Henrique França Diniz Oliveira

NANOAGREGADOS BASEADOS EM CICLODEXTRINAS EM ASSOCIAÇÃO COM A TETRACICLINA: CARACTERIZAÇÃO FÍSICO-QUÍMICA E AVALIAÇÃO ANTIMICROBIANA.

NANOASSEMBLIES BASED ON CYCLODEXTRIN AS CARRIER FOR TETRACYCLINE: PHYSICOCHEMICAL CHARACTERIZATION AND ANTIMICROBIAL EVALUATION.

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LISTA DE ABREVIATURAS E SIGLAS

δ	Chemical Shift
β-CD	β-cyclodextrin
$\Delta_{\beta\text{-CD}}H°$	Partial molar enthalpy of β-CD
1:1	β-cyclodextrin:tetracycline 1:1 molar ratio
2:1	β-cyclodextrin:tetracycline 2:1 molar ratio
3:1	β-cyclodextrin:tetracycline 3:1 molar ratio
4:1	β-cyclodextrin:tetracycline 4:1 molar ratio
A.a., A. Actinomycemcomitans	*Actinobacillus actinomycetemcomitans*
ATCC	American Type Culture Colection
BHI	Brain Heart Infusion
CD	Cyclodextrin
CDs	Cyclodextrins
CFU	Colony Forming Units
D_2O	Heavy water
DLS	Dynamic Light Scattering
DSC	Differential Scanning Calorimetry
FTIR	Fourier Transform Infrared
ITC	Isothermal Titration Calorimetry
MIC	Minimal Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy

P.g., P. gingivalis	*Porphyromonas gingivalis*
PLGA	DL-co-polymers of lactic and glycolic acid
PM 1:1; 2:1; 3:1 e 4:1	Physical mixtures in 1:1; 2:1; 3:1 and 4:1 β-cyclodextrin:tetracycline molar ratio
PVA	Polyvinyl Alcohol
SEM	Scanning Electron Microscopy
TC	Tetracycline
TG	Thermogravimetric Analysis
UV	Ultra Violet
XRD	X-ray diffraction
ν	Vibration Modes

SUMÁRIO

RESUMO

A auto agregação molecular tem se mostrado como um método eficiente para produzir estruturas medindo alguns nanômetros em tamanho. Esses compostos moleculares sintetizados a partir da associação entre β-ciclodextrina:tetraciclina foram caracterizados por espalhamento de luz dinâmico, calorimetria isotérmica de titulação, difração de raios-X, espectroscopia em infravermelho, termogravimetria, calorimetria exploratória diferencial e ressonância nuclear magnética. Além disto, esses compostos supramoleculares foram testados para a determinação de sua atividade antimicrobiana contra *A. actinomycetemcomitans* e *P. gingivalis* em solução e associados à nanoesferas poliméricas. Utilizando-se das técnicas de caracterização, a formação do composto de inclusão entre TC e β-CD na razão molar de 1:1 foi evidenciada e, ao se aumentar a concentração de β-CD, ocorreu uma auto agregação supramolecular espontaneamente, resultando em compostos de maior razão molar de β-CD:TC. Estes complexos apresentaram propriedades fisico-químicas diferentes entre si e diferentes da β-CD e TC puras, tamanho nanométrico, além de potenciarem a atividade antimicrobiana do fármaco. Os compostos de razão molar 2:1 β-CD:TC mostraram uma atividade antimicrobiana significativamente maior em solução ($p<0.05$). Dentre os outros compostos, 4:1 foi mais eficiente contra *P. gingivalis* (zona de inibição = 41.67±1.4mm, MIC 0.25μg/mL, $p<0.05$). As nano esferas poliméricas, preparadas utilizando-se os nanoagregados, mostraram liberação controlada do fármaco por 10 dias, em uma concentração superior à concentração inibitória mínima das bactérias testadas.

Palavras chave: Tetraciclina, Ciclodextrinas, Nanoagregados, Nanoesferas, Liberação Controlada.

ABSTRACT

Molecular self-assembling has been shown to be an efficient method to produce structures of few hundreds of nanometers in size. The supramolecular compounds made from β-cyclodextrin:tetracycline in aqueous solution were evaluated. The physicochemical interactions between β-cyclodextrin:tetracycline were characterized by dynamic light scattering (DLS), isothermal titration calorimetry (ITC), X-ray diffraction (XD), infrared spectroscopy (FTIR), thermogravimetric analyses (TG), differential scanning calorimetry (DSC), and nuclear magnetic resonance (NMR). The supra-molecular interaction with *A. actinomycetemcomitans* and *P. gingivalis* in solution and in association with polymeric nanospheres were determined. Using the characterization techniques, it was demonstrated that the formation of inclusion complex takes place at a 1:1 β-CD:TC molar ratio and, increasing β-CD concentration, supramolecular spontaneous aggregation occurred. The resulting complexes showed different physicochemical properties, nanometric size and improved antimicrobial activity. The 2:1 β-CD:TC showed significantly higher antimicrobial activity in nsolution ($p<0.05$). Among the other compounds, 4:1 was the most effective against *P. gingivalis* (inhibition zone = 41.67±1.4mm, MIC 0.25μg/mL, $p<0.05$). Polymeric nanospheres were then manufactured using these nanoassemblies. The nanospheres showed controlled TC release for 10 days, and concentrations above minimum inhibitory concentrations of tested bacteria.

Key words: Tetracycline, Cyclodextrin, Nanoassemblies, Nanospheres, Controlled Release.

APRESENTAÇÃO

Recentemente, estudos reportam o uso de nano agregados utilizando-se polissacarídeos cíclicos para a alteração das características fisico-químicas de substâncias. Esses poderiam solubilizar fármacos pouco hidrossolúveis em água, através de interações em seus microdomínios hidrofóbicos. A auto organização molecular tem se mostrado um fenômeno útil para a produção de estruturas com o tamanho de poucos nanômetros. Devido às propriedades especiais apresentadas pelas nanopartículas, que incluem propriedades químicas e biológicas entre outras, elas exibem uma performance superior em relação aos materiais tradicionais [1]

Estruturas supramoleculares auto organizadas podem também ser alcançadas utilizando-se as ciclodextrinas. Estas são moléculas cíclicas que atuam como hospedeiras, apresentando características anfifílicas. Apesar de a molécula como um todo ser hidrosolúvel, o interior da sua cavidade é relativamente apolar, criando um microambiente hidrofóbico. Sua parte exterior é predominantemente hidrofílica. Estas características são responsáveis pela solubilidade destas moléculas em meio aquoso e ainda por apresentar a capacidade de interagir com substâncias hidrofóbicas em solução, mudando suas propriedades farmacológicas no caso de medicamentos, incluindo o aumento de sua biodisponibilidade [2-5].

Compostos supramoleculares, nos quais as ciclodextrinas se associam à fármacos para a formação de compostos de inclusão, estão sendo desenvolvidos com sucesso. Os compostos de inclusão são freqüentemente descritos em estequiometrias fixas (1:1). No entanto, o caráter

anfifílico das ciclodextrinas e a capacidade desta molécula para formar ligações de hidrogênio e de Van der Waals, sugerem que, aumentando-se a concentração de β-ciclodextrinas no sistema, novos complexos de razões molares diferentes poderiam ser formados. Esta nova conformação espacial poderia conferir diferentes propriedades ao fármaco incluído. Os processos de auto organização podem ser pré-determinados para que as condições experimentais se tornem favoráveis para a formação e a estabilização desse novo composto [6-8].

Estudos prévios mostraram vantagens ao se usar o composto de inclusão β-ciclodextrina:tetraciclina (1:1) em solução em relação à tetraciclina pura, resultando em melhores propriedades antimicrobianas e menores concentrações, *in vitro*. Esse composto de inclusão mostrou ainda um padrão de liberação de tetraciclina de maneira mais lenta e gradual em comparação ao fámaco puro [9]. Além disso, as ciclodextrinas podem agir como conservantes em formulações, protegendo o fármaco da degradação, aumentando seu tempo de estoque. Esta propriadade foi também descrita para as formulações contendo hidroxipropil-β-ciclodextrina:tetraciclina. Os autores sugerem que esta ciclodextrina poderia proteger alguns radicais mais sensíveis presentes na molécula do agente farmacológico [10].

Como a tetraciclina possui um amplo espectro de ação antimicrobiano além de propriedades antiinflamatórias, e, vem sendo testada ultimamente em sistemas de liberação controlada de fármacos [11-16] com diversas aplicações tais como na odontologia [17-25], com diferentes veículos [11-12; 26-34], foi levantada a hipótese que a utilização do fármaco em novas conformações espaciais associado às ciclodextrinas poderia levar a novas propriedades e formulações. A formação dos nano agregados poderia ser vantajosa mudando as características do fármaco e neste caso, reforçando suas propriedades antimicrobianas.

Assim, o objetivo deste trabalho foi desenvolver uma nova abordagem para o desenvolvimento dos compostos entre β-ciclodextrina e tetraciclina, combinando as vantagens das ciclodextrinas em uma técnica de nano organização. Então, a caracterização fisico-química dos compostos foi realizada, assim como a medida de suas atividades antimicrobianas através de suas interações biológicas com microrganismos anaeróbicos em solução e em associação a nanoesferas poliméricas. Estas foram ainda testadas em relação a suas características de liberação controlada do fármaco.

NANOASSEMBLIES BASED ON CYCLODEXTRIN AS CARRIER FOR TETRACYCLINE: PHYSICOCHEMICAL CHARACTERIZATION AND ANTIMICROBIAL EVALUATION.

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Abstract:

Molecular self-assembling has been shown to be an efficient method to produce structures of few hundreds of nanometers in size. The supramolecular compounds made from β-cyclodextrin:tetracycline in aqueous solution were evaluated. The physicochemical interactions between β-cyclodextrin:tetracycline were characterized by dynamic light scattering (DLS), isothermal titration calorimetry (ITC), X-ray diffraction (XD), infrared spectroscopy (FTIR), thermogravimetric analyses (TG), differential scanning calorimetry (DSC), and nuclear magnetic resonance (NMR). The supra-molecular interaction with *A. actinomycetemcomitans* and *P. gingivalis* in solution and in association with polymeric nanospheres were determined. Using the characterization techniques, it was demonstrated that the formation of inclusion complex takes place at a 1:1 β-CD:TC molar ratio and, increasing β-CD concentration, supramolecular spontaneous aggregation occurred. The resulting complexes showed different physicochemical properties, nanometric size and improved antimicrobial activity. The 2:1 β-CD:TC showed significantly higher antimicrobial activity in nsolution ($p<0.05$). Among the other compounds, 4:1 was the most effective against *P. gingivalis* (inhibition zone = 41.67±1.4mm, MIC 0.25μg/mL, $p<0.05$). Polymeric nanospheres were then manufactured using these nanoassemblies. The nanospheres showed controlled TC release for 10 days, and concentrations above minimum inhibitory concentrations of tested bacteria.

Key words: Tetracycline, Cyclodextrin, Nanoassemblies, Nanospheres, Controlled Release.

1. Introduction

In recent years many studies reported that the physicochemical and biochemical stability of drugs were altered by the formation of nanoagregates using cyclic polysaccharides. Those were able to solubilize, in water, poorly hydrophilic drugs entrapped into their hydrophobic microdomains. Molecular self-assembling has been shown to be a useful property to produce structures at a few hundreds nanometers in size. Because nanoparticles exhibit new chemical and biological properties, their performance are usually improved comparing to traditional materials [1].

The supramolecular self-assembled species may also be achieved by using cyclodextrin (CDs). Indeed, cyclodextrins are host cyclic carbohydrates with amphiphilic characteristics. Although the entire molecule is water soluble, the interior part is relatively apolar, creating a hydrophobic microenvironment, being the outer part of the molecule more hydrophilic. These properties are responsible for their aqueous solubility and ability to interact with hydrophobic and hydrophilic substances in solution, changing drug pharmacological properties, including improving their bioavailability [2-5].

The supramolecular assemblies, in which CDs were associated to drug molecules in order to form inclusion compounds, are being successfully developed. Fixed stoichiometries (1:1) are often assumed for β-cyclodextrin (β-CD) inclusion compounds. However, CDs amphiphilic behavior makes possible the formation of different types of chemical interactions such as Van der Waals and hydrogen bondings, and suggests that by increasing β-CD molecules ratio in the system, new complexes could be formed. Thus, spontaneous molecular self-assemblies with different β-CD ratios are possible. The new spatial conformation could change pharmacokinetics and bioavailability of this antibiotic. These self-assembling processes may

be pre-determinate to make the favorable conditions for new compound organization and stabilization [6-8].

Previous studies showed advantages of using 1:1 β-CD:Tetracycline (TC) inclusion compounds solutions resulting in improved *in vitro* antimicrobial activity when compared to pure tetracycline. This inclusion compound showed also sustained release of the drug when compared to pure tetracycline [9]. Moreover, CD cyclic molecules protected the antibiotic from degradation, increasing the formulation shelf life. This property was also described for hydroxypropyl-β-cyclodextrin:TC (HP-β-CD) pharmaceuticals formulation. The authors suggested that this cyclodextrin could protect some especially labile radicals of the TC molecule [10].

Since TC is a drug with wide antibiotic spectrum and anti-inflammatory activity and is being tested in controlled release devices [11-16] with several applications, for example in dentistry [17-25], with different vehicles [11-12; 26-34], it is hypothesized that new spatial drug conformations using CDs could lead to new formulations for tetracycline, with new biological properties. The nanoassemblies could be advantageous changing drug properties, in this case, enhancing antimicrobial action.

Hence, the aim of this work was to develop a new approach for TC inclusion compound combining advantages of the CD properties and nanoassembly strategy. Thus, the physicochemical interactions between β-CD:TC were characterized and the biological supra-molecular interactions with anaerobic microorganism in solution and in association with polymeric nanospheres were determined. Polymeric nanospheres were also tested for TC release.

2. Materials and Methods

2.1 Materials

β-cyclodextrin (β-CD) was purchased from Cerestar® (USA), tetracycline hydrochloride (TC) (Merck, Darmstadt, Germany), DL-co-polymers of lactic and glycolic acid 50:50 (PLGA) were supplied by Birmingham Polymers, Inc., USA (MW 60000 g/mol^{-1}). The Brain Heart Infusion (BHI) and Blood Agar from Biobrás S.A. (Minas Gerais, Brazil); *Actinobacillus actinomycetemcomitans* (Y4-FDC) from Universidade Federal de Rio de Janeiro (Rio de Janeiro, Brazil) and *Porphyromonas gingivalis* strains were purchased from ATCC (ATCC 49417). All other chemicals were reagent grade and used without further purification.

Solid state inclusion compounds of TC were prepared by freeze-drying method from β-cyclodextrin and tetracycline aqueous solutions in 1:1 [35]. The different molar ratios (2:1; 3:1 and 4:1) were also prepared using the same methods described before for 1:1 inclusion compounds. The physical mixtures were prepared using a mortar and a pestle at the same molar ratios and used as comparison groups.

2.2 The cyclodextrin:tetracycline complexes characterization and antimicrobial activity

The compounds and free agents were characterized by the following physicochemical methods: dynamic light scattering (DLS), isothermal titration calorimetry (ITC), X-ray diffraction (XD), infrared spectroscopy (FTIR), thermogravimetric analysis (TG), differential scanning calorimetry (DSC), and nuclear magnetic resonance (NMR).

In order to further analyze the possible interactions between β-CD and TC, as well as size distribution of these complexes as a function of concentration in aqueous solution, a titration experiment was carried out. Zetasizer Nano ZS (Malvern) equipment was used for

measurements and hydrodynamic diameter was determined by Dynamic Light Scattering (DLS). The solutions were prepared by dissolution of the substances in milli-Q water at 25°C. A TC solution was prepared at a concentration of 0.5 x 10^{-3} mol/L and a 12.3 x 10^{-3} mol/L β-CD solution was used for titration [8]. The β-CD solution was added to the 1.5mL cuvette containing TC solutions in 10μL increments increasing the β-CD concentration in steps of 8.14 x 10^{-7} mol/L. At each increment, the cuvette was positioned in the Zetasizer chamber and hydrodynamic diameter was recorded. A total of 25 (250μL) increments were added for each TC solution.

The hydrodynamic diameters of the complexes were measured using the Zetasizer Nano ZS simultaneously to the titration experiment. The average sizes and standard deviations were recorded.

The isothermal titration calorimetry was accomplished in duplicate in a Microcal Microcalorimeter VP-ITC. The solutions were prepared by dissolution of the chemicals in milli-Q water. Titration was accomplished by injecting solutions of 5μL of β-CD (12.3 x 10^{-3} mol/L) into TC solution (0.5 x 10^{-3} mol/L) after the electrical and chemical calibration of the calorimeter. The initial 1 μL injection was discarded from each dataset in order to eliminate the effect of titrant diffusion across the syringe tip during the pre-equilibration process. The initial cell volume was 1.6mL. The concentration correction as well as the integration of the heat flow peaks involved in partial molar enthalpy of β-CD were made with software Microcal Origin 5.0 for ITC [8].

The X-ray powder diffraction patterns of the samples were recorded on a Rigaku X-ray diffractometer. The samples were irradiated with monochromatized Cu K_α radiation and

analyzed with 2θ angles between 5 e 40^{o} C. The voltages, current, and time per step were 30Kv, 5 mA, and 1 min, respectively.

FTIR spectra of the pure TC, β-CD, and their complexes at different β-CD:TC ratios 1:1, 2:1, 3:1, 4:1 and their respective physical mixtures (PM 1:1, PM 2:1, PM 3:1 and PM 4:1) were recorded in a Perkin Elmer spectrometer model Spectrum GX (Perkin Elmer, Boston, MA, USA) at 4000 – 400cm^{-1} in KBr pellets at a resolution of 4cm^{-1} using 32 scans per sample.

The Thermogravimetric analysis (TG) curves were recorded on a Shimadzu TGA-50H at a scan rate of 10°C/min, among 25-750°C, under nitrogen atmosphere by using a platinum crucible. The samples mass were 2mg each. The Differential Scanning Calorimetric curves (DSC) were recorded on a Shimadzu DSC-50. The samples mass were 3.5mg in an aluminum pan with lid using a heating rate of 10°C/min, under nitrogen atmosphere.

NMR spectra were recorded at 27ºC on a Bruker DRX 400 – AVANCE spectrometer operating at 400MHz, equipped with a 5mm inverse probe with z-gradient coil. ^{1}H NMR experiment was achieved using the WATERGATE technique for suppression the residual water signal. NOESY spectra were acquired using a standard experiment from the spectrometer library with a mixing time of 600ms. All NMR experiments were used to confirm the assignments of the protons signals of the TC molecule and its inclusion compound.

The β-CD:TC system at the 1:1, 2:1, 3:1 and 4:1 molar ratio were dissolved in D_2O (Cambridge Isotope Laboratories, Inc – 99.9% of isotopic purity) to confirm the interactions between the species. The δ = 4.70 water signal was used as reference.

The minimal inhibitory concentration (MIC) was determined using broth dilution susceptibility test against reference strains of *A. actinomycemcomitans* (Y4-FDC) and *P. gingivalis* (ATCC 49417). Serial two fold dilutions of antimicrobial agents were prepared in Brain Heart Infusion agar (BHI) supplemented with 0.5% yeast extract, menadione and haemin. After 24 hours of incubation, 100μL of bacterial strain cultures with density adjusted to 10^8/mL colony-forming units (CFU) were inoculated. Serial fresh solutions of 1:1; 2:1; 3:1; 4:1 β-CD:TC compounds were used. TC and β-CD alone, plain broth or inoculate with bacteria were used as control groups. The TC concentrations ranged from 128 to 0.25μg/mL. The cultures were placed at 37°C in an incubator for 24h in anaerobic conditions (75% N_2; 25% CO_2). Tubes absorbance were then measured and compared to the controls by spectrophotometry and analysed by ANOVA.

Moreover, based on the MIC found in the previous test (Table 1) agar diffusion susceptibility test was carried out to determine the antimicrobial activity. Solutions were prepared with the same groups listed above at different drug concentrations (1, 10, 30 μg/mL). Pure TC solution was prepared as positive control groups, and cyclodextrin solutions and plane blank discs as negative control groups were used. The plates with blood agar were swabbed with the microorganisms *A.a.* and *P.g*). Blank discs (6 mm diameter) were immersed in the solutions and placed on the culture media. The plates were then incubated for 48h at 37°C under anaerobic conditions. Afterward, the inhibition zone diameters were measured. The results were analysed by Kruskal-Wallis with Tukey test.

2.2 Nanospheres preparation, release study and antimicrobial activity

The TC, β-CD and the β-CD:TC compounds (1:1; 2:1; 3:1 and 4:1) loaded in the polymer nanospheres were prepared by a solvent evaporation process by water-oil-water emulsion. An aqueous phase containing 10mg of TC, β-CD or the β-CD:TC compounds (1:1, 2:1, 3:1 and 4:1) in 100μL of water was injected into an organic phase solution containing 100mg of the polymer dissolved in 1mL of dichloromethane. The mixture was immediately ultra-sonicated for 2 minutes. This first emulsion was added drop wise inside 50mL of a 1% polyvinyl alcohol (PVA) solution under constant homogenization for 2 minutes. The emulsion was left under magnetic stirring for 2 hours for solvent evaporation. Following, the particles suspension was concentrated to the desired final volume by sedimentation using centrifuge under a speed of 13000rpm for 20 minutes and removal of the supernatants. This procedure was repeated three times and then, freeze dried for 48 hours. Control nanospheres were prepared without drug.

Drug loading in the nanospheres was determined using 10mg samples. They were dissolved in 50mL of 3M NaOH solution and filtered through a 0.2μm filter. The aqueous solutions containing extracted drug were assayed using UV-visible spectrophotometry in the 365-370nm region. The calibration curve was prepared diluting TC in 3mol/L NaOH at known concentrations. The experimental drug loading efficiency was calculated.

The nanospheres size and morphology were determined by scanning electron microscopy (SEM). No chemical fixation or freezing methods were used in the preparation of samples for SEM. Samples were placed on round brass stubs and sputter coated under an argon atmosphere with gold. Samples were examined using a Jeol JFM 840A scanning electron microscope. Moreover, nanospheres size distribution was assessed by suspending 1mg of each

group in 2mL milli-Q water inside a plastic transparent cuvette. The cuvette was placed in the Zetasizer Nano-ZS (Malvern) and measured by DLS. The measurements were performed using a low intensity Helium-Neon laser (4mW), at 25°C. The particles hydrodynamic diameters were obtained from analysis of resultant size histogram.

TC release profiles were obtained by soaking the test samples resulting from the inclusion compound nanoencapsulation process, in 1mL of distilled water with constant stirring of 70rpm at 37°C. In spite of different weight percentage of TC, the drug was normalized at 1mg/mL. The tubes were placed in a greenhouse incubator at a shaking speed of 70rpm at 37°C. At pre-determined intervals, the samples were centrifuged at 5000rpm for 5 minutes and the entire media was removed from the tubes and replaced with fresh distilled water. The concentration of released drug in media was evaluated by UV spectrophotometry at 365-370nm wavelengths using a HP 8452A-diode array UV-visible spectrophotometer.

Nanospheres antimicrobial activity was assessed by agar diffusion susceptibility test according to the NCCLS standards [36]. In addition, antimicrobial activity was determined from the media of the release experiment to asses nanospheres released drug for all tested groups at the same period test. Blank disks were immersed into the released aliquots and placed previously inoculated plates using the same bacteria and culture medium as described above. As a control groups were used TC alone or drug empty nanospheres. Then the plates were incubated for 48h at 37°C. The inhibition zone diameters were measured.

3. Results and Discussion

3.1 The cyclodextrin:tetracycline complexes characterization and antimicrobial activity

The mean hydrodynamic diameter of the β-CD:TC compounds measured by the dynamic light scattering is shown in Figure 1. The titration of β-CD solution in TC solution showed an increase of this parameter as a function β-CD concentration. The experiment was designed so as to include all the fixed molar ratios used in the physicochemical characterizations. The average hydrodynamic diameter of the associations was of 433.3±41.9nm, 492.3±46.1nm, 542.3±57.5nm and 861.0±236.8nm for the 1:1, 2:1, 3:1 and 4:1, respectively. The size of the complex increased upon β-CD concentration increment. An increase of dispersity could be due to a non-geometric shape of these growing aggregates [37]. TC seems to have seeding properties among β-CD molecules, inducing these self-assembling phenomena [8].

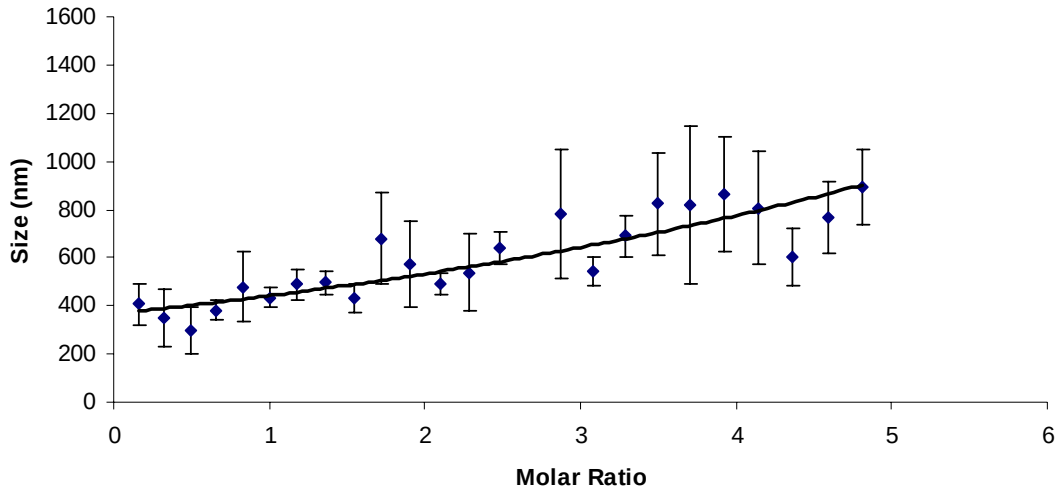

Figure 1. Size measurements obtained by DLS titration of a 0.5 x 10^{-3} solution of TC in a 12.3 x 10^{-3}mol/L β-CD solution.

ITC experiments were also performed using the same conditions as those of the DLS experiment. The curves showed, by linear regression, two domains with the increase of β-CD in TC solution. The first domain was characterized by a higher inflection, with high $\Delta_{\beta\text{-CD}}H°$

modulus, attributed to host-guest interactions, which may be attributed to the formation of hydrogen bonds between TC aromatic hydrogens with β-CD cavity, releasing highly energetic water molecules. This suggested the formation of an inclusion compound (Figure 2). The second domain was also exothermic but showed that as β-CD concentration increased the $\Delta_{\beta\text{-}CD}H°$ modulus became smaller. This reduction could be related to the complexation and desolvation of outer water molecules of the high stoichiometry complexes, suggesting the formation of nano assemblies [8].

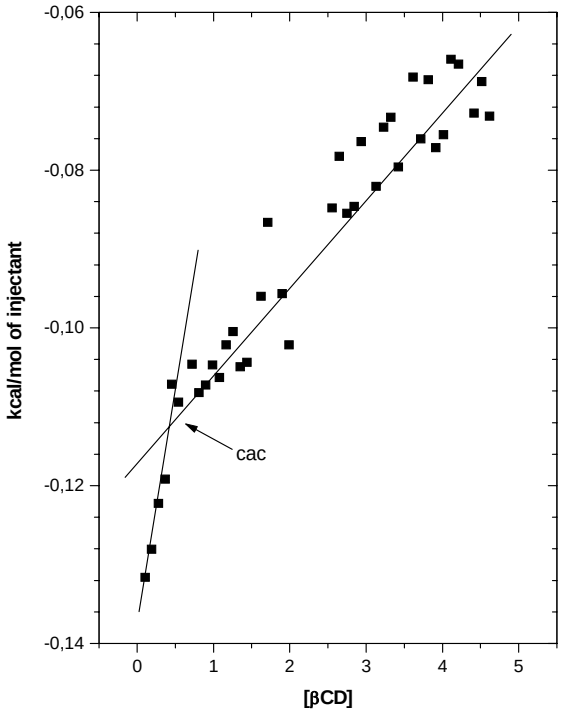

Figure 2. ITC experiments expansion to β-CD 12.3 x 10^{-3} mol/L in TC 0.5 x 10^{-3} mol/L.

The NMR spectrum of pure TC and the inclusion compound as in the 1:1 molar ratio are presented in Figure 3a and 3b respectively. Some modifications in the profile of the spectrum of pure TC are observed in the presence of β-CD. These modifications in the chemical shits are caused by the change in the electronic density upon the inclusion of the TC in the β-CD cavity.

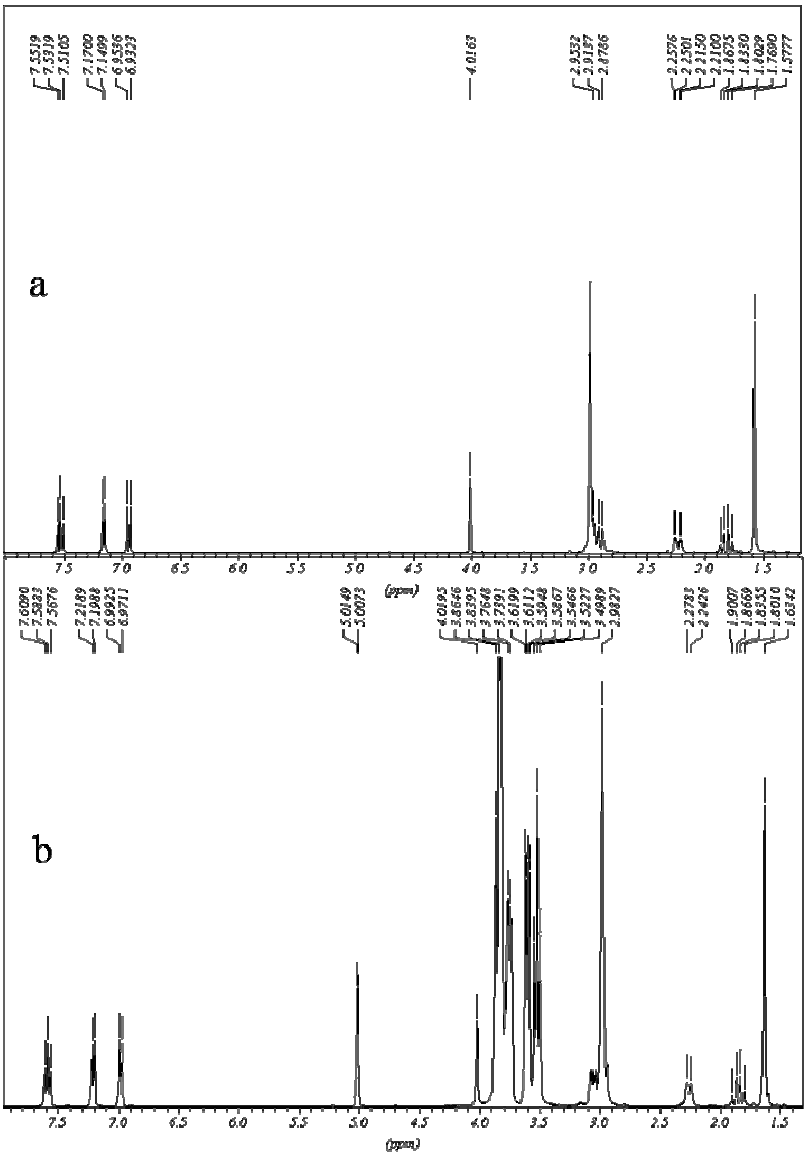

Figure 3. ^{1}H NMR (400MHz) in D_2O at 25 ºC of: a) pure TC and b) IC at 1:1 TC β-CD molar ratio.

To confirm which region of TC molecule was included into the β-CD cavity, NOESY experiments were conducted. The 1:1 molar ratio NOESY experiment showed a cross peak correlation, at short distance (less than 5 Å) between the aromatic protons of the TC, H_e (δ 6.98), H_f (δ 7.59) and H_g (δ 7.21), with the cavity protons of β-CD, H_3, H_5 and H_6 (region at δ 3.62 to δ 3.87) (Figure 4).

In addition, the correlation between the protons H_b, H_d (δ 3.01), H_h and H_i (δ 3.98) of TC were determined with all β-CD protons. Also the H_j (δ 1.63) of TC were correlated to protons H_3, H_5 and H_6 from the β-CD, Figure 4b.

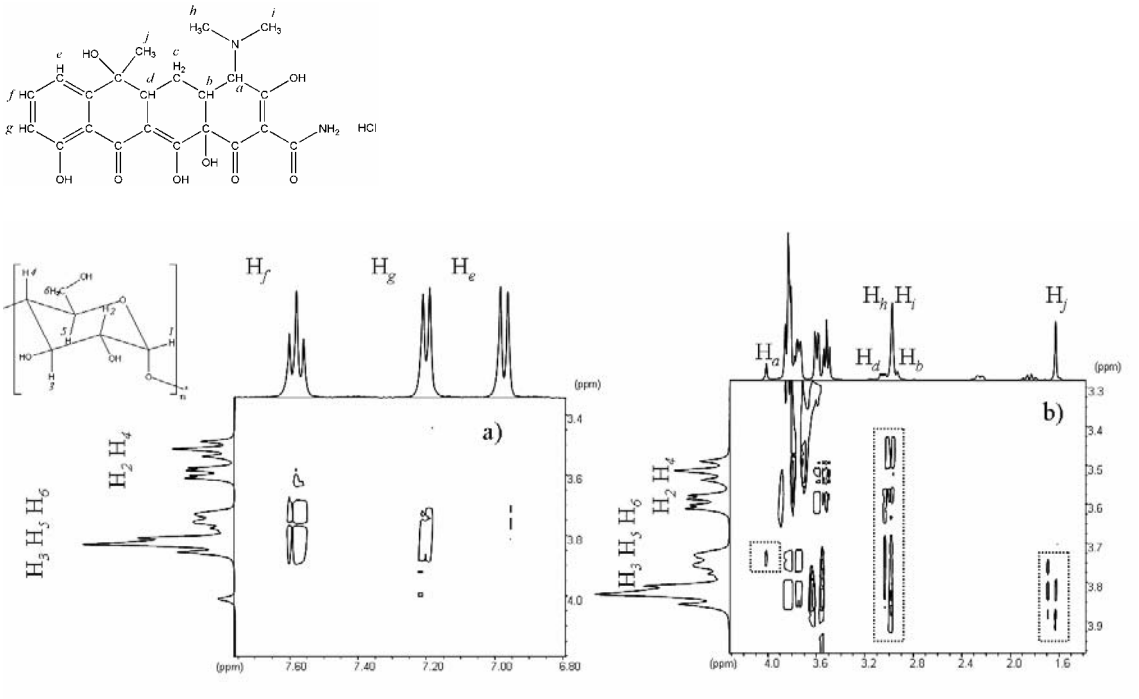

Figure 4. Expansion of the NMR NOESY contour map (400MHz, mixing time 600ms) in D_2O of IC at molar ratio of 1:1, a) to aromatic region and b) to other correlations between TC and β-CD.

These results suggested that multiple associations were present in this system. The correlations between the outer part of the molecule hydrogens (H_2 and H_4) and TC non-aromatic protons, besides not being less than 5Å interactions, could be interpreted as proximity between these molecules. No change in the chemical shifts at the higher molar ratios 2:1, 3:1 and 4:1 were observed when these are compared to the 1:1 β-CD:TC system and similar cross peak correlations between the aromatic region of TC with internal protons of the β-CD are also observed.

In order to further characterize the supramolecular complexes, solid state physicochemical analysis were also performed.

X-ray free substances and mechanical mixtures spectra showed high crystallinity (Figure 5) [38; 39], a pattern that changed to an amorphous profile as long as the β-CD concentration also increased, relatively. This could suggest new molecular organization phenomenon upon interaction.

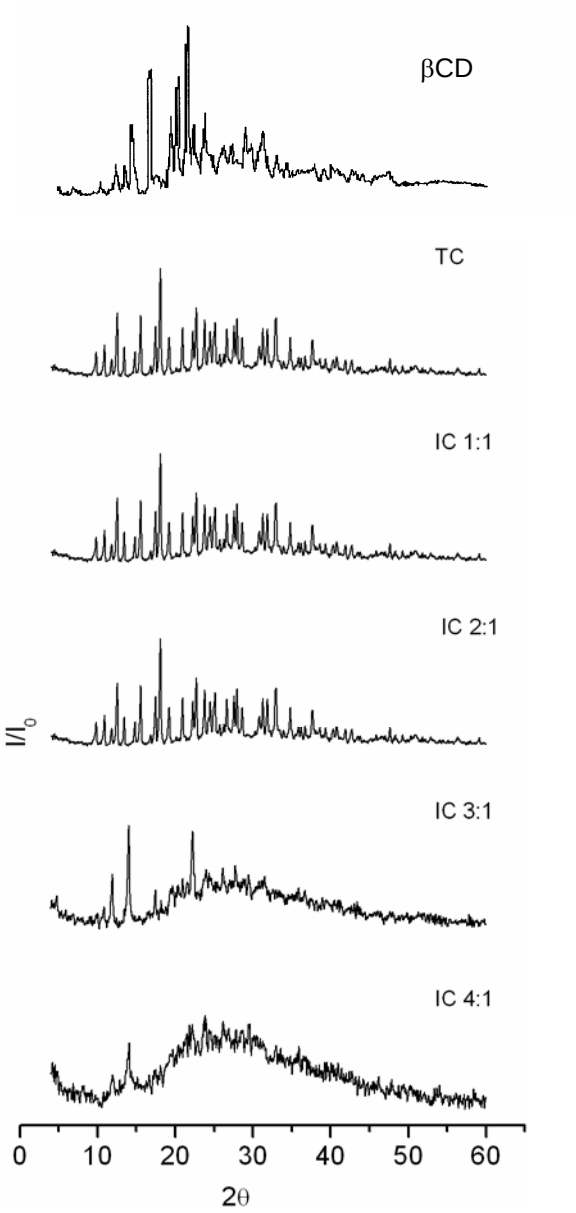

Figure 5. X-ray powder diffraction of the β-CD:TC compounds (in the labels TC represents tetracycline and IC represents β-CD:TC respectively molar ratio of 1:1; 2:1; 3:1 and 4:1).

A host:guest interaction is also suggested by infrared analysis. The infrared spectra of β-cyclodextrin, tetracycline, physical mixture and the supramolecular compounds are shown in Figure 6. The FTIR spectrum of pure TC is presented, and the most important vibrations modes are: ν (C-N) at 3365 cm^{-1}, ν (C=O) and ν (C=C) of aromatic ring between 1674 to

1580 cm^{-1}, at 1460 – 1310 cm^{-1} related to δ (OH), δ (C-C) and ν (C-C) and finally the bands at 1250 – 1200 cm^{-1} corresponding to δ (N-H) and ν (C-N) [40].

A sharpening of the ν (O-H) at 3300 cm^{-1} and ν (C-O-C) at 1070 cm^{-1} modes are observed in the spectra of the inclusion compounds at 1:1 to 4:1 molar ratio, when compared to the spectrum of pure β-CD. These modifications may be attributed to the reduction in the hydrogen bond forming upon the inclusion of the TC as result of the loss of intra cavity water molecules [41]

In addition, a strong modification in the vibration mode relating to the ν (C=O) and ν (C=C) of aromatic ring between 1674 to 1580 cm^{-1} are observed with the gradual increase of the β-CD concentration in the inclusion compound. Modifications in the bands below 900 cm^{-1} are also observed in all the inclusion compounds. These results suggested the formation of a new species in solid state when the freeze-drying method is used to prepare the inclusion compounds.

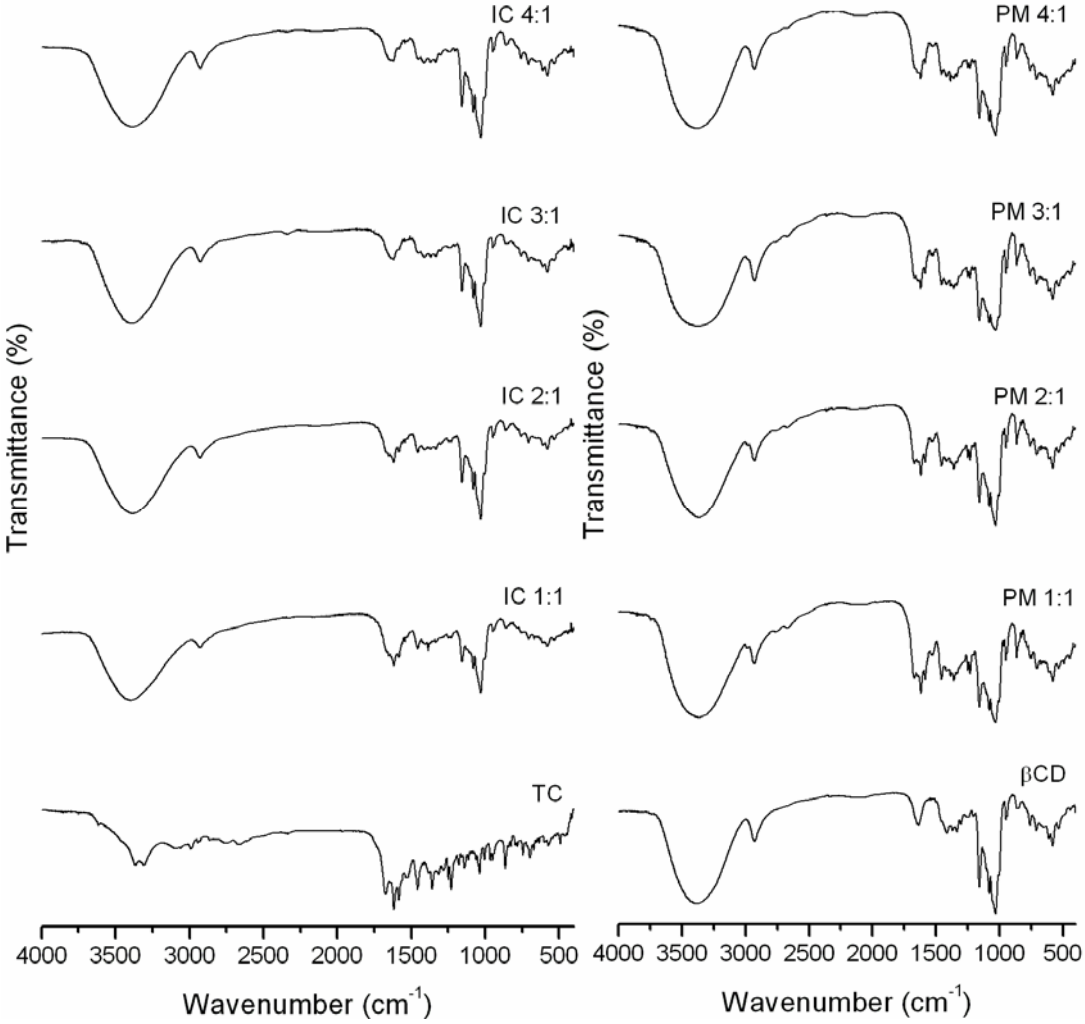

Figure 6. FTIR spectra at 4000 – 400cm^{-1} of pure TC, β-CD:TC (IC) 1:1, 2:1, 3:1, 4:1, pure β-CD, and physical mixtures (PM) 1:1, 2:1, 3:1 and 4:1.

The TG and DSC curves are presented in Figure 7 and 8. Tetracycline hydrochloride showed great thermal stability between 25 and 200°C and mass losses in two stages, between 200 and 738°C, as shown in its TG and DSC curves. The first event occurred at 287°C, a fast process of 23.81% mass loss; and the second stage (287-737°C) showed 76.57% mass loss, due to the thermal decomposition with the formation of carbonaceous products. TC DSC curve showed an endothermic event at 225°C and exothermic event at 235°C. This result suggested fusion followed by thermal decomposition. The exothermic point at 235°C was probably due to

oxidation, corresponding to the first mass loss showed at the TG curve. The exothermic events after this point were due to the pirolysis of the carbonaceous products [38].

β-cyclodextrin showed mass losses in two main phases between 25 and 700°C. The first, from 27.2 to 111.56°C, corresponded mainly to water loss (12%) from β-CD cavity. The second phase (111.56 to 682.94°C) represented the substance degradation and mass loss of 88%. The respective DSC curves showed two endothermic events at the same temperature ranges and an endothermic event at 320°C [39].

In the case of 1:1 inclusion compound, the first event in TG curve showed mass loss with less intensity (4.5% at 25-96.5°C) comparing to β-CD courve. Therefore, it suggested that water and TC occupied β-CDs' cavities. This event could be confirmed with less pronounced endothermic peak when compared to β-CD alone DSC curve at the same temperature range; and lower β-CD and TC stability between 96.5-282.9°C. This mass loss (12.3%) corresponded an endothermic event in the DSC curve, which mainly represented TC fusion. The third event showed 55% mass loss (283-405°C), and an endothermic peak at the same region of β-CD alone, representing the final thermal decomposition.

In relation to 2:1 compound TG curve, it showed water mass loss in the first stage of degradation in the range of 25-107°C (6.9%). The endothermic event showed in DSC curve confirms this finding. The water mass loss was more pronounced in this case when compare with 1:1 inclusion compound due to β-CD excess in the system. The second event at 107-280°C had lower intensity (7%) and corresponded to probably TC degradation. The DSC curves showed an endothermic event with lower intensity than in TC alone and 1:1 IC curves corroborating the TG proposed interpretation. The following event showed mass loss of 65%

in the 280-424.6°C range and was higher than in 1:1 endothermic event registered in DSC curves that could be related to excess of β-CD in the system.

In the case of the 3:1 compound, TG curve showed mass loss of 8.2% at the first stage of degradation (25-102°C), higher than in 2:1 compound group, due to the increased β-CD concentration in the system. The endothermic peak in the DSC was more pronounced, corroborating that interpretation. The following stage of degradation showed in 1:1 and 2:1 groups was not seen in this group. This fact can be related to a higher protection of TC molecule inside the β-CD aggregate, providing higher thermal stability to the drug. In consequence, the second event of thermal degradation in this group showed an increased mass loss of 74% (103-423°C). A higher endothermic event was observed in the same temperature range.

In the case of 4:1 compound, TG curves showed mass loss of 9.1% at the first event of degradation (25-102°C). This curve resembled to the β-CD TG curve due to the excess of this molecule in the system. The next stages of degradation showed mass loss of 78% (103-430°C) and 13% (430-748°C). The 4:1 DSC curve was similar to the 3:1 compound showing the equivalent endothermic events described above.

The β-CD:TC continuous thermal decomposition process was different from TC and/or β-CD alone. It could be noticed that with the increase of β-CD molar ratio, the thermal decomposition profile of the compounds became similar to β-CD alone profile. Then, thermal instability of TC and β-CD occurred after forming the inclusion compounds, evidencing the interaction between the two molecules. Furthermore, the mechanical mixtures showed the first

β-CD degradation event near 100°C, TC fusion and thermal decomposition. The second β-CD endothermic event near 320°C was not observed [35; 38; 39].

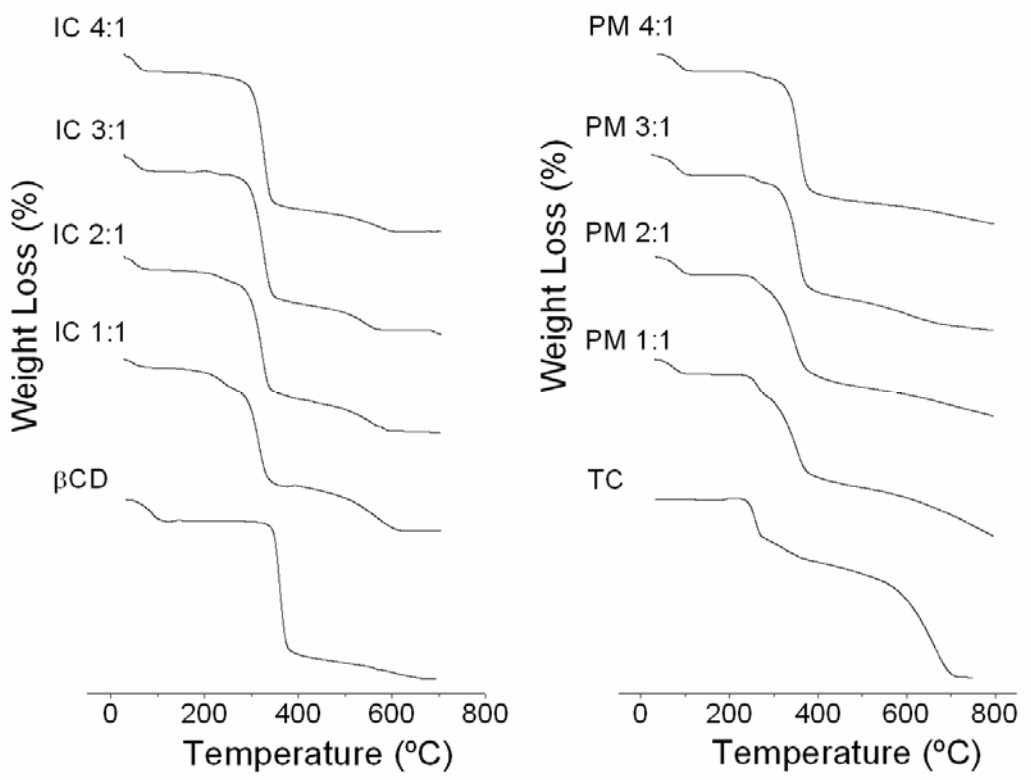

Figure 7. TG of tetracycline (TC), β-cyclodextrin (βCD) and the β-CD:TC compounds at different molar ratios (IC 1:1; IC 2:1; IC 3:1 and IC 4:1) and their physical mixtures (PM 1:1; PM 2:1; PM 3:1 and PM 4:1).

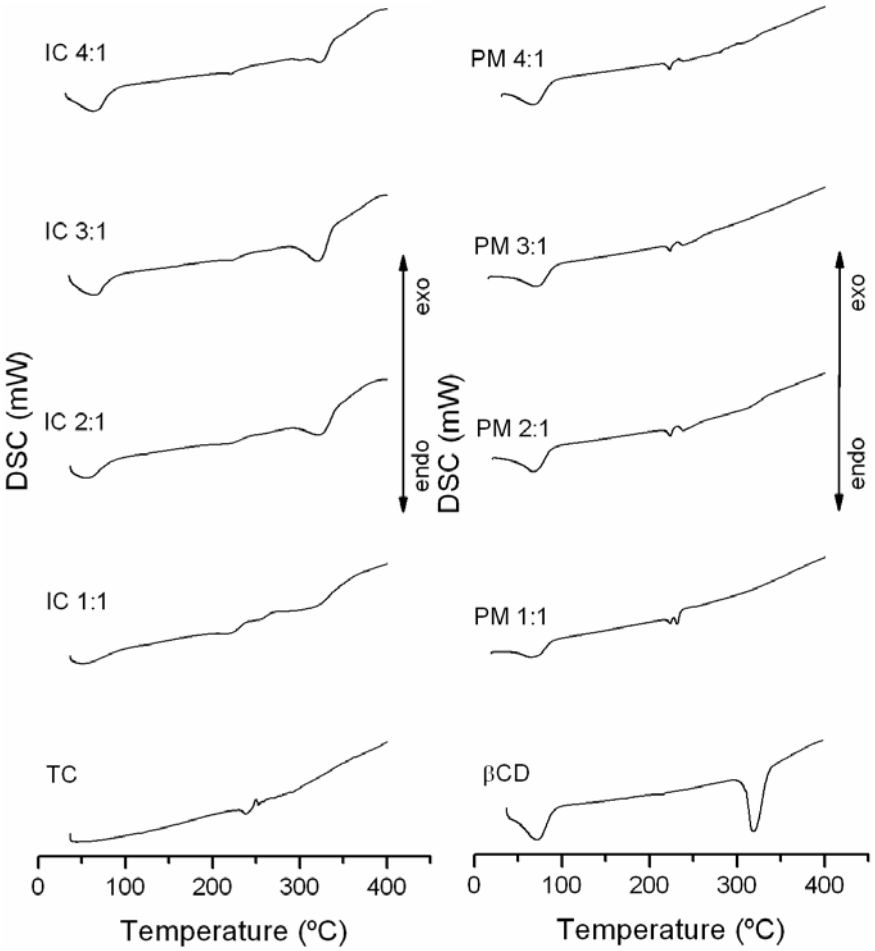

Figure 8. DSC curves of tetracycline (TC), β-cyclodextrin (βCD) and the β-CD:TC compounds at different molar ratios (IC 1:1; IC 2:1; IC 3:1 and IC 4:1) and their physical mixtures (PM 1:1; PM 2:1; PM 3:1 and PM 4:1).

The physicochemical characterization strongly suggested important structural changes in the molecules characteristics, revealing the formation of an inclusion compound and, with the gradual increase of β-CD amount in the system, these compounds could self assemble as supramolecular complexes. This interpretation differs from other studies that suggested that the interactions take place with the outer part of the β-CD molecule which is more hydrophilic, and also contradicts the affirmative that TC molecule seems to be too large to fit inside β-CD cavity (4.5-8Å) as previously stated in literature [10; 42].

The structural changes that happened upon molecules interaction reflected in TC antimicrobial properties. The antimicrobial tests showed that 2:1 β-CD:TC compound exhibited significant antimicrobial activity against *A. actinomycetemcomitans* with MIC of 0.25μg/mL of TC concentration(1:1; 2:1 groups), followed by 1μg/mL (4:1 and TC control group) and 2μg/mL (3:1) (Table 1). In spite of TC concentrations used in the test solutions being normalized with the same TC concentration, the results suggested different interactions between this bacteria strain and the different β-CD:TC compounds in solution. *A. actinomycetemcomitans* is frequently resistant to TC showing MIC values between 0.8-1.2μg/mL [43; 44]. These findings have also to be confirmed against rough-type microorganisms [45]. The MIC values found for *P. gingivalis* were 0.25μg/mL (2:1; 3:1 and 4:1 groups), followed by 1μg/mL (1:1 and TC control groups). Other studies showed MIC_{100} values for TC of 1-4μg/mL against the same bacteria, isolated from humans or reference strains [46; 47]. β-CD showed no activity against the tested bacteria. Thus, nanoassemblies antimicrobial properties evidenced that MIC concentrations could decrease four times and show the same inhibition as non-complexed drug.

Table 1. Minimal inhibitory concentrations values (MIC in μg/mL) of the studied compounds against tested reference bacteria strains *in vitro*.

Bacteria	Condition	Strains	Minimal inhibitory concentration μg/mL					
			TC	β-CD	1:1	2:1	3:1	4:1
A. actinomycetemcomitans	Anaerobic	Y4-FDC	1	>64	0.25	0.25	2	1
P. gingivalis	Anaerobic	ATCC 49417	1	>64	1	0.25	0.25	0.25

Agar diffusion susceptibility test revealed that all compounds successfully inhibited growth of *A. actinomycetemcomitans*, disclosing that the inclusion process did not affected negatively TC antimicrobial action. No significant difference between β-CD:TC compounds and TC

were found against this strain. β-CD:TC compounds and TC alone showed comparable growth inhibition of *P. gingivalis*, at the concentration of 30μg/mL. The exception was the 1:1 compound that demonstrated significantly lower inhibition ($p<0.05$). For the concentration of 10μg/mL, all groups showed similar inhibition. 1μg/mL of TC showed inhibition in all tested groups, being 4:1 with larger inhibition zone when compared to 2:1 group ($p<0.05$).

Table 2. Inhibition of growth of β-CD:TC compounds (1:1, 2:1, 3:1 and 4:1) and against *A. actinomycemcomitans* and *P. gingivalis*.

Bacteria	Tetracycline Concentration (μg/mL)	Mean inhibition zone diameter (mm ± S. D.)					
		Sample					
A. actinomycemcomitans		β-CD	TC	1:1	2:1	3:1	4:1
	1	-	21.16±3.0	21.10±3.6	16.40±3.2	16.00±2.1	15.75±2.6
	10	-	27.33±3.0	24.00±3.4	23.00±3.5	23.00±4.3	22.25±3.5
	30	-	30.5±0.6	25.60±2.3	25.10±2.4	25.50±3.4	23.60±1.4
P. gingivalis							
	1	-	27.17±2.9	27.67±1.8	24.00±3.1	28.33±1.3	31.33±1.3
	10	-	37.33±6.0	39.67±1.3	39.67±1.3	38.67±1.4	41.67±1.4
	30	-	39.67±1.2	13.33±1.6	14.33±1.4	14.67±1.6	19.50±2.2

3.2 Nanospheres preparation, release study and antimicrobial activity

The TC encapsulation efficiency of nanospheres prepared with PLGA by double emulsion process showed values of 46.80% w/w (1:1), 16.73% (2:1 group), 19.07% (3:1), 25.63% (TC alone) and 42.90% (4:1) (Table 2). Thus, different proportions of β-CD influenced the TC encapsulation efficiency. The encapsulation efficiency obtained in this study was higher than other values related in the literature obtained by spray drying technique (PLGA-TC) [48] and

double emulsion [49; 50]. The SEM images disclosed nanospheres morphology and also a smooth surface (Figure 9).

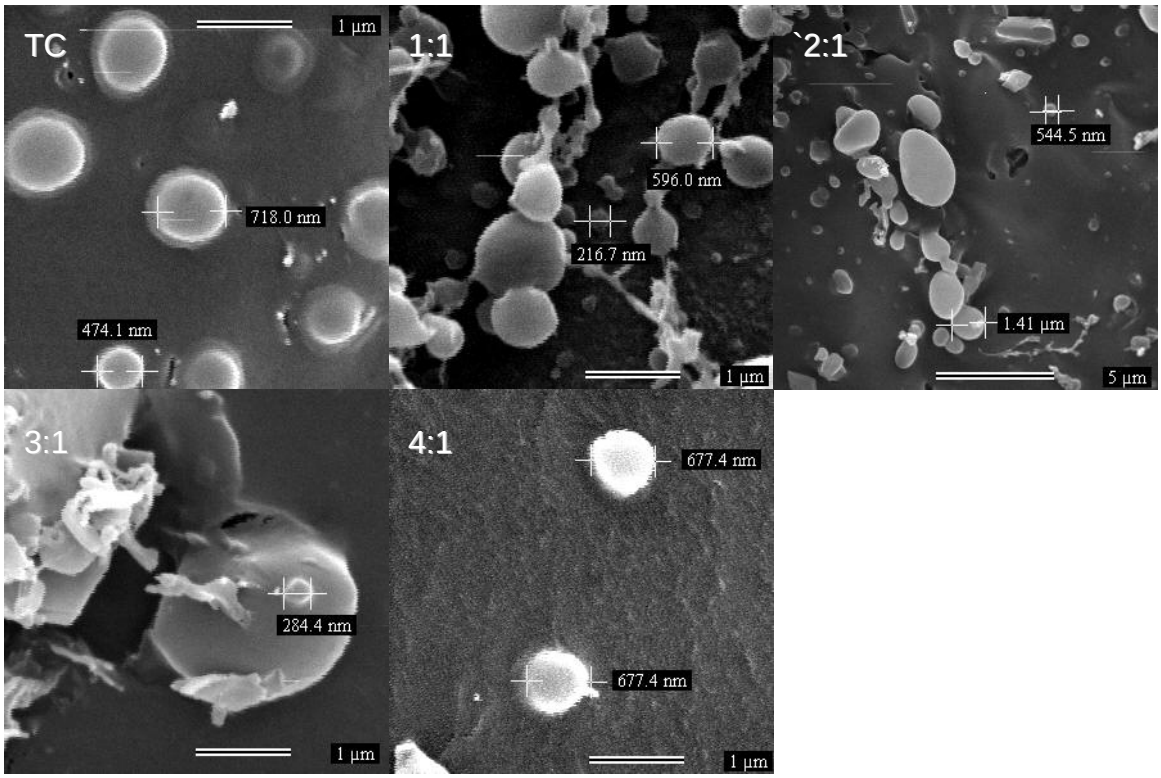

Figure 9. SEM images from the PLGA nanospheres in association with TC; β-CD:TC 1:1; 2:1; 3:1; 4:1.

Nanospheres size distribution is shown in Table 3 and also reflected the influence of β-CD in the encapsulation process. Nanospheres measuring 285 to 396nm were observed when the cyclic molecule was present in the process comparing to 958nm with TC alone group.

Table 3. Characteristics of PLGA/TC particles prepared in the presence of β-CD with respect to drug encapsulation efficiency and size distribuition.

Group	TC Loading (%)	Size distribution		
		(%)	(nm)	Pdi
TC	25.63	82.9	958	0.5
		8.60	5400	
		8.40	309	
1:1	46.80	100	307	1.0
2:1	16.73	100	396	0.7
3:1	19.07	82.90	285	0.7
		17.10	5240	
4:1	42.90	100	360	0.6

Tetracycline release kinetics from the tested compounds in association with PLGA for 10 days is shown in Figure 10. All the groups were prepared with the same TC initial amount. β-CD influenced drug release from nanospheres with the 2:1 group nearer to a zero order release. This sample showed a burst effect of 12.74±1.4% (78.9μg/mL) of the total released drug. The other groups showed a significantly higher 24h release relative to the total amount of released drug, including TC (23.84±1.0% - 105.3μg/mL), 1:1 (29.39±11.3 - 46.2μg/mL), 3:1 (28.08±0.7 - 52.7μg/mL) and 4:1 (22.23±0.2% - 64.2μg/mL) ($p<0.01$). These results showed more constant release kinetics than Actisite®, the commercially available TC local release device [51]. PLGA degradation is mainly by bulk erosion and this could explain the drug delivery for the subsequent days. β-CD showed to have great influence in TC release profile.

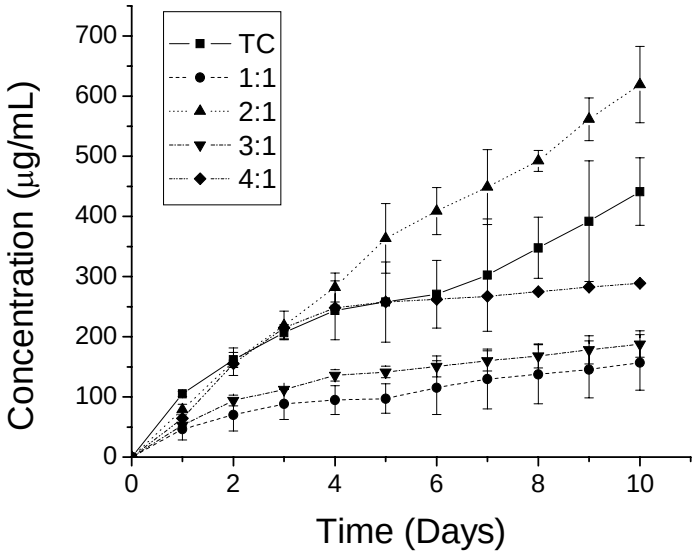

Figure 10. *In vitro* cumulative TC release from PLGA nanospheres in water at 37°C.

The in vitro release study therefore revealed that the concentration of the drug released at each time point throughout the 10 days test period was above 4μg/mL. This amount of the drug released was enough to provide appropriate TC levels that correspond to MIC values in vivo [43-47]. This fact associated to the initial high release and prolonged subsequent release was favorable to increase antibiotic potency

Agar diffusion susceptibility test revealed that the TC concentrations released from nanospheres produced satisfactory growth inhibition. The groups showed efficient antimicrobial activity during 10 days of release. 2:1 group revealed higher inhibition compared to the other groups in days, which correlated with the release findings ($p<0.05$). These results suggest that the drug can be released in a controlled fashion and exert efficiently its biological activity .

5. Conclusion

The physicochemical characterization and drug release data obtained in this study confirms the potential of this system for optimizing antimicrobial activity.

The interactions between β-CD and TC suggested by X-ray diffraction, thermal analysis, IR were strongly confirmed by ITC and DLS. NMR analysis also disclosed short distance interactions between these two molecules in different molar ratios and, TC seems to act as a seeding molecule capable of inducing self-assembly phenomena with β-CD. The supramolecular conformation in stoichiometries of 2:1, 3:1 and 4:1 increased four times TC efficiency against *P. gingivalis*. 1:1 and 2:1 complexes were also more efficient against *A. actinomycetemcomitans*.

The water-oil-water emulsion technique used was efficient to produce PLGA nanospheres containing β-CD:TC compounds. The cyclodextrin influenced the encapsulation efficiency, size distribution and also increased the antimicrobial effectiveness of the antibiotic.

The nanoparticles showed a controlled release profile mainly with the 2:1 molar ratio, releasing TC over minimum inhibitory concentration of *A. actinomycetemcomitans* and *P. gingivalis*.

Acknowledgments

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Table 1. Minimal inhibitory concentrations values (MIC in μg/mL) of the studied compounds against tested reference bacteria strains *in vitro*.

Bacteria	Condition	Strains	Minimal inhibitory concentration μg/mL					
			TC	β-CD	1:1	2:1	3:1	4:1
A. actinomycetemcomitans	anaerobic	Y4-FDC	1	>64	0.25	0.25	2	1
P. gingivalis	anaerobic	ATCC 49417	1	>64	1	0.25	0.25	0.25

Table 2. Inhibition of growth of β-CD:TC compounds (1:1, 2:1, 3:1 and 4:1) and against *A. actinomycemcomitans* and *P. gingivalis*.

Bacteria	Tetracycline Concentration (μg/mL)	Mean inhibition zone diameter (mm ± S. D.)					
		Sample					
A. actinomycemcomitans		β-CD	TC	1:1	2:1	3:1	4:1
	1	-	21.16±3.0	21.10±3.6	16.40±3.2	16.00±2.1	15.75±2.6
	10	-	27.33±3.0	24.00±3.4	23.00±3.5	23.00±4.3	22.25±3.5
	30	-	30.5±0.6	25.60±2.3	25.10±2.4	25.50±3.4	23.60±1.4
P. gingivalis							
	1	-	27.17±2.9	27.67±1.8	24.00±3.1	28.33±1.3	31.33±1.3
	10	-	37.33±6.0	39.67±1.3	39.67±1.3	38.67±1.4	41.67±1.4
	30	-	39.67±1.2	13.33±1.6	14.33±1.4	14.67±1.6	19.50±2.2

Table 3. Characteristics of PLGA/TC particles prepared in the presence of β-CD with respect to drug encapsulation efficiency and size distribuition.

Group	TC Loading (%)	Size distribution		
		(%)	(nm)	Pdi
TC	25.63	82.9	958	0.5
		8.60	5400	
		8.40	309	
1:1	46.80	100	307	1.0
2:1	16.73	100	396	0.7
3:1	19.07	82.90	285	0.7
		17.10	5240	
4:1	42.90	100	360	0.6

Figure Legends

Figure 1. Size measurements obtained by DLS titration of a 0.5 x 10^{-3} solution of TC in a 12.3 x 10^{-3}mol/L β-CD solution.

Figure 2. ITC experiments expansion to β-CD 12.3 x 10^{-3} mol/L in TC 0.5 x 10^{-3} mol/L.

Figure 3. ^{1}H NMR (400MHz) in D_2O at 25 ºC of: a) pure TC and b) IC at 1:1 TC β-CD molar ratio.

Figure 4. Expansion of the NMR NOESY contour map (400MHz, mixing time 600ms) in D_2O of IC at molar ratio of 1:1, a) to aromatic region and b) to other correlations between TC and β-CD.

Figure 5. X-ray powder diffraction of the β-CD:TC compounds (in the labels TC represents tetracycline and β-CD:TC represents respectively molar ratio of IC 1:1; IC 2:1; IC 3:1 and IC 4:1). PM 1:1; PM 2:1; PM 3:1 and PM 4:1 represents the physical mixtures.

Figure 6. Infrared spectra of tetracycline (TC), β-cyclodextrin (βCD), the β-CD:TC compounds at different molar ratios (IC 1:1; IC 2:1; IC 3:1 and IC 4:1) and their physical mixtures (PM 1:1; PM 2:1; PM 3:1 and PM 4:1).

Figure 7. TG of tetracycline (TC), β-cyclodextrin (βCD) and the β-CD:TC compounds at different molar ratios (IC 1:1; IC 2:1; IC 3:1 and IC 4:1) and their physical mixtures (PM 1:1; PM 2:1; PM 3:1 and PM 4:1).

Figure 8. DSC curves of tetracycline (TC), β-cyclodextrin (βCD) and the β-CD:TC compounds at different molar ratios (IC 1:1; IC 2:1; IC 3:1 and IC 4:1) and their mechanical mixtures (PM 1:1; PM 2:1; PM 3:1 and PM 4:1).

Figure 9. SEM images from the PLGA microspheres in association with TC; β-CD:TC 1:1; 2:1; 3:1; 4:1.

Figure 10. *In vitro* cumulative TC release from PLGA microspheres.

Figure 1

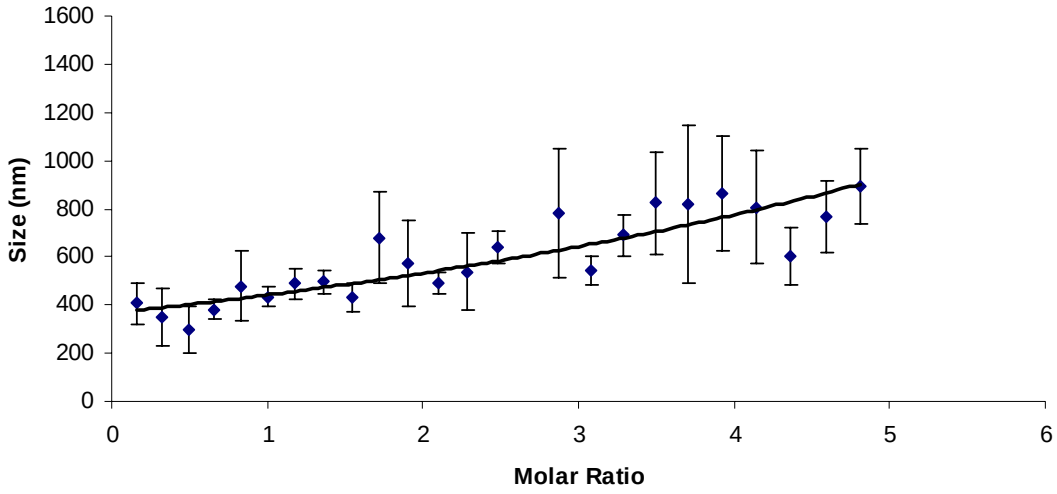

1600
1400
1200
1000
800
600
400
200
0
Size (nm)
0
1
2
3
4
5
6
Molar Ratio

Figure 2

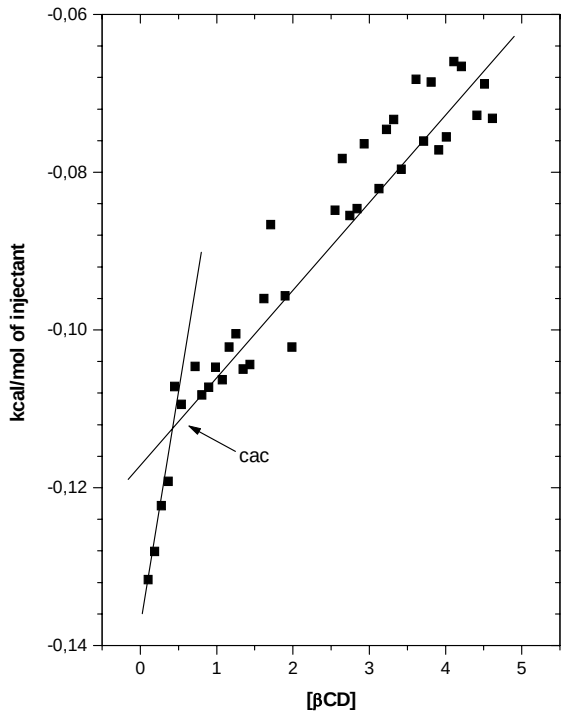


Figure 3

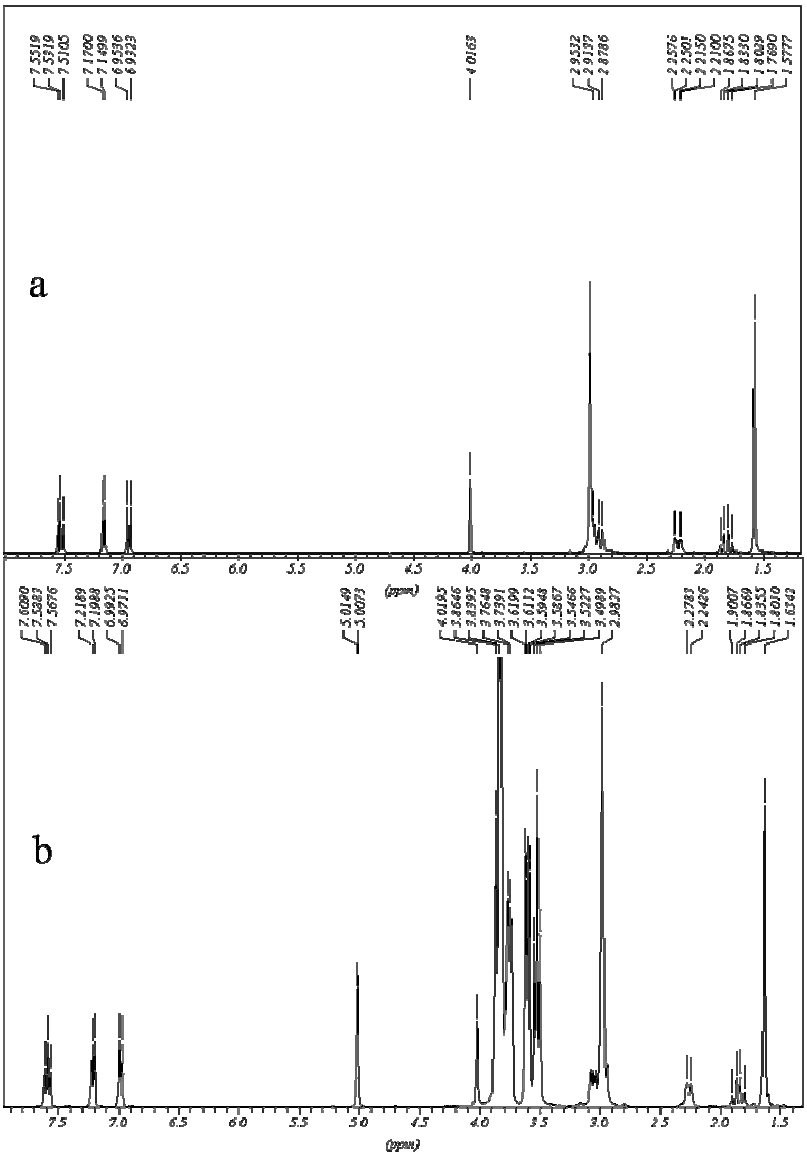


Figura 4

Figure 5

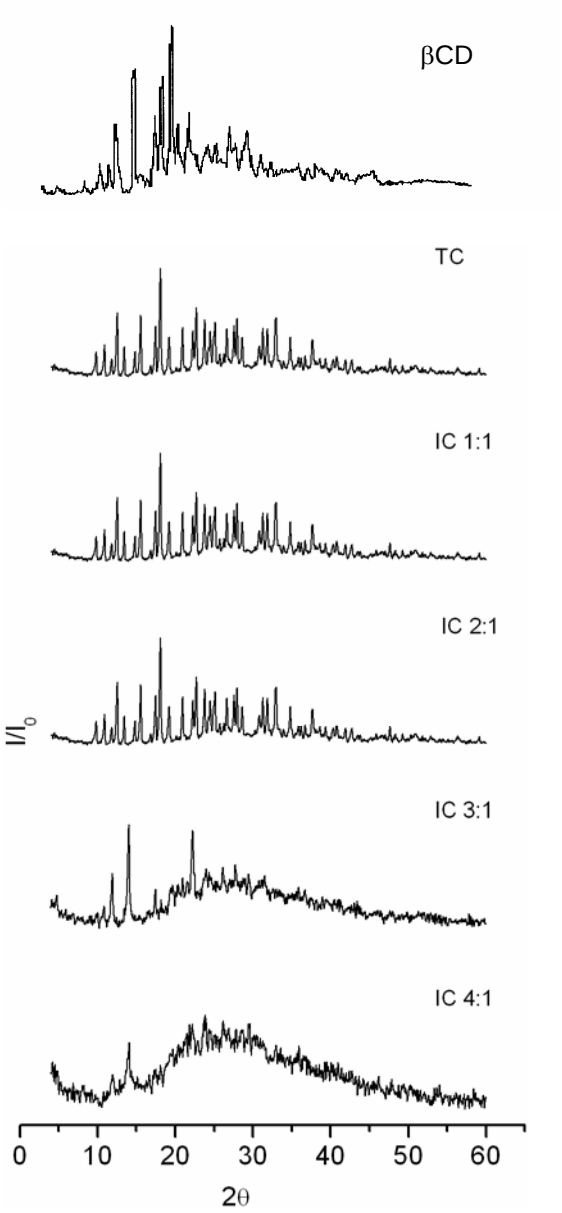


Figure 6

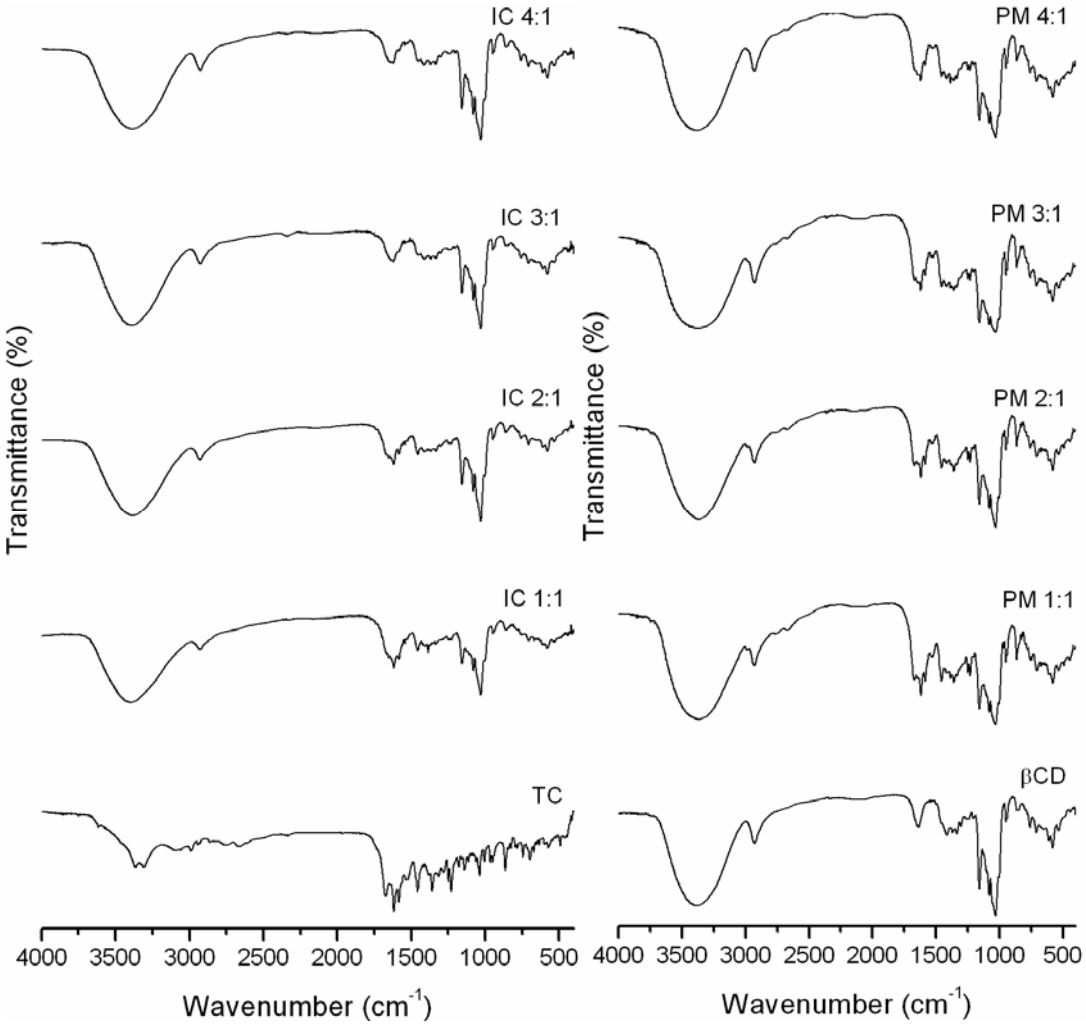

IC 4:1
IC 3:1
IC 2:1
IC 1:1
TC
PM 4:1
PM 3:1
PM 2:1
PM 1:1
βCD
Transmittance (%)
Transmittance (%)
4000 3500 3000 2500 2000 1500 1000 500
Wavenumber (cm-1)
4000 3500 3000 2500 2000 1500 1000 500
Wavenumber (cm-1)

Figure 7

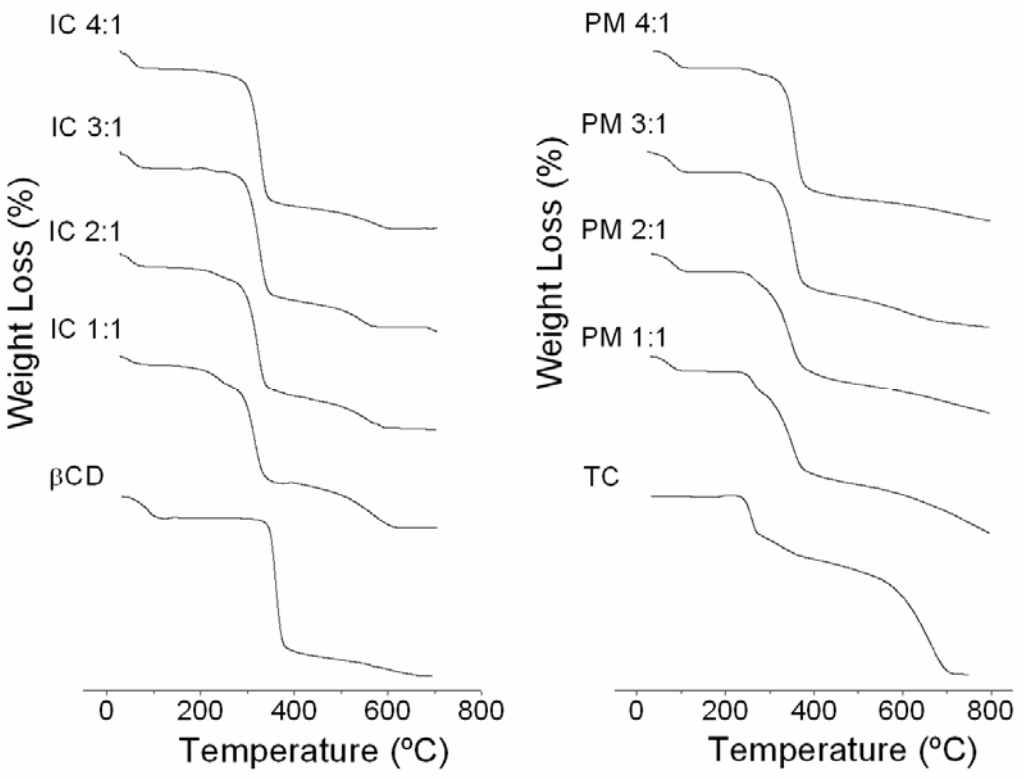

IC 4:1
IC 3:1
IC 2:1
IC 1:1
βCD
Weight Loss (%)
0
200
400
600
800
Temperature (ºC)
PM 4:1
PM 3:1
PM 2:1
PM 1:1
TC
Weight Loss (%)
0
200
400
600
800
Temperature (ºC)

Figure 8

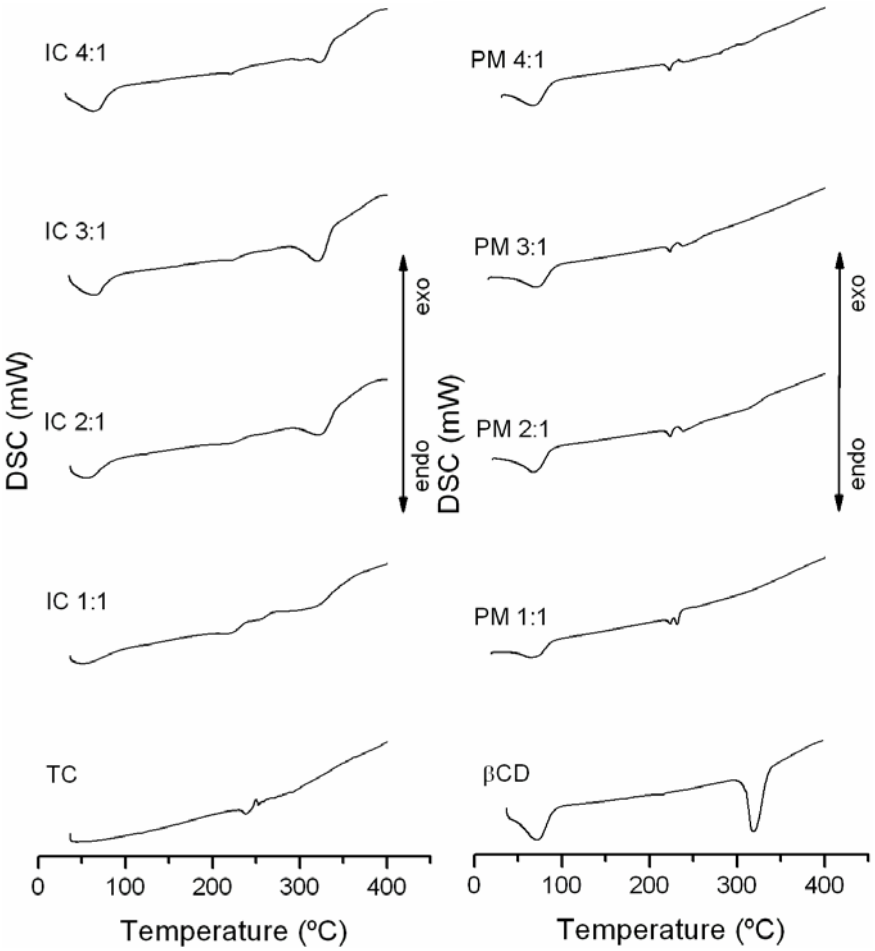

IC 4:1
IC 3:1
IC 2:1
IC 1:1
TC
PM 4:1
PM 3:1
PM 2:1
PM 1:1
βCD
DSC (mW)
exo
endo
0
100
200
300
400
Temperature (ºC)

Figure 9

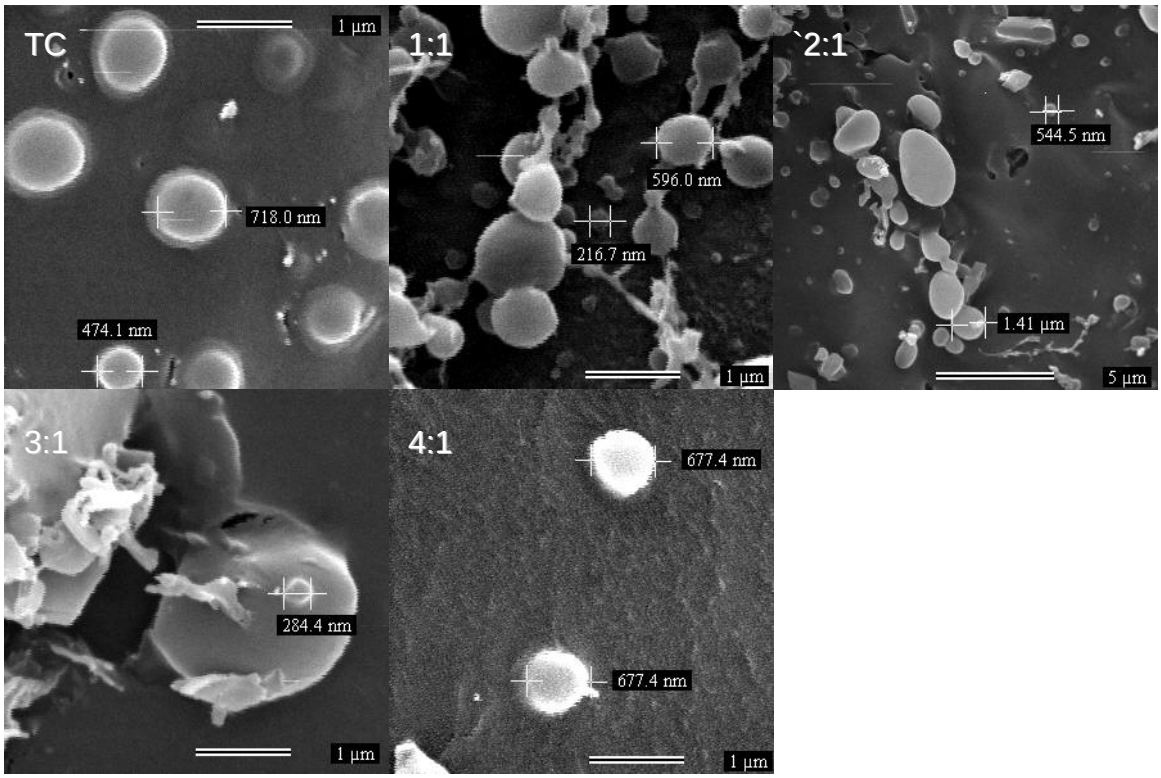
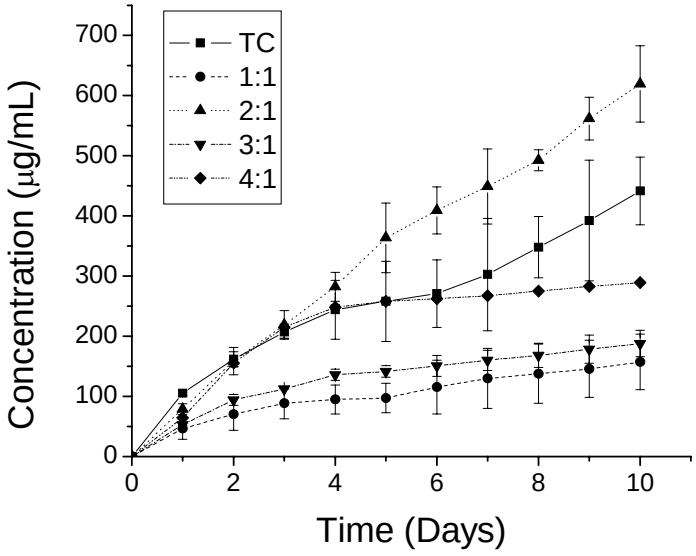

TC
1 µm
718.0 nm
474.1 nm
1:1
596.0 nm
216.7 nm
1 µm
`2:1
544.5 nm
1.41 µm
5 µm
3:1
284.4 nm
1 µm
4:1
677.4 nm
677.4 nm
1 µm

Figure 10


TC
1:1
2:1
3:1
4:1
Concentration (μg/mL)
Time (Days)
0
100
200
300
400
500
600
700
0
2
4
6
8
10

COMENTÁRIOS FINAIS

O presente trabalho teve como intenção a fabricação e a caracterização de compostos supramoleculares entre β-ciclodextrinas e tetraciclinas. A elucidação das interações entre as duas moléculas é de fundamental importância para o futuro desenvolvimento de formulações e produtos que as contenham.

A caracterização físico-química revelou interação, mudanças estruturais e conformacionais entre as duas moléculas, relacionadas ao aumento da concentração de β-ciclodextrina no sistema. Na medida em que a razão molar de β-ciclodextrina cresceu no sistema, foi observado um aumento de tamanho das partículas formadas pelos compostos em solução, devido a interações dentro e fora da cavidade, além de mudanças na cristalinidade e em relação às suas propriedades de estabilidade térmica.

Essas interações tiveram impacto na atividade antimicrobiana desses compostos. Foi mostrados que esses nano agregados, quando em solução, apresentaram maior eficácia antimicrobiana contra as bactérias testadas em determinadas concentrações. Estas novas propriedades antimicrobianas abrem novos questionamentos à cerca de como esses nano agregados interagem com as bactérias, porque nestes experimentos, todos os compostos apresentavam a mesma concentração de tetraciclina, diferindo apenas na razão molar de β-ciclodextrina. Possivelmente as β-ciclodextrinas poderiam modular a liberação do fármaco de uma maneira mais controlada por um período mais prolongado, diferindo da ação da tetraciclina pura e melhorando suas propriedades [33; 38]. Outra possibilidade seria uma diferente interação dos nanoagregados com a parede celular bacteriana o que poderia explicar uma maior eficiência antimicrobiana do fármaco.

A técnica de nano encapsulamento no polímero PLGA mostrou-se eficaz para a produção de nano esferas de tetraciclina. A presença da β-ciclodextrina mostrou influenciar o percentual de encapsulamento de tetraciclina no polímero além de ter marcante influência no tamanho das nano esferas.

As nano esferas poliméricas exibiram uma liberação controlada por cerca de 10 dias. Foi observado que a liberação obtida das nano esferas sintetizadas a partir do composto na razão molar de 2:1 β-ciclodextrina:tetraciclina esteve perto de uma liberação de ordem zero. As concentrações da droga obtidas a partir da concentração inicial de 1mg/mL mostraram-se acima das concentrações inibitórias mínimas para as bactérias testadas *A. actinomycetemcomitans* e *P. gingivalis*.

Assim, foi demonstrado que é possível a formação de nano agregados com tetraciclina e β-ciclodextrina e que estes podem ser utilizados para a formulação de nano esferas de PLGA para a liberação controlada de fármacos.

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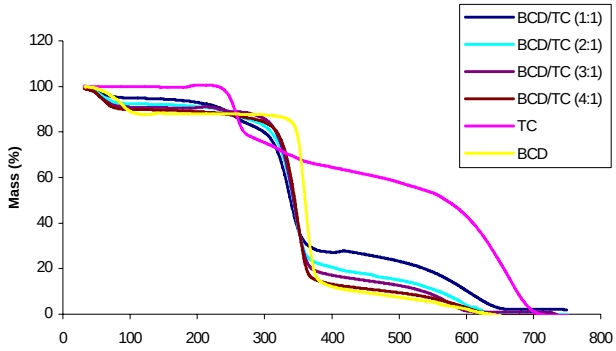
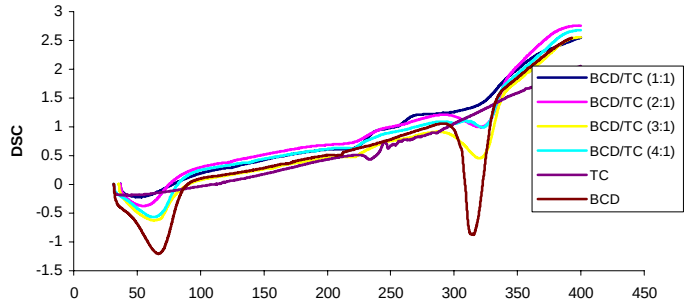
ANEXOS

Anexo A – Gráficos de TG (1) e DSC (2) dos compostos de β-CD e TC nas razões molares de 1:1; 2:1; 3:1 and 4:1 juntamente com os fármacos puros.

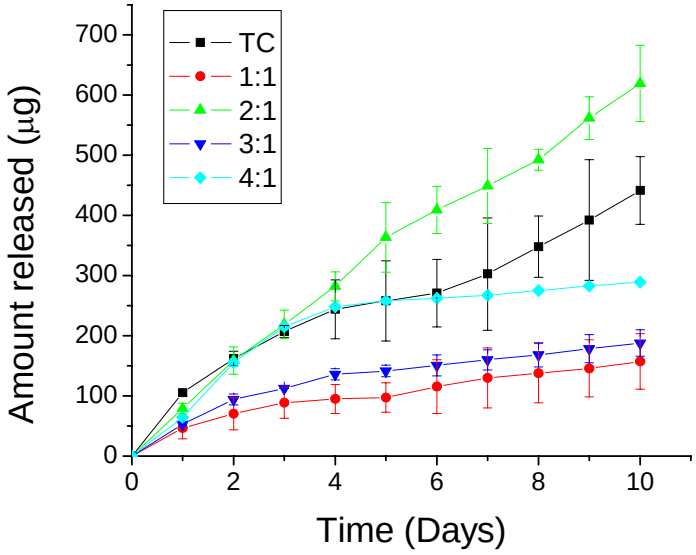
1)



2)



Anexo B – Liberação controlada de TC a partir das nanoesferas poliméricas.



Anexo C - Instruções aos autores (normas da revista que foram seguidas)

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[4] J.B. Phipps, D.F. Untereker, Iontophoresis apparatus and methods of producing same, U.S. Patent 4, 744, 787, May 17, 1988.

Report:

[5] N.F. Cardarelli, K.E. Walker, G. Zweig, Development of registration criteria for controlled release pesticide formulations, U.S. Environmental Protection Agency, Washington, DC 20460, EPA-504/9077-916, January 1978.

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