addition to increased particle mucoadhesive properties, the release kinetics of RIF are modified.

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## 1. Introduction

The use of polymeric microparticles (MPs) for the delivery of drugs which are intended to act topically, by nebulization was recently investigated [1]. Polylactide-co-glycolide (PLGA), chitosan (CHT) and PLGA/CHT microparticles (MP) were prepared by emulsion or precipitation techniques, and their ability to encapsulate RIF (EE%) or to be nebulized (NE%) [as well as their stability during freeze-drying or/and nebulization (NEED%)], were evaluated. MP cytotoxicity and mucoadhesive properties were also investigated. It was found that in all cases, freeze-drying prior to nebulization did not affect EE%, NE or NEED%. In CHT MP's RIF encapsulation efficiency (EE%) decreased with increasing CHT concentration (and thereby viscosity) and CHT-MP NEED% was higher when the polymer was crosslinked by glutaraldehyde. PLGA MPs, exhibited both higher RIF EE% and also higher nebulization ability and NEED%, compared to the CHT ones, but also slightly higher cytotoxicity.

However, when the two polymers were combined in the PLGA/CHT MPs, EE%, NE% and NEED% increased with increasing MP CHT-content. PLGA/CHT MPs with 0.50% or 0.75% CHT exhibited highest EE% for RIF and also best nebulization ability and stability, compared to all other studied MP formulations. Additionally, they had good mucoadhesive properties and comparable low cytotoxicity.

However, the release kinetics of the drug from those MPs, was not studied and since drug release from polymeric MPs is an important characteristic that has great impact on their applicability as drug carriers, as reported in many instances [2–5], we investigated herein the release of RIF from RIF-loaded PLGA, CHT and CHT-coated PLGA microparticles at two different pH values 4.40 and 7.40, in order to have a feel of the release kinetics of the drug from the different types of particles under acidic (pH 4.40) and physiological (pH 7.40) conditions.

## 2. Materials and methods

### 2.1. Materials

Medium molecular weight chitosan [with a deacetylation grade of 87%], poly(lactide-co-glycolide) having a monomer ratio

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**Table 1**  
Preparative parameters of MPs constructed

A. Chitosan MP's			
Formulation	Chitosan (mg/ml)	Acetic acid (ul)/Na <sub>2</sub> SO <sub>4</sub> (ml)	Glut (25%)
CHT-1	2.5	200/2	–
CHT-2	5.0	200/4	–
CHT-3	7.5	200/6	–
CHT-4	2.5	200/2	+
CHT-5	5.0	200/4	+
CHT-6	7.5	200/6	+
B. PLGA MP's			
Formulation	PLGA (mg/ml)	PVA (% w/v)	RIF addition
PLGA	2	4	In organic phase
C. PLGA/CHT MP's			
Formulation	PLGA (mg/ml)	PVA (% w/v)	CHT (% w/v)
Non-coated	2	1	0
0.1% CHT	2	1	0.10
0.25% CHT	2	1	0.25
0.5% CHT	2	1	0.50
0.75% CHT	2	1	0.75

In all cases RIF was included in the formulations (during their preparation) at a concentration of 2 mg/ml.

[lactic acid/glycolic acid] of 75/25, and MW 85.2 kDa, rifampicin (RIF), sodium sulphate, glutaraldehyde, acetic acid and polyvinyl alcohol (PVA) with average MW 30–70 kDa, were purchased by Sigma–Aldrich (Athens, Greece), as well as all other reagents used which were always of analytical grade. Water was always demineralised and distilled.

## 2.2. Preparation of CHT microspheres

RIF-loaded CHT particles were formulated as previously described [1] by the precipitation method [6]. The amounts of components used for each type of MPs formulated are reported in Table 1. In brief, RIF and CHT, at different concentrations, were initially dissolved in acetic acid (2%, v/v). Sodium sulphate (20% w/v) was subsequently added dropwise during vigorous stirring with Ultraturax® T8, IKA (Germany) at 500 rpm and concurrent bath sonication (Branson 1200). After the addition of the full amount of sodium sulphate, stirring and sonication continued for 30 min. In some formulations a solution of glutaraldehyde (25%, w/w) was also added, at this point, in order to evaluate the influence of cross-linking agent on the drug release kinetics of the particles prepared. In all cases, stirring and sonication continued for an additional 30 min period.

Microparticles were purified by centrifugation for 15 min at 6000 rpm in a Biofuge 28RS (Hereaus). The obtained sediment was re-suspended in water and washed again twice. Finally, particles were freeze-dried and stored in dry form refrigerated (5 °C) until evaluation.

## 2.3. Preparation of PLGA microspheres

RIF-loaded PLGA microspheres (MP) were prepared by an O/W solvent evaporation method adapted from Prieto et al. [7]. In brief, 20 mg of PLGA and RIF (appropriate amount for 20 mg/ml in final preparation) were dissolved in 1 ml dichloromethane. This was dispersed in 10 ml of an aqueous phase containing PVA (4%, w/v). The emulsion formed was homogenised for 10 min with an Ultraturax® T8 IKA homogeniser at 800 rpm. Subsequent evaporation of dichloromethane was carried out with mechanical stirring for 6 h at room temperature. The formed microspheres,

were collected by centrifugation and washed by dispersion in water and subsequent centrifugation. This final step was repeated three times, in order to separate the MPs from non-associated drug.

Finally, the particles were freeze-dried and stored in dry form refrigerated (5 °C) until evaluation.

## 2.4. Preparation of PLGA/CHT MPs by emulsion solvent diffusion

For the preparation of CHT-coated PLGA microspheres the emulsion solvent diffusion method in water [8] was used. Chitosan was dissolved (in different concentrations [0.1, 0.25, 0.5 and 0.75%, w/v]) in 50 ml of acetic acid buffer, pH 4.4, which also contained PVA (1%, w/v). The PLGA (100 mg) was dissolved in 5 ml dichloromethane, which was poured into the coating polymer (CHT) aqueous solution (prepared beforehand) at 2 ml/min under Ultraturax® stirring (400 rpm), at room temperature. RIF (20 mg/ml) was dissolved in the organic phase together with PLGA. The PVA presence in the aqueous solution of the coating polymer (CHT) prevented aggregation of the emulsion droplets and sticking of the polymers to the propeller shaft during agitation. After particle formation the entire dispersed system was centrifuged (10,000 rpm 15 min) and the sediment was resuspended in distilled water. This process was repeated (in order to wash the particles from non-associated drug) and the resultant dispersion was subjected to freeze-drying overnight. Control PLGA particles of this type (with no CHT) were also prepared for comparison with the other PLGA particles. The compositions of the different types of formulations prepared are presented in Table 1.

## 2.5. Measurement of amount of RIF in microparticles

The RIF content of each lot of microspheres was determined after extraction of RIF from the particles with acetonitrile. Quantification of the amount of drug was done spectrophotometrically; for this, a series of (known concentration) RIF solutions in acetonitrile (0.5–20 ppm) were prepared and their optical density (485 nm) was measured for the preparation of a standard curve. Unknown concentrations of RIF in the acetonitrile extracts (from the MPs) were then calculated by the standard curve, and finally the amount of RIF entrapped per amount (mg) of MP was determined for each MP type.

The encapsulation efficiency of RIF of the microparticles was calculated by extracting and quantifying the amount of RIF in a known amount of particles both prior to, and after separation of non-entrapped RIF (by centrifugation assisted repeated washings, as mentioned above). Finally, the drug EE% was calculated as the percentage of drug entrapped in microspheres compared to the initial amount of drug recovered from unpurified samples.

## 2.6. RIF Release from MPs

**In vitro release of RIF from CHT, PLGA and CHT-coated PLGA microparticles was determined using the USP-30 (XXX) dissolution apparatus 2 (Paddle).** As dissolution medium we used both phosphate buffer (0.1 mM) pH 7.40 to simulate the pH in the lungs, and acetate buffer pH 4.40 (0.1 mM) in order to evaluate the stability of the various MPs in acidic medium. Freeze-dried formulations were suspended in 500 ml of the dissolution medium, and the amount of microspheres was varied in order to keep the amount of drug in the aqueous medium constant and equal to 25 mg. It has to be clarified, at this point, that the release of RIF was evaluated in a high volume of solute (500 ml) in order to ensure that sink conditions would prevail for the full experimental period, since the purpose of this study was to compare the various types of MPs

and not to simulate *in vivo* conditions (in which case the use of a much lower volume of solute would be required). The experiments were carried out at  $37 \pm 0.3$  °C at a rotation speed of  $100 \pm 2$  rpm. Samples of 1 ml were withdrawn at appropriate time intervals and centrifuged at 10,000 rpm. Supernatants were diluted suitably with acetonitrile and absorbance of the resulting solution was measured at 485 nm. The residue (after centrifugation) was redispersed in 1 ml of the fresh dissolution medium and replaced back into the dissolution apparatus. The cumulative amount of RIF was obtained from the calibration curve of RIF in acetonitrile. A stock solution of RIF (2 mg/ml) was prepared by dissolving the drug in acetonitrile and storing at 4 °C. A standard calibration curve was prepared by using standard solutions that were obtained by appropriate dilution of the stock solution (with acetonitrile).

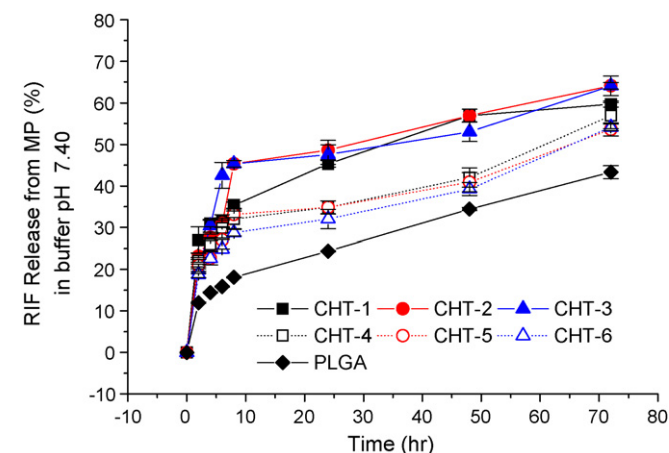
## 2.7. Statistical analyses

All experiments were repeated at least three times. Results are expressed as means  $\pm$  standard deviation. A difference between means was considered significant if the *p*-value was less than or equal to 0.05.

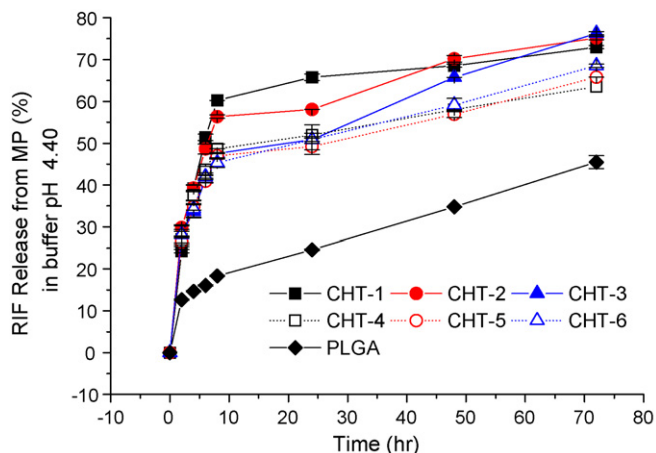
## 3. Results and discussion

The release profiles of RIF from the CHT and plain PLGA microparticles are presented in Figs. 1 and 2, for pH 7.40 and 4.40, respectively. As seen, in general the CHT particles always release RIF faster, compared to those composed by PLGA.

At pH 7.40 (Fig. 1), the burst release measured for PLGA particles at the first time point measured (2 h) was 12%; while for the various types of CHT MPs formulated, it ranged between 20–27% for the MPs with no crosslinker (CHT-1–CHT-3) and 19–22% for the particles with glutaraldehyde in their composition (CHT-4–CHT-6). In fact, the addition of crosslinker in the CHT particles resulted in a marketed decrease of RIF release, proving that the particle stability was increased by the crosslinking reaction. Indeed, it has been suggested earlier, that glutaraldehyde addition can be used as a method to modulate the release kinetics of drugs from chitosan particles, as demonstrated for theophylline [9]. The slower release of RIF from the crosslinked MPs is also in good agreement with the increased stability during nebulization demonstrated previously



**Fig. 1.** RIF release profile (% of initially encapsulated RIF which is released from the particles) from PLGA and CHT MPs, at different time points of incubation in Phosphate buffer pH 7.40. Each point is the mean value calculated from release experiments carried out, as described in detail in methods Section 2.6, in at least three different samples of each MP type studied and standard deviation of the mean is presented as bars. The key for data points is presented in the figure insert.



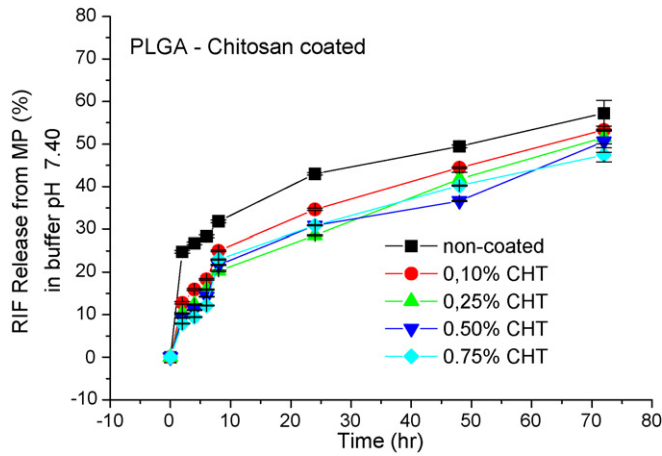
**Fig. 2.** RIF release profile (% of initially encapsulated RIF which is released from the particles) from PLGA and CHT MPs, at different time points of incubation in acetate buffer pH 4.40. Each point is the mean value calculated from release experiments carried out, as described in detail in Section 2.6, in at least three different samples of each MP type studied and standard deviation of the mean is presented as bars. The key for data points is presented in the figure insert.

for these (crosslinked CHT) MP's (compared to the ones without crosslinker).

At pH 4.40 (Fig. 2), the higher retention of RIF in the crosslinked CHT MPs was not as pronounced as in the case of pH 7.40. Indeed, in the more acidic media both types of CHT MPs released the drug considerably faster compared to the release profile observed for each MP type at pH 7.40. Under acidic conditions the burst release values (at the 2 h time point) were ranging between 25–30% for the MPs with no crosslinker (CHT-1–CHT-3), and 26–28% for those with glutaraldehyde in their composition (CHT-4–CHT-6). This faster release from the CHT MPs in media of acidic pH is attributed to the higher solubility of CHT at lower pH. In fact, as proposed earlier [10], CHT microspheres can provide a pH-responsive release profile for entrapped drugs, due to dissolution of the polymer in the acidic environment of the gastric fluid. In this case crosslinking reactions cannot substantially modulate the particle release kinetics, since the release of the drug under the acidic conditions prevailing is mainly attributed to the solubilization of the polymeric particles, and the diffusion of drug molecule through the polymeric matrix is a less important mechanism for drug release (compared to what happens at physiological pH).

Oppositely with what was observed for the CHT MPs, the release of RIF from PLGA MPs is independent of pH (at least in the pH range 4.40–7.40, which was evaluated herein). As reported before the release of RIF or other drugs from PLGA particles is influenced by several factors as PLGA molecular weight and monomer composition (lactid acid/glycolic acid) as well as solute pH [11], and polymer transition temperature (*T<sub>g</sub>*) [12,13]. However, as stated above, we did not see any significant influence of solute pH on RIF release from the PLGA MP's under the conditions applying in our experiments. This could be attributed to the fact that the experimental setup used herein was different from that used by others, resulting in substantially faster release compared with other studies. The most important difference is the solute volume, which was 500 ml in the present studies compared to 250 ml [13], or 5 ml [11] in others. As explained in the Methods section (Section 2.6) this specific setup was used in order to evaluate the release of RIF under sink conditions, since the purpose of this study was to compare the different MPs.

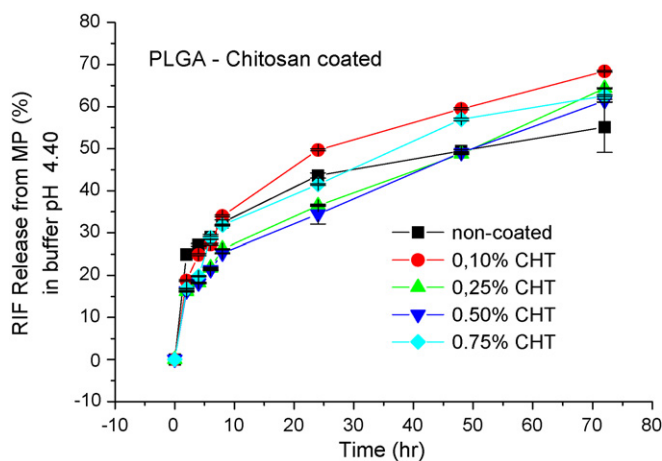
For the CHT-coated PLGA MPs, RIF release results are presented in Figs. 3 and 4 (for pH 7.40 and 4.40, respectively). As seen in Fig. 3,



**Fig. 3.** RIF release profile (% of initially encapsulated RIF which is released from the particles) from various types of CHT-coated PLGA MPs, at different time points of incubation in Phosphate buffer pH. 7.40. Each point is the mean value calculated from release experiments carried out, as described in detail in Section 2.6, in at least three different samples of each MP type studied and standard deviation of the mean is presented as bars. The key for data points is presented in the figure insert.

at pH 7.40, the burst release of the PLGA particles with no CHT is 24.7%; while when CHT is added to the particle composition, at a concentration of 0.10% (w/v) this is substantially reduced to 12.7%. The burst release of RIF from the MPs is furthermore reduced as the concentration of CHT added in the particles increases up to 0.75% (7.9%). This stabilizing effect of CHT on the PLGA MPs was abolished when the release of RIF from particles was studied under acidic conditions, as seen in Fig. 4. Indeed a much faster release of the drug from the CHT-coated MPs was observed in the acidic media (compared to their release profiles at pH 7.40), and the burst release of RIF was 18.8% and 16.9%, for the CHT-coated PLGA MPs containing 0.10 and 0.75% CHT, respectively. On the other hand, the PLGA MPs with no CHT were not affected by the pH change (as also observed for the PLGA MPs studied and presented in Figs. 1 and 2), since the burst release of RIF was 24.7% and 24.9% of the total encapsulated drug at pH 7.40 and 4.40, respectively.

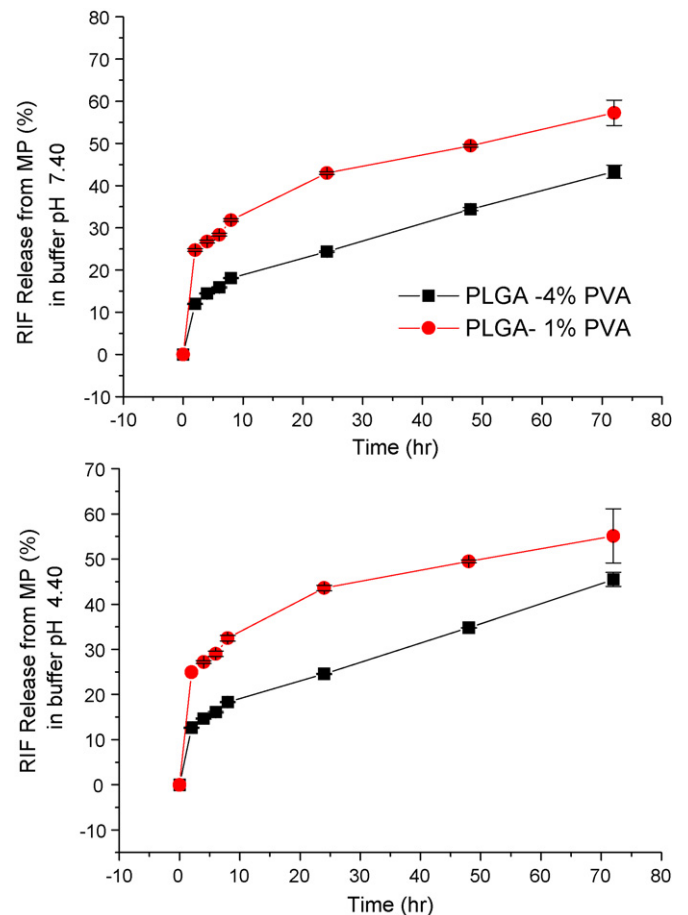
However at pH 4.40, the release profile of RIF from the various types of CHT-coated PLGA MPs is not in direct agreement with



**Fig. 4.** RIF release profile (% of initially encapsulated RIF which is released from the particles) from CHT-coated PLGA MPs, at different time points of incubation in acetate buffer pH. 4.40. Each point is the mean value calculated from release experiments carried out, as described in detail in Section 2.6, in at least three different samples of each MP type studied and standard deviation of the mean is presented as bars. The key for data points is presented in the figure insert.

the CHT content of each MP type, as seen when the RIF release profiles (especially during the first 8 h period of incubation) of the various MPs are compared. The MPs containing the lowest (0.10) and the highest (0.75%) amounts of CHT release RIF faster, while the MPs coated with intermediate CHT concentrations (0.25% and 0.50%) release the drug slightly slower. This difference was not due to a difference in burst release, which was around 16–18% for all the CHT-coated PLGA MPs studied, but mostly due to a different release rate during the period between 2 and 8 h (of incubation). This indicates that in the case of the CHT-coated PLGA MPs the release of RIF under acidic conditions is a more complex process (compared to the cases of plain CHT or plain PLGA MPs). Several concurrent processes, as interactions between RIF and CHT and/or between CHT and PLGA, most probably influence the release of RIF and therefore, depending on the ratios of the different components in each particle type, the influence of the increased CHT solubility in acidic media on the release of the drug, is not directly proportional to the CHT content of each MP.

Another interesting observation is that the release of RIF from the PLGA MPs that contain 4% PVA (the MPs constructed initially), is significantly slower compared to the ones (constructed as control particles together with the chitosan-coated ones) which contain 1% PVA (Fig. 5). Since the only difference in the composition between the two batches of PLGA particles is their PVA content, it is logical to



**Fig. 5.** Comparison of RIF release profile from PLGA MPs containing 1% and 4% of PVA. Percent of initially encapsulated RIF released from the microparticles at different time points of incubation in phosphate buffer pH 7.40 (upper graph) and acetate buffer pH. 4.40 (lower graph). Each point is the mean value calculated from release experiments carried out, as described in detail in Section 2.6, in at least three different samples of each MP type studied and standard deviation of the mean is presented as bars. The key for data points is presented in the figure insert.

conclude that this is the reason for the different RIF release profiles observed for the two MP types. In fact, this conclusion is in good agreement with many recent studies in which the stabilizing effect of PVA on PLGA micro and/or nanoparticles, has been reported. In a study in which the burst release of FITC-dextran loaded PLGA microspheres was investigated, the burst release decreased from 12.2% to 5.9%, by increasing PVA concentration in the continuous phase from 0.1% to 1% [14]. This was attributed to the fact that internal porosity of microspheres decreased considerably with increasing polymer concentration. Additionally [15], the amount of PVA used for formation of PLGA microspheres containing a staphylokinase variant K35R (DGR) by a double-emulsion solvent extraction technique, had an effect on the stability and release of DGR. When 2% PVA was co-encapsulated, DGR encapsulation efficiency was significantly increased from 7.1% to 78.1% and DGR was distributed uniformly throughout the microspheres. Additionally, in vitro release tests showed that a large amount of released DGR was inactive in the absence of co-encapsulated PVA. On the contrary, when 2% PVA was co-encapsulated, the released DGR was almost completely intact even after 9 days. In another study [16], it was observed that PVA content resulted in a significant effect on the particle size of PLGA microspheres (increase of PVA concentration with concomitant increase on stirring velocity produced microspheres with the lower size), while PVA content has also been observed to influence drug loading capacity of PLGA microspheres [17]. Indeed, when flurbiprofen sodium, microspheres were prepared from PLGA (by the solvent evaporation method), as the concentration of PVA increased, the drug loading of the microspheres increased. Although there was no correlation between microsphere size and amount of PVA, an optimum PVA concentration was essential to achieve narrower size distributions of microspheres.

In our case, although the PVA content had a significant influence on the release rate of RIF from the PLGA MPs, it did not influence the MP size (since as reported earlier both types of PLGA MPs had mean diameters around 2.5  $\mu\text{m}$ ), perhaps due to the fact that both types of particles were prepared using the same stirring velocity, however the encapsulation efficiency of RIF was higher in the case of the particles that contained 4% PVA (compared to those with 1% PVA) [1].

#### 4. Concluding summary

Concluding, we evaluated herein the release profile of RIF from various types of PLGA, CHT or CHT-coated PLGA microparticles which were loaded with the drug. The potential use of the constructed particles is to serve as carriers of RIF to alveolar macrophages after they have been nebulized. The release of the drug from CHT or CHT-containing MPs, was found to be pH dependent, while in the case of PLGA MPs the amount of PVA which was added as a stabilizer, had a profound influence on drug release; as PVA content increased drug burst release decreased. Another factor that decreased the burst release of drug from PLGA MPs was CHT-coating. Both of the previous factors, increase of PVA content and CHT-coating, were also previously found to (i) increase the particle stability during nebulization and (ii) decrease the cytotoxicity of the MPs towards alveolar cells [1]. Thereby, we conclude that since also RIF retention in the particles is influenced in a positive way by these parameters, depending on the need to have better mucoadhesive properties [18] or not, CHT-coated PLGA MPs (with a 0.75% of CHT) or PLGA MPS with high content of PVA, will be the com-

positions of choice for particulate-assisted RIF delivery to alveolar macrophages, respectively. Furthermore, it would be interesting to construct and evaluate the performance of CHT-coated PLGA MPs that contain also high amounts (at least higher than 1–5%) of PVA, in future studies.

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