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Development of cyclodextrin microspheres for pulmonary drug delivery

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ABSTRACT

Purpose. Microparticles of diameter < 5 μm were synthesized by interfacial cross-linking of 7.5% (w/v) β-cyclodextrins (β-CD) with 4.5% (w/v) terephtaloyle chloride in 1 M NaOH, in order to provide stable vector for drug encapsulation suitable for administration at the alveolar scale. **Methods.** Batches were prepared varying different parameters such as amount of monomer (β-CD) (5-30% w/v), NaOH concentration (0.5-4 M), reaction time (15-240 min), agitation rate (8000-24000 rpm), amount of cross-linking agent (terephtaloyle chloride: 1.25-10% w/v), surfactant percentage (2.5-10% of Span 85), studying the influence of the freeze-drying step. Microparticles were controlled with respect to their size by a laser diffraction technique, pH of the colloidal suspension, IR spectroscopy, Differential Scanning Calorimetry. After optimization of the microparticles size, complexation with amikacin sulfate was investigated comparing encapsulation efficiency and yield at each step of the preparation (solubilization, emulsification, cross-linking, freeze-drying), contact time and influence of the amount of amikacin. **Results.** An optimized method was obtained with 1 M NaOH, 4.5% (w/v) cross-linking agent and 5% (w/v) surfactant agent, a 30 min reaction time, a 24000 rpm agitation rate, conducting to microparticles whose size is inferior to 5 μm. Amikacin sulfate encapsulation in polycondensed β-cyclodextrin showed that better incorporation was obtained during the solubilization step or just before freeze-drying. **Conclusions.** Amikacin encapsulation in 5 μm diameter microparticles of β-CD is achievable for pulmonary drug delivery.

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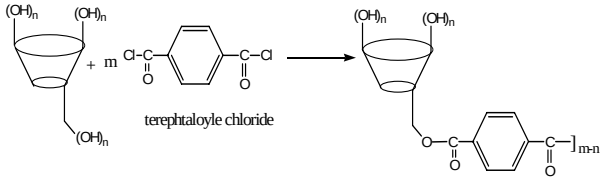
INTRODUCTION

Native cyclodextrins are polysaccharides made up of six to eight cyclic linked oligosaccharides of D-glucopyranose monomers connected by α-1, 4-indican bonds. These compounds form cone-shaped molecules with primary hydroxyl groups (6-OH) arranged in an inner hydrophobic cavity of 5.7, 7.8 and 9.5 Å respectively for α-, β-, γ-cyclodextrins, and secondary hydroxyl groups (2- and 3-OH) rendering external walls hydrophilic (1). These two microenvironments confer to the molecule the ability of forming inclusion complex with guest molecules. Cyclodextrins act as molecular hosts toward various, poorly water-soluble drugs, ranging from ion, very polar molecules to non-polar molecules, affecting advantageously their physicochemical properties (2, 3). Thus, they have found extensive applications in chromatography, catalysis, asymmetric reactions, food, cosmetic, pharmaceutical technology (4), medicinal applications. Partially or entirely encapsulation occurs by the intermediate of hydrophobic forces and van der Waals interactions, ion pairing, hydrogen bonding (5) participating in improving, through complexation, the aqueous solubility and stability of drugs (6-8), vitamins and food colorant, preventing molecules self-aggregation, ameliorating dissolution rate, bioavailability of the hydrophobic drugs, decreasing toxicity and controlling drug releasing (9-11).



Scheme 1. Preparation of the CD microparticles with terephtaloyle chloride.

Preparation of inclusion complexes (4, 12, 13) can be done by different methods namely, coprecipitation, freeze-drying, kneading, grinding or co-pulverizing, microwave heating (14). Inclusion complexes can be formed in solution or in a solid state (15). The advantage of the preparation in the crystalline state is the protection of the complexes formed against some type of reactions such as oxidation, hydrolysis and the role played in the decrease of their sublimation and volatility (16). Guests of varying size were tested by many authors, screening their physicochemical features in function of various stoechiometric ratios (17, 18).

Analytical methods for physicochemical characterization used are fluorescence spectroscopy (steady state fluorescence), NMR spectroscopy, IR

(19), differential scanning calorimetry (DSC), elemental analysis, power X-ray diffraction, thermogravimetric analysis (TGA). When complexed with CDs, modification in the photoreactivity (20, 21) of the guest molecule occurs and generally fluorescence efficiencies are enhanced (22, 23) due to a decrease in non-radiative and quenching processes observed in bulk solution (24, 25). Fluorimetric analysis is also used due to its sensibility and selectivity to evaluate association constants of complexes (26-28). NMR gives useful informations about the geometry of complexes (29-31). Therefore, cyclodextrin microspheres have been shown to be stable vectors for drug encapsulation (10, 30) and may have some applications in pulmonary drug delivery when deposited in the alveolar region.

Two methods for the preparation of drug-containing CD particles are described in the litterature. The first one involves cross-linking drug directly to CDs adsorbed on a porous inorganic oxide via a bifunctional agent, epichlorydrin (32) or sebacoyle chloride (33). The second one requires the synthesis of 10-35 μm microcapsules, in an emulsion system, and subsequent interfacial cross-linking of β-cyclodextrin (β-CD) with terephtaloyle chloride (34). In this paper, we describe our efforts to optimize the second method to produce 5 μm particles intended for alveolar drug delivery following inhalation. Some authors prepared microparticles, with a mean geometric size inferior to 5 μm, from an oil-in-water emulsion consisting of an aqueous phase containing cyclodextrin derivative (35). The first goal of our investigation was devoted to the preparation of microparticles from native cyclodextrins synthesized by interfacial cross-linking with terephtaloyle chloride. We measured the influence of reaction conditions on the resulting microparticle size. Once our objectives were reached, fabrication yield and pH of the colloidal suspension (nearest physiological pH) were optimized and microparticles characterized by granulometry, FT-IR and DSC. The second part of the study concerned the microparticle complexing properties with amikacin sulfate (Figure 1), a potential drug to be administered in pulmonary disease. The effects of variations in the preparation conditions of encapsulated microparticles were investigated. Amikacin is an antibiotic of the class of aminosides used in the treatment of severe infections, particularly those due to aerobic, Gram-negative bacilli (GNB). Their main drawback has been the occurrence of (reversible) nephrotoxicity and ototoxicity in a significant number of patients Nosocomial pneumonia with GNB is the first cause of infection mortality for patients requiring mechanical ventilation. The pulmonary targeting through encapsulation of this antibiotic into cyclodextrin microspheres seems to be interesting to optimize therapeutic efficacy and limit it's toxicity. Particular system based on cyclodextrin microspheres presents a great stability, a high encapsulation efficiency and allows drug spray-drying at the alveolar scale as compared with other drug delivery systems.

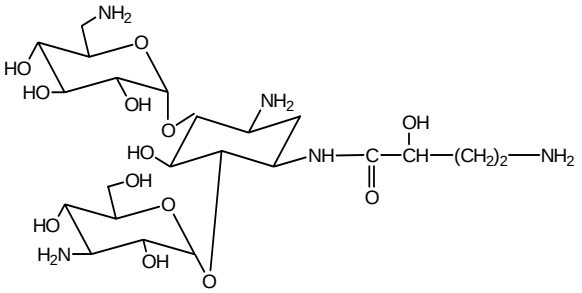


Figure 1. Amikacin sulfate.

Microspheres were synthesized by interfacial cross-linking of β-cyclodextrins (β-CD) with terephtaloyle chloride in 1 M NaOH (Scheme 1). A series of batches were prepared in which formulation parameters such as monomer concentration (5-30% w/v), NaOH concentration (0.5-4M), reaction time (15-240min), agitation rate (8,000-24,000 rpm), polymerization agent concentration (1.25-10% w/v), and surfactant content (2.5-10% of Span 85) were varied. The influence of freeze-drying on the particles size was also investigated. Microspheres size and pH of the colloidal suspension were optimized around two objectives. (1) The maximum yield of particles smaller than 5μm, measured by a laser diffraction technique, and (2) pH nearest to physiological pH. Following size and pH optimization, amikacin sulfate was encapsulated into the microspheres at four different stages of the fabrication process (solubilization, emulsification, cross-linking and freeze-drying) and the encapsulation efficiency (mass of amikacin sulfate in particles (μg)/ mass of β-CD (mg)) and yield (mass of amikacin sulfate in particles (μg)*100/mass of amikacin sulfate introduced (μg)) were determined by a HPLC method (36).

MATERIAL AND METHODS

Material

Amikacin sulfate was purchased from Bristol-Myers Squibb (France). β-cyclodextrin was obtained from Roquette Frères (Lestrem, France). Terephtaloyle chloride was supplied by Sigma Aldrich Chemie

(Allemagne). Sodium hydroxide was from SDS Peypin (France). Sorbitan 85 trioleate, Span®85 (HLB : 1.8), polysorbate 20 (HLB : 16.7), polysorbate 80 (HLB : 15) were provided by SEPPIC laboratory (Orsay). Cyclohexane from Fluka (Buchs, Suisse), chloroform from Prolabo (Fontenay sous Bois, France) and all other reagents were of analytical grade.

Preparation of microparticles

Polycondensation of β-cyclodextrin by terephtaloyle chloride was undertaken by the following standard method :

In a first step, 6 mL of β-CD (7.5% w/v) was solubilized in 1M NaOH and emulsified during 10 min, using a Heidolph RGL 500 stirring motor (Prolabo, France) at a stirring rate of 2000 rpm, in a 30 mL-cyclohexane solution containing 5% (v/v) sorbitan 85 trioleate, at ambiant temperature.-

-Cross-linking solution was prepared dissolving 5% (w/v) terephtaloyle chloride in a (1:4, v/v) mixture chloroform/cyclohexane.

- Microparticles were formed by addition of this organic phase to the emulsion, and mixing with 30 min stirring, the agitation speed being regulated to 2000 rpm. In this study, on contrary to the standard method, agitation rate was regulated to 8000 rpm (Ultra Turrax type TP 18/10 Janke et Kunkel). This cross-linking reaction was stopped by dilution with 40 mL cyclohexane.

The microparticles thus formed were separated by centrifugation (5 min, 3000 rpm), washed successively with cyclohexane, a 95% ethanol solution containing 2% (v/v) polysorbate 20, 95% ethanol and finally with distilled water.

In the last step, the colloidal suspension obtained was freeze-dried (Lyophilisator Virtis® Advantage, vacuum <200 mTorr, condenser <-40°C, plate +30°C).

Influence of different parameters was studied, introducing variations in the standard procedure : amount of monomer, NaOH concentration, reaction time, agitation rate, amount of cross-linking agent and amount of surfactant. All formulations were prepared three times.

Physicochemical characterization of the particles

Influence of these parameters was studied controlling the size of the particles formed by a laser diffraction technique (granulometer Coulter LS 100, Maurepas, France). Size distributions were displayed in term of volume versus particle size. Fabrication yield (mass of particles formed *100 / mass of active substances used), pH of the colloidal suspension measured by a pH meter (Mettler Toledo) were also controlled to optimize the fabrication method, the main goal being firstly the obtention of a granulometry inferior to 5 μm, the second objective consisting in the better fabrication yield possible and a pH nearest the physiological pH.

IR analysis

The FT-IR spectra acquired were taken from dried samples. A FT-IR (Spectrum One®, Lita detector, MIR source and FT-IR Spectrum® software from Perkin Elmer) was used for the analysis in the frequency range between 4000 and 600 cm^{-1}, a 8 cm^{-1} resolution and a 0.2 cm^{-1} rate. The results were the means of 16 determinations. Physical mixtures of microparticles and active drug (1/1) were used as blanks.

Differential scanning calorimetry (DSC)

Thermal analysis was performed using a DSC 6 calorimeter equipped with a Pyris® software (Perkin Elmer). All samples were heated at a 10°C min^{-1} scanning rate between 30-350°C after a 1 min stabilization plate at 30°C/20 mW, under nitrogen atmosphere. Thermograms are expressed in °C = f(mW). Physical mixtures of β-CD microparticles with amikacin (1/1) are used as blanks.

Encapsulation of hydrosoluble substances

Amikacin sulfate was encapsulated at two different steps of the fabrication :

-encapsulation during the microparticles fabrication process (solubilization, emulsification, cross-linking, freeze-drying),

- Encapsulation with the synthesized microparticles controlling the contact time and the influence of amikacin sulfate amount.

Influence of contact time

A series of experiments were conducted increasing contact time (15-60 min) between colloidal suspension (100 μL) and amikacin amount (500 μg). Results show that equilibrium is reached rapidly, the plate being reached after 30 min incubation time (Figure11).

Influence of amikacin sulfate

The volume of colloidal suspension is maintained at 50 μL, with a contact time fixed at 60 min. Encapsulation efficiency and yield increase linearly with the amount of amikacin introduced (Figure 11).

Statistical Analysis

Statistical data analyses were performed using the

Student's t-test at $p < 0.05$.

RESULTS AND DISCUSSION

Microparticles synthesis

Influence of reactions parameters on the size of microparticles

Elaboration of 5 µm microparticles size by interfacial polycondensation of β-CD with the cross-linking agent, terephtaloyle chloride, was undertaken and assayed varying different parameters, from the standard procedure.

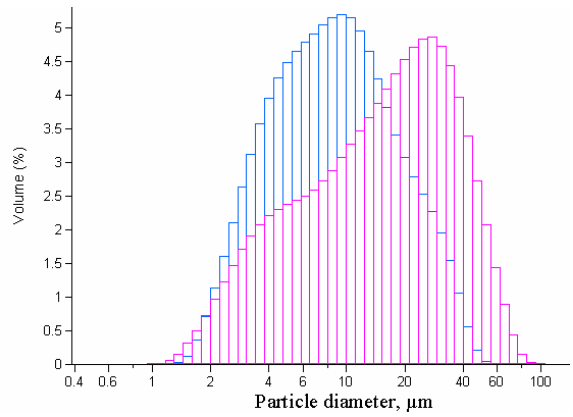


Figure 2. Granulometric features of the obtained particles by the standard method with 0.5 M (right) and 1 M NaOH (left) (β-CD: 7.5%w/v, 1M NaOH, 30 min reaction time, 8000 rpm agitation rate, polymerization and surfactant agent: 5% w/v). For unimodal repartition (1M NaOH: n=3, mean=8.549±2.089µm).

The size of the particles formed increased respectively with the β-CD amount, with NaOH concentration (Table 1), only with concentration above 1M and the step of freeze-drying. NaOH concentration lower than 1 M produced a bimodal repartition of the particles size (Figure 2). Freeze-drying increased the median microspheres size from 8.7 to 15.3 µm, in a bimodal repartition (Figure 3) modifying the studied population granulometry features due to particles agglomeration. On contrary, the size of the particles was minimal at 5-10% (v/v) of surfactant and at maximal agitation rate (24000 rpm) (Table 1). The surfactant amount and the agitation rate were determinant in the emulsification step and crucial for the formation of the smallest particles. An optimized method was elaborated, modifying the agitation speed to 24 000 rpm and decreasing the concentration of cross-linking agent to 4.5% (w/v), a concentration that don't affect the microspheres size, yield and unimodal repartition. This new preparation method allowed the formation of particles with size inferior to 5 µm (Figure 4) with a high reproductibility.

Stability assay, on the form of colloidal suspension were undertaken at ambiant temperature and at 4°C. Results obtained showed a physical stability of uncharged particles for at least three months at ambiant temperature. At lower temperatures, conservation of colloidal suspension was more difficult due to an increased sensitivity to crystallization phenomenon.

FT-IR spectra

The spectrum of microparticles formed with the optimized method was compared with the spectrum of original β-CD. Three bands appeared in microparticle spectrum at 1711, 1263 and 730 cm^{-1}, due to the formation of esters from hydroxy groups of the β-CD (Figure 5). The mechanisms have been studied by reaction of β-CD treated with 1 equivalent of terephtaloyle chloride in pyridine (37).

Table 1. Influence of preparation parameters on mean microparticle size, colloidal suspension pH and yield, when varied independently from the standard formulation (β-CD: 7.5%w/v, 1M NaOH, 30 min reaction time, 8000 rpm agitation rate, polymerization and surfactant agent: 5% w/v) (n=3). The optimized formulation is shown in bold.

Parameter	Value	pH	Mean size (µm) ±SD	Mean yield (%) ±SD
Monomer amount (% w/v)	5	2.68	7.89±1.10	26.7 ± 3.3
	7.5	2.74	6.31±1.23	26.5± 3.0
	10	2.89	8.92±1.20	25.5 ± 3.1
	20	2.67	10.64±0.51	27.7 ± 2.9
	30	2.95	12.78±0.45	28.4 ± 2.8
NaOH (M)	0.5	1.95	15.78±0.97	18.1 ± 2.5
	1	2.69	8.52±1.12	28.9 ± 3.5
	2	3.80	10.82±0.74	34.7 ± 2.6
	4	5.60	12.79±0.88	53.1 ± 4.2
Reaction time (min)	15	2.84	17.19±1.51	27.5 ± 2.5
	30	2.70	8.43±0.57	29.4 ± 2.1
	60	2.64	8.38±0.35	32.8 ± 2.4
	120	2.95	8.71±0.52	35.5 ± 2.6
	240	2.67	8.11±0.76	36.6 ± 2.5
Surfactant amount (% v/v)	2.5	2.68	12.97±0.81	24.9 ± 2.4
	5	2.71	8.25±0.42	26.8 ± 2.9
	10	2.61	8.59±0.59	27.9 ± 2.5
Cross-linking agent (% w/v)	1.25	6.73	13.48±0.61	18.8 ± 1.5
	2.5	5.10	9.56±0.52	21.9 ± 2.8
	3.75	3.90	8.56±0.53	29.9 ± 2.7
	5	2.69	8.69±0.46	28.7 ± 3.4
	6.25	1.91	8.98±0.47	27.4 ± 2.6
	7.5	1.56	8.68±0.41	28.6 ± 2.5
	8.75	1.69	8.90±0.34	29.5 ± 2.4
	10	1.09	8.80±0.46	27.5 ± 2.7
Agitation rate (rpm)	8,000	2.64	8.68±0.65	25.6 ± 2.7
	9,500	2.79	8.29±0.74	27.9 ± 2.5
	13,500	2.84	6.15±0.34	28.1 ± 3.5
	24,000	2.83	4.54±0.22	29.8 ± 1.2

DSC

No observable signal was present in the temperature range 30-115°C (Figure 6). At 172.4°C, an endotherm peak responsible for the cyclodextrin melting was observed whereas it was displaced at 162°C for reticulated cyclodextrin. At 300°C, an additional exothermic peak was attributed to terephtaloyle chloride.

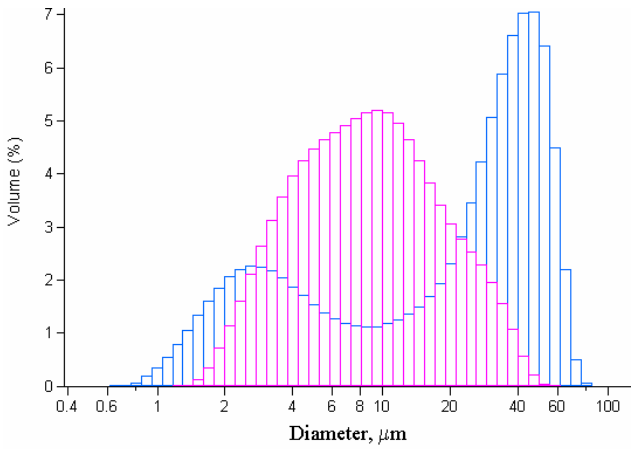


Figure 3. Granulometric features of the obtained particles by the standard method before freeze-drying step (unimodal) and after freeze-drying step (bimodal) (β-CD: 7.5%w/v, 1M NaOH, 30 min reaction time, 8000 rpm agitation rate, polymerization and surfactant agent: 5% w/v). For unimodal repartition (before freeze-drying: n=3, mean=8.549±2.089µm).

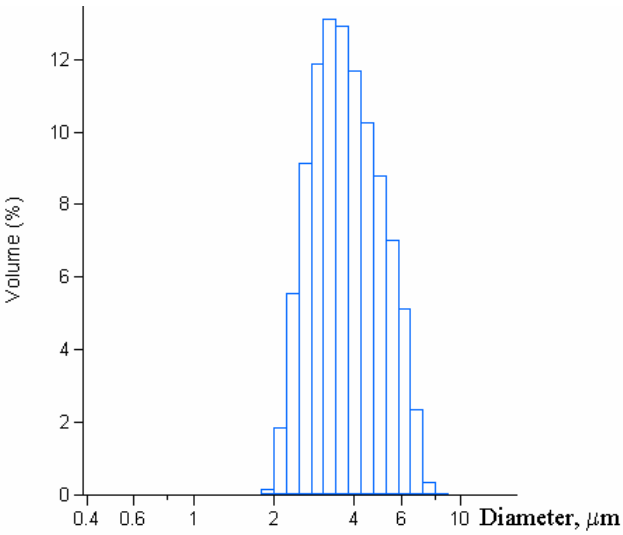


Figure 4. Granulometric features of the obtained particles for the optimized formulation (n=5 ; pH = 3.65±0.13; size=3.72±1.34 µm ; yield =28.5±1.4%) (β-CD: 7.5%w/v, 1M NaOH, 30 min reaction time, 24000 rpm agitation rate, polymerization agent: 4.5% w/v and surfactant agent: 5% w/v).

Amikacin sulfate encapsulation in β-CD microparticles

Encapsulation during microparticles fabrication process

Encapsulation efficiency (mass of amikacin sulfate in particles (µg)/ mass of β-CD (mg)) (Figure 7) increased linearly with the amikacin sulfate incorporated due to the fact that β-CD must be in excess over the amikacin sulfate incorporated. Encapsulation was more pronounced for solubilization and freeze-drying step. Encapsulation yield (mass of amikacin sulfate in particles (µg)*100/mass of amikacin sulfate introduced (µg)) (Figure 7) increased for the step of solubilization and freeze-drying reaching a plate from 20 µg amikacin. On contrary, yields were constant from 10 µg for emulsification step and even decreased from 10 µg for cross-linking step.

Encapsulation with microparticles

Ganulometry measurements were represented in function of% (m/v) amikacin introduced in the microsphere solution (Figure 8). Until 50% (m/v) amikacin, a bimodal repartition of the complexed amikacin was observed with a maximum size of particles around 3 µm (1% 3.136±1.689 µm ; 10% 2.433±1.541 µm ; 30% 2.970±1.603 µm). When 50% amikacin was put into contact with microspheres, a maximum size centered on 4.968±1.689 µm with an unimodal repartition was obtained reaching a limit for pulmonary drug delivery at alveolar scale.

Freeze-drying of a β-CD colloidal suspension and amikacin sulfate gave a chemical entity whose thermic features superpose to the host particle (Figure 9).

The thermogram (Figure 9) and IR spectra (Figure 10) of amikacin charged freeze-dried cyclodextrin and that corresponding to physical mixture compared with amikacin sulfate did not show the reapparition of the signal characteristic of amikacin sulfate. Therefore, this phenomenon can be attributed to dilution of the active substance in the powder. The nature of the interactions between amikacin and cyclodextrins remains unknown. Two hypotheses can be emitted : either the amikacin molecule forms partially or wholly an inclusion complex with cyclodextrins or one of the reaction functions of amikacin reacts with hydroxyl groups from cyclodextrin to create an hydrogen bond.

The first hypothesis seems unlikely because inclusion complexes are observed with hydrophobic compounds which insert inside the hydrophobic

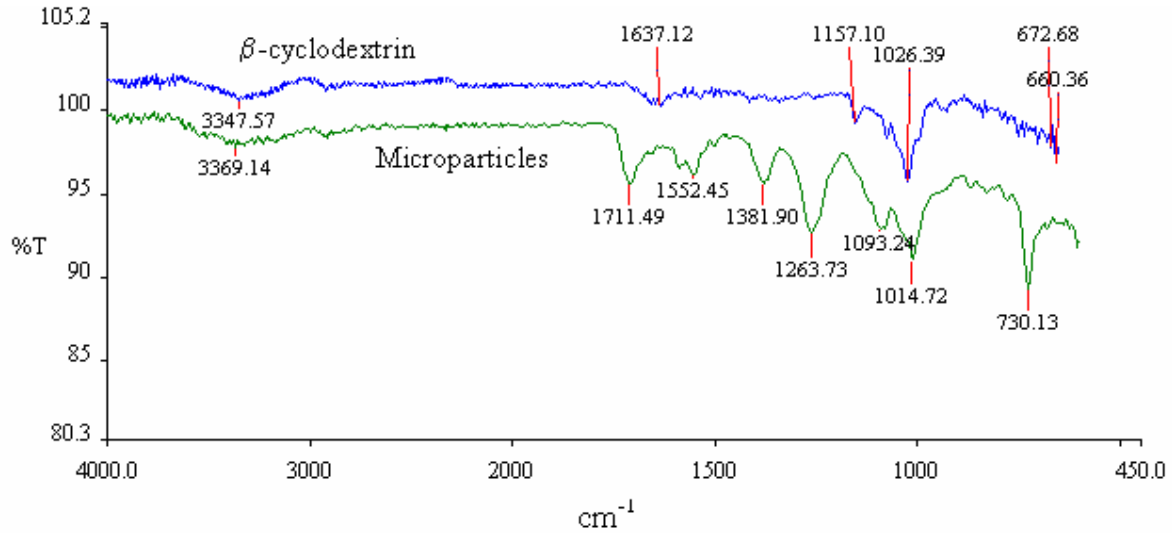


Figure 5. FTIR spectra from β-cyclodextrins and microparticles.

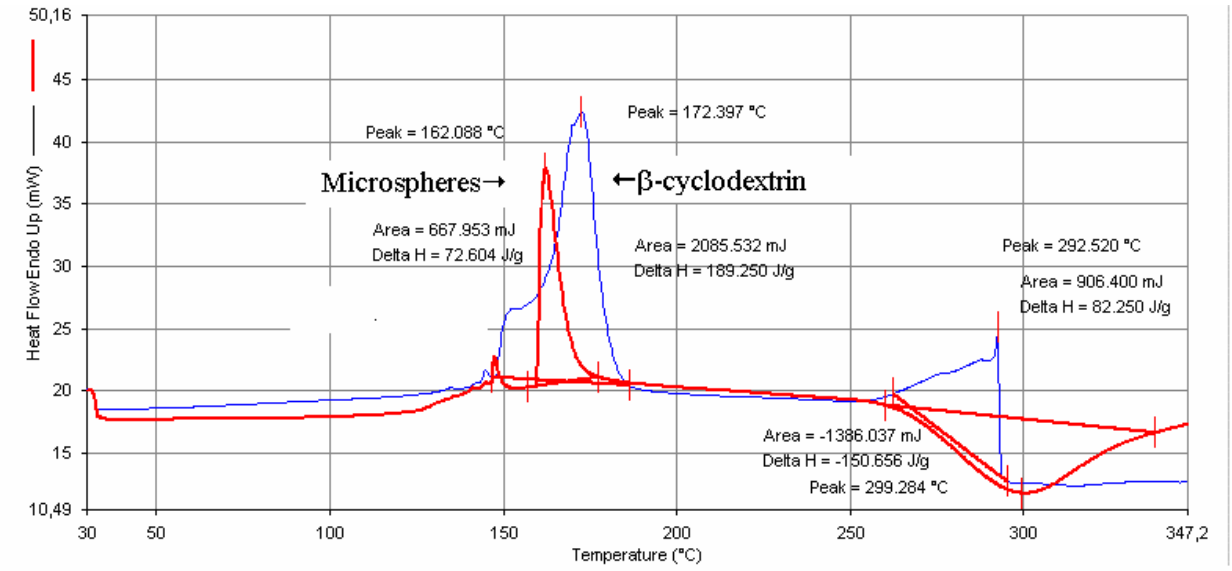


Figure 6. Comparative thermograms of β-cyclodextrins and microparticles.

cavity. Amikacin which is an hydrophilic compound would not be included in the cavity. The second hypothesis is more probable, encapsulation consisting in drug incorporation into the microsphere network and not inside the cyclodextrins cavity.

Encapsulation efficiency and encapsulation yield (Figure 7) varied respectively between 5-59 µg amikacin/mg β-CD and 0.17 to 59% proving the ability of cyclodextrin microsphere to encapsulate amikacin. This retention of active principe was influenced by the incorporation step. Amikacin sulfate encapsulation in polycondensed β-cyclodextrins showed that better incorporation was obtained during the solubilization step or just before freeze-drying. This result is certainly due to a better dispersion of the substance in aqueous medium. Nevertheless, encapsulation efficiency and yield remained lower when amikacin sulfate was introduced in the solubilization step than just before freeze-drying. Two hypothesis may explain this observation: the first one consists in the possible elution phenomenon of the active molecule during the successive particles washing steps by methanol and water, in the step following solubilization, resulting in a decrease of encap-

sulation yield and efficiency. The second one is based on the mechanical action of the freeze-drying which forces the amikacin sulfate inside the host particle increasing encapsulation parameters.

When the guest molecule was introduced with the synthesized microparticles, after freeze-drying, encapsulation efficiency and yield increased with contact time (Figure 11), proving the importance of passive absorption in the retention mechanism, and the substance being encapsulated (Figure 11) provided an amikacin concentration saturation plateau was not being reached.

A 60 days-incubation time for each preparation step resulted in a 30 to 50% loss of encapsulated amikacin (Figure 12) for all the steps except for the freeze-drying step, for which the amount of active substance was maintained until 120 days.

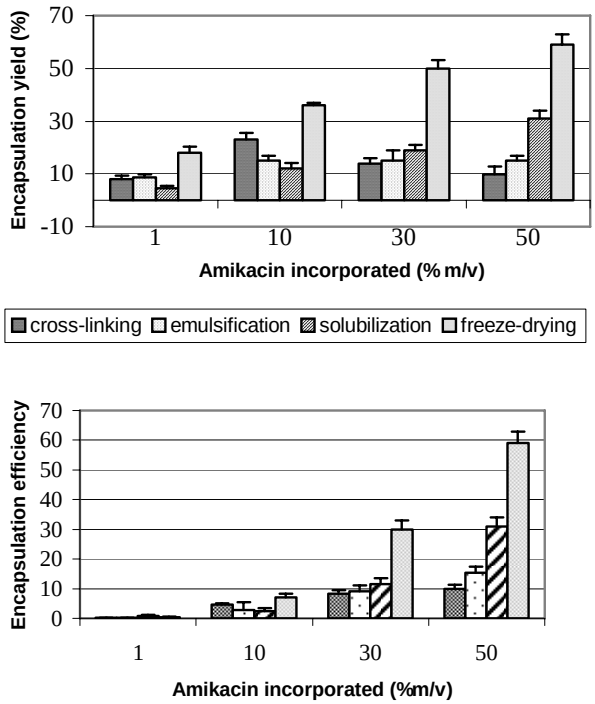


Figure 7. Encapsulation efficiency of amikacin (mass of amikacin sulfate in particles (μg)/ mass of β-CD (mg)) and encapsulation yield (%) of amikacin in particles (mass of amikacin sulfate in particles (μg)*100/mass of amikacin sulfate introduced (μg)) at different steps in preparation of the optimized formula (n=3).

In conclusion, this work showed that 5 μm microparticles, can be prepared at room temperature, by interfacial cross-linking with terephtaloyle chloride. Thus, the cross-linked microparticles formed are able to encapsulate hydrophilic compounds destinated to pulmonary delivery, with an encapsulation yield higher than 50%. These results confirm the encapsulation possibility and allow to foresee some other potential applications with similar active substance having a structural homology. Amelioration could be brought to increase the pH for administration in physiological conditions and to see its effect on the stability of the system. The elaboration process showed that encapsulation method consisting in incorporating the active substance before the freeze-drying step gives higher yields than after freeze-drying, proceeding that tends to agglomerate particles. Finally, it could be interesting to study amikacin sulfate complexes with cyclodextrins and their derivatives to have information on the nature of the interactions between the host-guest complexes.

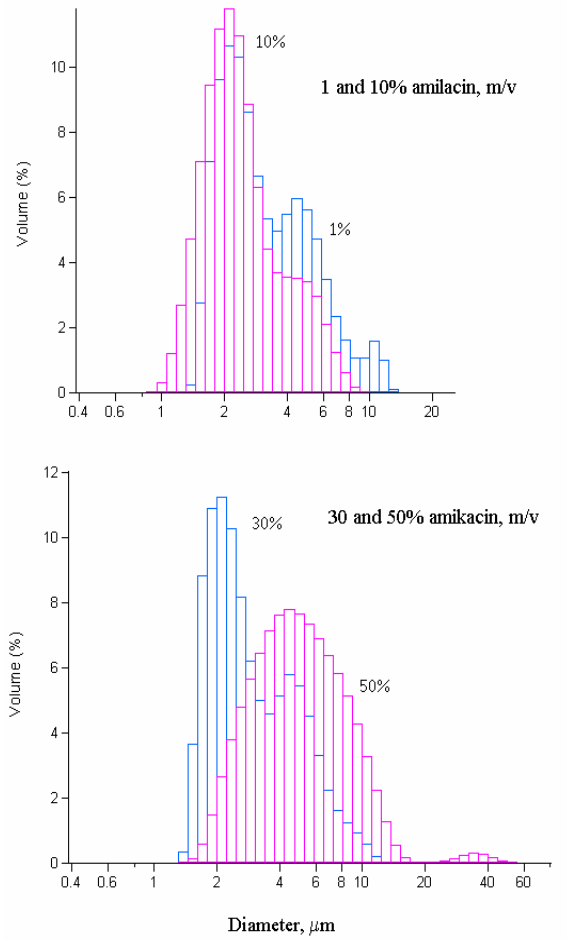


Figure 8. Granulometric features of the obtained particles for the optimized formulation charged in amikacin (β-CD: 7.5%w/v, 1M NaOH, 30 min reaction time, 24000 rpm agitation rate, polymerization agent: 4.5% w/v and surfactant agent: 5% w/v). (n=3,%amikacin/mean: 1%/3.136±1.635 μm; 10%/2.433±1.541 μm; 30%/2.970±1.603 μm; 50%/4.968±1.689 μm).

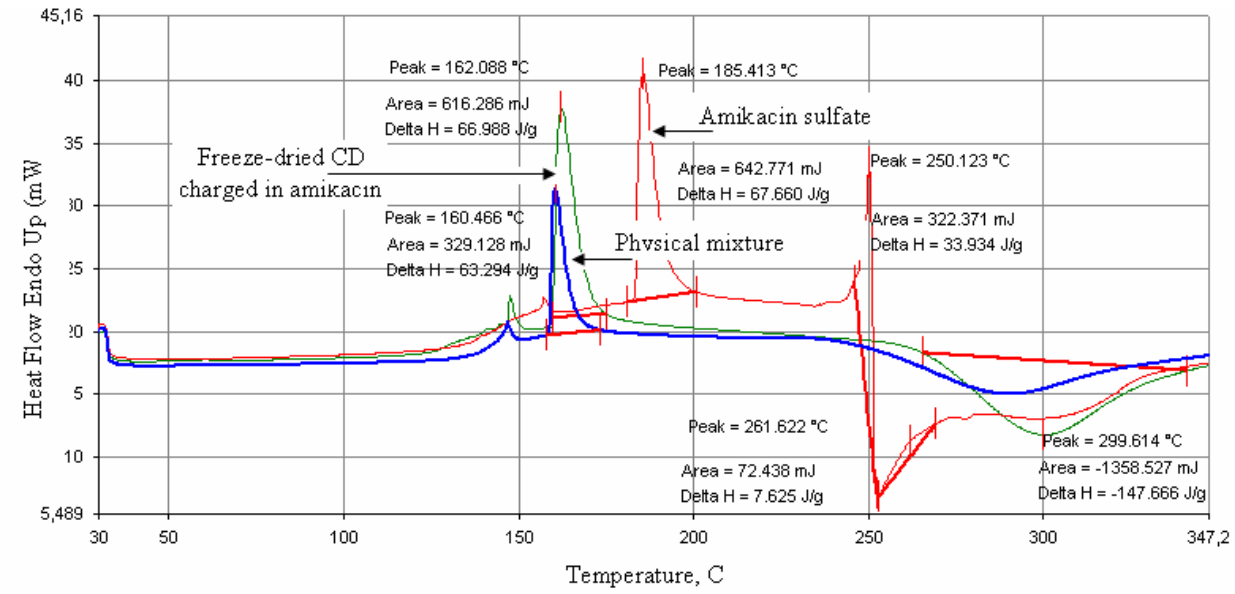


Figure 9. Comparative thermograms of amikacin sulfate, freeze-dried cyclodextrin charged in amikacin and corresponding physical mixture.

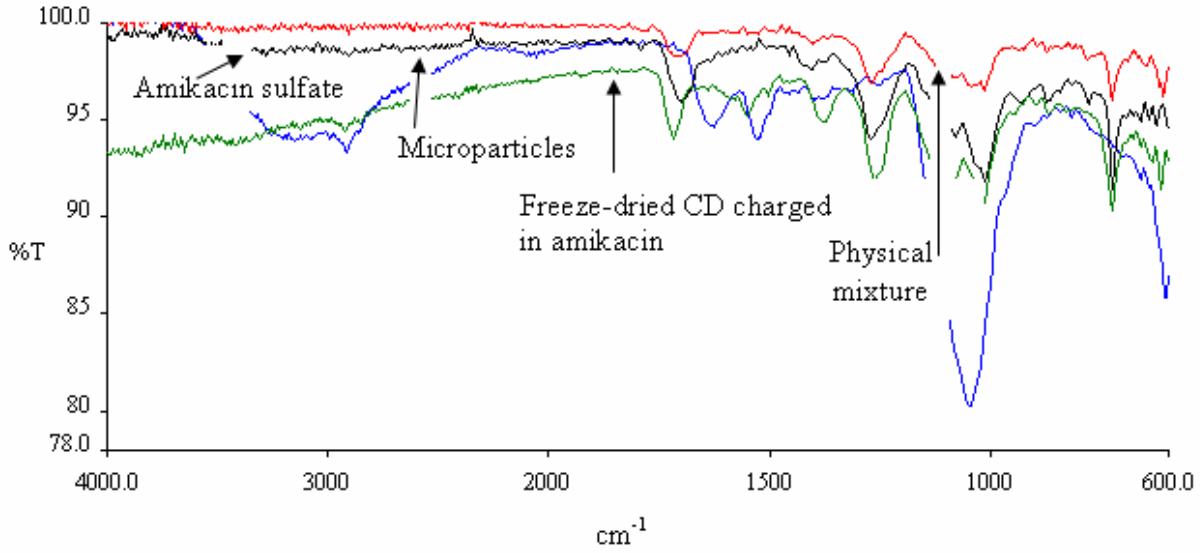


Figure 10. FTIR spectra from amikacin sulfate, microparticles, freeze-dried cyclodextrin charged in amikacin and physical mixture (1/1).

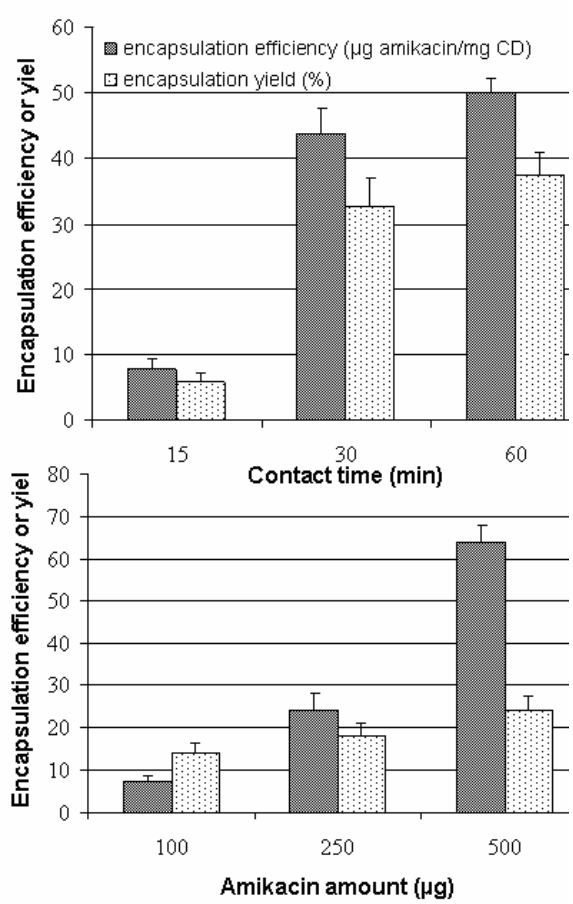


Figure 11. Influence of the contact time (min) and the amount of amikacin on encapsulation efficiency and yield (n=3).

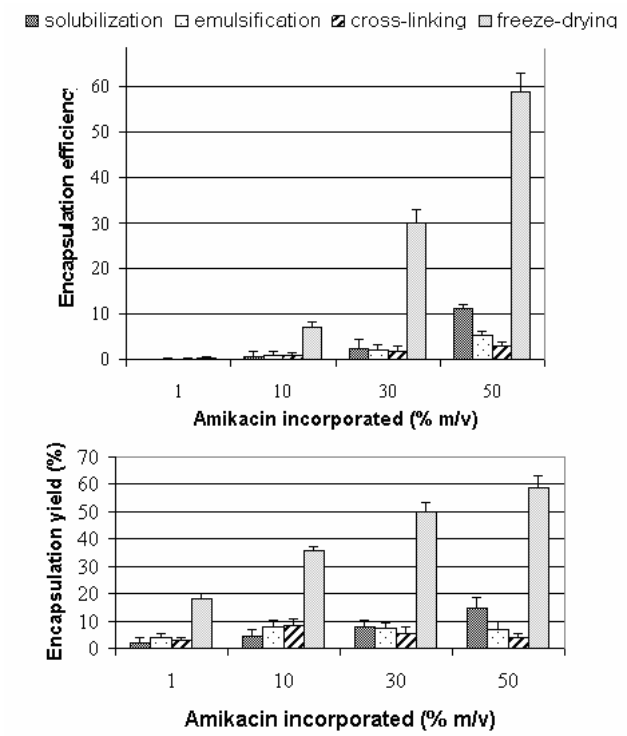


Figure 12. Encapsulation efficiency of amikacin (mass of amikacin sulfate in particles (µg)/ mass of β-CD (mg)) and encapsulation yield of amikacin in particles (mass of amikacin sulfate in particles (µg)*100/mass of amikacin sulfate introduced (µg)) when particles were maintained in suspension during 60j).

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