Article:
Richter, AR, Carneiro, MJ, de Sousa, NA et al. (9 more authors) (2020) Self-assembling cashew gum-graft-polylactide copolymer nanoparticles as a potential amphotericin B delivery matrix. International Journal of Biological Macromolecules, 152. pp. 492-502. ISSN 0141-8130

https://doi.org/10.1016/j.ijbiomac.2020.02.166

International Journal of Biological Macromolecules 152 (2020) 492–502 Date of acceptance: 15 February 2020

https://doi.org/10.1016/j.ijbiomac.2020.02.166

SELF-ASSEMBLING CASHEW GUM-GRAFT-POLYLACTIDE COPOLYMER NANOPARTICLES AS A POTENTIAL AMPHOTERICIN B DELIVERY MATRIX

Ana Rosa Richter [a],Maria J. Carneiro[a], Nayara A. de Sousa [b],Vicente P. T. Pinto [b],Rosimeyre S. Freire, R.[c], Jeanlex S. de Sousa[c], Josilayne de Fátima Souza Mendes[d], Raquel Oliveira dos Santos Fontenelle[d], Judith P. A.Feitosa [a], Haroldo C. B. Paula[a], Francisco M. Goycoolea[e], Regina C. M. de Paula.[a]*

[a]Departament of Organic and Inorganic Chemistry – UFC – CEP, Caixa Postal 6021 – Humberto Monte s/n, Campus do Pici, 60455-760 Fortaleza, Brazil.

[b]Facultyof Medicine– UFC – CEP 62.042-280 – Comandante Mauro Célio Rocha Pontes, 100, Derby, Sobral, Brazil.

[c] Department of Physics – UFC, Fortaleza, Brazil

[d]Curso de Ciências Biológicas, Centro de Ciências Agrárias e Biologicas, Universidade Estadual do Vale do Acaraú.

[e]School of Food Science and Nutrition, University of Leeds, Leeds LS16 7PA, U.K.

ABSTRACT

Amphotericin B is an antibiotic used in the treatment of fungal disease and leishmania; however, it exhibits side effects to patients, hindering its wider application. Therefore, nanocarriers have been investigated as delivery systems for amphotericin B (AMB) in order to decrease its toxicity, besides increase bioavailability and solubility. Amphiphilic copolymers are interesting materials to encapsulate hydrophobic drugs such as AMB, hence copolymers of cashew gum (CG) and L-lactide (LA) were synthesized using two different CG:LA molar ratios (1:1 and 1:10). Data obtained revealed that copolymer nanoparticles present similar figures for particle sizes and zeta potentials; however, particle size of encapsulated AMB increases if compared to unloaded nanoparticles. The 1:10 nanoparticle sample has better stability although higher polydispersity index (PDI) if compared to 1:1 sample. High amphotericin (AMB) encapsulation efficiencies and low hemolysis were obtained. AMB loaded copolymers show lower aggregation pattern than commercial AMB solution. AMB loaded nanoparticles show antifungal activities against four *C. albicans* strains. It can be inferred that cashew gum/polylactide copolymers have potential as nanocarrier systems for AMB.

Keys words: Poly(lactic acid), cashew gum, nanoparticles, amphotericin B, antifungal activity.

Correspondent author: Regina Célia Monteiro de Paula rpaula@dqoi.ufc.br

Departament of Organic and Inorganic Chemistry – UFC – CEP, Caixa Postal 6021 – Humberto Monte s/n, Campus do Pici, 60455-760 Fortaleza, Brazil

1. Introduction

Amphiphilic polymers have been widely investigated as drug delivery system due to their ability to form nanoparticles by self-assembly. The hydrophobic core of amphiphilic nanoparticles can incorporate hydrophobic drugs and delivery them to specific targets [1,2].

Polysaccharides have been used as matrix building blocks to produced amphiphilic materials through the insertion of hydrophobic groups or the grafting of hydrophobic monomers onto polysaccharide chains. The choice of polysaccharide is based on its properties (biodegradability and non-toxicity) and the source material (renewable resource) as well as the presence of several chemical groups, such as hydroxyl, sulfate, amino and carboxyl, which can be used to promote the synthesis.

Lactic acid is obtained by microbial fermentation and source materials include corn syrup, sucrose from sugar cane, lactose cheese pulp and paper [3,4]. Poly (lactic acid) (PLA) is biocompatible, bio-absorbable and biodegradable and it has been approved by the US Food and Drug Administration (FDA) as a biomaterial [5,6]. Copolymers of PLA and polysaccharides, such as cellulose, starch, chitosan and pullulan have been reported [7-16]. Recent studies have addressed synthesis methods and applications as well as the development of potential delivery systems for hydrophobic drugs, due to the capacity to form amphiphilic micelles through self-organization [7-16].

Cashew gum (CG) is an exudate polysaccharide obtained from the *Anacardium occidentale* tree, which shows the potential for commercial use due to the large plantations of these trees in Brazil and in other countries [17]. The number of articles published on cashew gum has increase significantly in the past decade. The gum is a branched macromolecule mainly composed of three types of galactan units within the core, linked by C-1 and C-3; C-1 and C-6; and C-1, C-3 and C-6 [18,19]. Copolymers of CG with monomers such as acrylamide [20-24]; acrylic acid [25] and isopropylacrylamide [26]have been reported.

CG gum has also been cited as a potential drug delivery system for epirubicin [26], astaxanthin [27], diclofenac diethyl amine [28], indomethacin [29,30], bovine serum albumin (BSA) [31], pilocarpine [32], isoxsuprine HCl [33], alkaloid epiisopiloturine [34] and insulin [35].

Richter et al, 2018 [36] reported proof-of-concept of the feasibility to prepare Pickering emulsion systems based on copolymer derivative of cashew gum and L-polylactide. The systems offered the possibility to associate AMB with encapsulation efficiencies up to 47 %, however the synthesis and characterization of copolymer neither the production and characterization of nanoparticles by self-assembling were reported.

Amphotericin B (AMB) was the drug selected to be loaded into the self-assembled nanoparticles. AMB is a potent fungistatic and fungicide drug produced by the actinomycetes *Streptomycetes nodosus* [37] that was approved for clinical use by FDA in 1959 [38]. AMB is also prescribed in the treatment of visceral leishmaniasis. It is a lipophilic drug that binds to lipids and intercalates into lipid bilayers that then associate to form transmembrane pores [39]. AMB also shows very low oral bioavailability due to its structural features that violate Lipinsky's rule (e.g., low Log P, high Mw, large polar surface area). Hence, novel pharmaceutical formulations of AMB are of great interest with a view to contribute to increase its pharmacological bioavailability for oral and other routes of administration, while exerting control on its drug release.

Many authors have proposed new encapsulation techniques for AMB in order to decrease its side effects and toxicity, such as: nanoparticles [40-43], micelles [44,45], conjugates [46] and emulsions [36,47,48].

In this study, cashew gum/polylactide copolymers (CGPLAP) with different CG:PLA molar ratios have been synthesize and characterized by resonance magnetic nuclear (RMN) and infrared spectroscopy. The effect of copolymer composition on physicochemical characteristics of AMB loaded and unloaded nanoparticles obtained by self-assembling were evaluated and compared with Pickering emulsion system previously reported [36].

2. Experimental

2.1. Materials

Cashew tree (*Anacardium occidentale*) exudate was kindly donated by EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) and the cashew gum (CG) was isolated as described in a previously paper [18]. L-Lactide (3,6-dimethyl-1,4-dioxane-2,5-dione), triethylamine (TEA) and Amphotericin B solution were purchased from Sigma-Aldrich

and used without further purification. The cashew gum used in this study has an average molar mass, obtained by size exclusion chromatography, of 6.9 $x10^4$ $g.mol^{-1}$ and the molar sugar ratio for galactose:glucose:arabinose:rhamnose:glucuronic acid was determined as 1.00:0.20:0.08:0.05:0.06.

2.2. Cashew gum/lactide copolymer (CGPLAP)

The copolymers were synthesized as previously reported by Seo et al. [13]. Briefly, 1.0 g of CG was dissolved in DMSO in the temperature range of 70-75°C,under magnetic stirring. L-Lactide and TEA were added to the flask (the amounts of reagents and solvent are given in Table 1) and the reaction was kept under N_2 atmosphere for 2 h. After this time, the N_2 flow was interrupted and the reaction was left to proceed for 10 h. At the end of the reaction time, the copolymer solution was filtered, dialyzed against water and lyophilized. The graft reaction was performed in two CG:PLA molar ratios (1:1 and 1:10). In this methodology, the concentration of lactide and TEA was kept at 10 and 2% (w/v), respectively. To remove residual monomer and/or homopolymer formed during the reaction, hexane was added to the lyophilized graft copolymers dispersed in distilled water and the system was kept under magnetic stirring for 48 h [49]. After this period of time, the aqueous phase was removed and lyophilized. The purified samples were named CGPLAP.

2.3. Characterization of cashew gum copolymer

The Fourier transform IR (FT-IR) spectra were recorded on an FTLA 2000, ABB Bomem spectrometer, in the range of 400 - 4000 cm^{-1}, with the samples as KBr pellet. ^1H NMR spectra were recorded in DMSO-d_6 at 353 K on a Fourier transform Bruker Advance DRX 500 spectrometer with an inverse multinuclear gradient probehead equipped with z-shielded gradient coils and a Silicon Graphics workstation. The molar substitution (MS), degree of substitution (DS) and degree of polymerization (DP) were calculated based on procedures reported in the literature by Guo et al. [5] and Teramoto and Nishio [50] with modifications:

$$MS = \frac{(A+B)/3}{H-1} \quad (1)$$

$$DS = \frac{A/3}{H-1} \quad (2)$$

$$DP = \frac{MS}{DS} \quad (3)$$

where A and B are the area of terminal and internal methyl groups, respectively, and H-1 is the area of anomeric protons (5.04 to 5.17 ppm).

2.4. Self-assembled nanoparticles

Self-assembled nanoparticleswere prepared by the dialysis method. GCPLAP (10 mg) was dissolved in 10 mL of dimethyl sulfoxide (DMSO) and the solution was dialyzed against distilled water using a dialysis membrane (cut off 14,000 $g.mol^{-1}$) for 3 days. The resulting nanoparticles were stored in a freezer until characterization.

2.5. Particle size

The size and zeta potential nanoparticles were characterized by dynamic light scattering with non-invasive back scattering (DLS-NIBS), at 25ºC,with irradiation of the sample using a 4 mW helium/neon red laser (λ=633 nm). The detection was carried out at an angle of 173°. The zeta potential was measured by mixed laser Doppler velocimetry and phase analysis light scattering (M3–PALS). A Nanosizer ZS 3600 system (Malvern Instruments Ltd., Worcestershire, UK) was used for both determinations.

2.6 Stability Study

The stability experiment was performed with blank nanoparticles. The stability was carried out in phosphate buffer solution (pH 7.4) at 37 ºC. The buffer (950 µL) was added to the nanoparticle solution (50 µL), and the system was left in an incubator at 37

ºC with stirring at 50 rpm. After 20 min, the particle size was measured and this analysis was repeated every 20 min until 360 min.Measurements were made in triplicates.

2.7. Morphology

The morphology of CGPLAP nanoparticles was examined using scanning electron microscopy (SEM) and atomic force microscopy (AFM). CGPLAP nanoparticles were examined using scanning electron microscopy (Quanta FEG 450 - FEI) at a voltage of 20 kV. The nanoparticles solutions were fixed in stubs with carbon tape and metallized with gold (Quorum QT150ES).

Blank NP´s were analyzed using Asylum MFP3D-Bio microscopy (AFM). The images were taken in tapping mode, using an EconoLTESP with nominal spring constant of 5n/m and resonance frequency of 138 Khz. Images acquired in intermittent contact mode (tapping mode). 10 µL of diluted samples (1:200 v/v) was spread onto freshly cleaned mica and vacuum dried.

2.8. AmphotericinB encapsulation and aggregation state

An amount of 50 mg of the copolymer and 5 mg of AMBwere solubilized in 5 mL of DMSO. The solution with the drug was dialyzed for 72 h against distilled water in a light-protected vessel. For a determination of encapsulation efficiency(EE%), NP's were frozen and lyophilized. The material in powder form was resuspended in DMSO and centrifuged at 40,000 G for 0.5 h so that the matrix of the nanoparticle ruptured and released the encapsulated drug. After centrifugation the AMBconcentration was determined by spectrophotometry in visible ultraviolet (UV-vis) without wavelength of 391 nm, using a Shimadzu® UV-1800 spectrophotometer.

The amount of AMBencapsulated was calculated using a calibration curve to determine the ratio of absorbance to concentration (R^2 = 0.9988). The following formula was used to calculate EE%:

$$EE\% = \frac{\text{AMB mass in NP}}{\text{initial AMB add to NP}} x\ 100\%$$

To determinate the state of aggregation of AMB in nanoparticles before extraction, the AMB loaded nanoparticles were diluted in deionized water and the UV/vis spectrum registered. The experiment was also performed with a commercial Sigma AMB solution (with sodium desoxycholate).

2.9 Hemolysis assay

To evaluate the toxicity of self-assembling nanoparticles, its hemolytic activity was tested using human red bloods cells (RBCs). Human bloods was collected in EDTA tubes, washed three times and re-suspended with sterile saline solution (0.9 %). The samples were tested at different AMBconcentration from 62.5 to 250 $\mu g.mL^{-1}$. Triton X and saline solution were used was positive and negative hemolysis control, respectively. The mixture was incubated for 30 min at 37 °C and centrifuged at 8,000 rpm for 1 min. After centrifugation time, the absorbance of samples supernatant was measured at 492 nm. The results were statistically compared by Tukey test ($p < 0.05$), using Statistical 10.0 program.

2.10 In vitro drug release

The release profiles of the drug loaded nanoparticles were obtained using a dialysis system. Each nanoparticle sample (20 mg) were introduced into cellulose acetate membranes (cut off 14,000 $g.mol^{-1}$) and dialyzed against 30 mL of PBS buffer solution containing 0.25% sodium lauryl sulfate, at pH 7.4, and 37 °C. Aliquots were taken at certain time intervals and analyzed by spectrophotometry in the UV–vis region.

2.11In vitro antifungal activity

The minimum inhibitory concentrations (MIC) for blank and AMB-loaded nanoparticle and the Sigma-Aldrich AMB solution were determined via the broth micro dilution method using 96-well plates according to document M27-A3, from the Clinical and Laboratory Standards Institute-CLSI (formerly NCCLS) [51]. Stock solution of nanoparticles (64 $\mu g.mL^{-1}$) in sodium lauryl sulfate (0.150 mL) were serially diluted to

range 0.007 – 16 μg.mL^{-1} in RPMI 1640 medium. AMB (0.007 - 16 μg.mL^{-1}) was used as a standard. The microplates were incubated at 35°C and fungal growth/inhibition was observed after 48 h. The MIC was defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism. The results were read visually as recommended by CLSI [51]. Each experiment was performed in duplicate. Four *Candida albicans* strains were used in the experiment, one obtained from the American Type Culture Collection (ATCC 90028) and three from clinical isolates of *C. albicans* obtained from Santa Casa de Misericórdia Hospital, Sobral (Ceará, Brazil).

3. Results and discussion

The grafting of L-lactide onto CG chains involves a nucleophilic attack by the hydroxyl groups of the polysaccharide. Since the hydroxyl groups are not sufficiently nucleophilic to initiate the reaction, the production of a reactive alkoxide is required. This wascarried out through the addition of triethylamine (TEA), as suggested by Cho et al. [13]. TEA also acts as a catalyst for the lactide ring-opening reaction.

3.1. Cashew gum graft reaction yield

The reaction yield was higher for CGPLA 1:1 than for CGPLA 1:10 (Table 2), indicating that an increase in the amount of L-lactide in the reaction medium leads to a decrease in the yield. The CGPLA was purified in order to remove the homopolymer produced in a side reaction. Purification of the copolymer was carried out by polylactide extraction with a non solvent. Several authors have reported the use of organic solvents, such as chloroform [15], toluene [7,52,53], acetone [54] and hexane [49,55], to remove the polylactide, or a solvent mixture like toluene/methanol [56]. In this study, hexane was chosen to remove the homopolymer. CGPLA copolymers are not soluble in this solvent but polylactide is soluble. The yield of the purified copolymer, in relation to the

unpurified copolymer, also decreased with increasing of CG:PLA molar ratio. An increase in the proportion of lactide in the reaction favored the formation of the homopolymer. The percentage (%) of grafting observed for the chitosan-g-poly(lactic acid) derivative at a chitosan:PLA ratio of 10:1 was higher (92.49%) than that observed for CGPLA in the same feed ratio [10]. Cellulose graft with PLA copolymers, with a feed ratio of 1:1, show an increase in the grafting from 27.88 to 32.63 % on varying the reaction temperature from 130 to 110˚C [5]. These values were also higher than that obtained with a CG:PLA ratio of 1:1. In both cited articles, stannous octoate was used as the catalyst and high temperatures were employed (110-140 ˚C). In the CGPLA synthesis triethylamine (TEA) was used as a catalyst with the temperature ranging from 70-75°C.

3.2. FTIR analysis

FTIR spectrafor the PLA, CG and copolymers are shown in Fig. S1-Supplementary material.The PLA spectrum shows intense bands in the range from 2971 to 2853cm^{-1} and at 1739 cm^{-1}. The bands at 2971and 2853cm^{-1} are due to the asymmetric and symmetric stretching vibration of CH_3, while the band at 2853 cm^{-1} is related to the symmetric vibration of aliphatic CH groups. The intense band at 1739 cm^{-1} is attributed to the C=O stretching vibration of aliphatic ester groups [56]. The cashew gum spectrum (in salt form) shows a broad band at 3379 cm^{-1} due to O–H stretching vibrations. Strong bands at 1150, 1080 and 1030 cm^{-1}are due to stretching vibrations of C–O–C from glycosidic bonds and the bending of O–H from alcohols, characteristic of polysaccharide structures. A weak band at 2937 cm^{-1} was attributed to C–H stretching vibrations. The absorption at 1647 cm^{-1} is due to the O–H scissor vibrations of bonded water molecules and the shoulders at ~1600 and 1414 cm^{-1} can be attributed to asymmetrical and symmetrical –COO^- vibrations of the uronic acid present in the gum [57-59].

In the copolymers, the presence of a new band at 1739 cm^{-1}, as well as other bands characteristic of CG polysaccharide (e.g., 3379, 1150, 1080, 1030 cm^{-1}) confirms the insertion of PLA in the CG chains.

3.3. NMR spectroscopy

The NMR spectrum for cashew gum without modification shows characteristic anomeric protons in the region of 4.4 to 5.0 ppm using D_2O as a solvent [73]. The signals in this region are reported to be due to α-D-glucose (4.95 ppm), α-L-rhamnose (4.81 ppm), β-D-galactose (1→3) (4.69 ppm and 4.43 ppm) and β-D-glucuronic acid (4.51 ppm). The H-2 to H-5 signals are overlapped in the regions of 3.4 ppm to 4.3 ppm and a quartet signal in the region of 1.26 ppm is due to the methyl protons of rhamnose [60].

Several polysaccharide/PLA graft copolymers have been investigated using ^{1}H-NMR spectroscopy. The insertion of PLA leads to signals characteristic of terminal and internal backbone methyl groups at 1.3 ppm (A structure in Fig. 1) and 1.4 ppm (B structure in Fig. 1), respectively. The signals at 5.2 ppm (C structure in Fig. 1) and 4.2 ppm (D structure in Fig. 1) were attributed to internal and terminal methine protons of PLA, respectively [7,9,11,61].

Fig.1 also shows the ^{1}H- NMR spectra for the CGPLAP copolymers in DMSO-d_6. Signals due to the lactide insertion into the cashew gum structure, at 1.31, 1.44, 4.2 and 5.4 ppm, and signals associated with the anomeric protons of cashew gum in DMSO-d_6 (in the region of 5.04 to 5.17 ppm) were observed. The signal associated with the terminal methyl groups at 1.31 ppm is overlapped with that of the methyl groups of rhamnose present in the CG. The presence of the PLA signals related tointernal and terminal groups indicates that the PLA chain is grafted onto the CG structure[11] and the absence of a signal at 1.66 ppm, characteristic of the lactide monomer [12], suggests the absence of residual monomer in the purified copolymers.

The values for MS, DS and DP, based on equations 1 to 3, are reported in Table 2. These values show that the increase in lactide content does not increase the percentage of grafting, since only a small difference can be observed in these values. However, a slight increase in the DP was observed for a CGPLAP ratio of 1:10. The variation in the DP values may lead to different nanoparticle aggregation pattern. This result differs from that obtained for the pullulan graft PLA[12,13], where an increase in the PLA:glycosidic repeating unit ratio from 5:1 to 20:1 led to an increase in the DS (from 0.45 to 0.65). However, the DS values obtained for the CGPLAP copolymers are higher than those reported for pullulan:PLA copolymers[12,13].

3.4. Particle size

The nanoparticles were prepared via self-organization using DMSO as the solvent. The hydrodynamic diameter, in an aqueous medium, was determined by DLS at 25 ºC. Nanoparticle (NP) size distributions for both copolymers are monomodal (see Fig. S2-Supplementary material). The Z-average diameters in distilled water for NP sobtained with CGPLAP molar ratios of 1:1 and 1:10 were 230.4 ± 7.7 and 243.6 ± 10.7 nm, respectively. The PDI values were 0.272 ± 0.002 and 0.307 ± 0.018, respectively for NPs obtained with ratios of 1:1 and 1:10, indicating a narrow size distribution. The CGPLAP 1:10 NPs had a higher PDI value than the CGPLAP 1:1 NPs. An increase in the feed ratio does not affect significantly the hydrodynamic size of the self-assembled CGPLAP nanoparticles. The zeta potentials for the two copolymers were also similar (-26.1 ± 1.9 and -24.3 ± 2.3 mV, respectively, for the CGPLAP copolymers 1:1 and 1:10).

In our previously work [36] we produced Pickering emulsion with monomodal distribution with CGPLAP 1:1 sample using Miglyol as oil phase. The particle size value of this Pickering emulsion was closer to that obtained by self-assembling (241 ± 5 nm). Graft copolymer of PLA with other polysaccharide such chitosan [18], pullulan [11,12], and chondroitin sulfate [61] have been synthesized and characterized by DLS. Chitosan-g-PLA nanoparticles were produced by dialysis and dialysis plus ultrasonification [16]. Dialysis methods led to a particle size of 569 nm, however, when these particles were submitted to ultra sonification with different amplitude and time, particles size ranging from 200 to 350 nm were obtained. For pullulan-g-PLA copolymers nanoparticles prepared with similar methods used in the present work, the particle size decrease with the increase of degree of substitution (DS) ranging from 341 to 202 nm depending on DS [11]. Chondroitin sulfate-g-PLA copolymers nanoparticle were prepared by emulsification (O/W emulsion) with particle size ranging from 92.1 to 108.5 nm,when the of proportion chondroitin sulfate increase in the copolymer the particle size decrease [62].

The stability of the nanoparticles in physiological buffer (pH 7.4 at 37 ˚C) over 6 h was determined by measuring the particle size (Fig.2) to observe whether aggregation occurred during this period. It can be observed that the copolymers nanoparticles in buffer pH 7.4 (231.4 ± 17.3 and 226.5 ± 18.2 nm for CGPLAP 1:1 and 1:10), respectively have similar size to that observed in distilled water.

The particle size remained almost constant for up to 240 min for both copolymers (Fig. 2a), and the CGPLAP 1:1 NPs are slightly larger compared with the CGPLAP 1:10 NPs. After this time, the CGPLAP 1:1 NPs started to aggregate, as observed by the increase in the particle size, however,size distribution is still monodisperse (Figs 2b and 2c). The higher stability of the CGPLAP 1:10 NPs may be related to the DP or different substitution patterns at the CG chain.

3.5. Nanoparticles Morphology

SEM and AFM micrographs show that the nanoparticles have spherical shape with a smooth surface (Fig. 3a, 3b, 3c and 3d) and the average nanoparticle sizes are significantly smaller than that obtained by DLS, approximately 70 nm for both CGPLAP 1:1 and CGPLAP 1:10 (Fig 3e and 3f). These differences may be due to the fact that AFM and SEM determine the diameter of the dry particles, and will probably cause them to shrink, whereas the DLS determines the hydrodynamic diameter in aqueous solution, which leads to swelling of the samples and consequently larger particles sizes.

Amplitude and phase images were show in Fig 4. The CGPLAP 1:1 samples are extremely homogeneous, showing no phase difference (Fig 4a and 4c), whereas CGPLAP 1:10 NP´s have texture in phase (Fig 4b and 4d). This can be explained by the slight increase in DP, observed for CGPLAP 1:10, that increase PLA chain. The variation on the hydrophobicity may lead to phase difference on the AFM images of CGPLAP 1:10.

3.6. Amphotericin B (AMB) encapsulation and loading efficiency (LE)

The other important aspect to evaluate for the CGPLAP nanoparticles was their capacity to associate AMB, a drug of low water solubility.The use of nanocapsules is reported for protection of different systems in pharmaceutical or cosmetic applications, especially for substances that degrade at temperatures above 40 °C or are sensitive to oxidation in the presence of water, pH variation or ultraviolet light[63].The loading efficiency were 89.7 and 82.7 (%) for CGPLAP 1:1 and 1:10, respectively. It can be seen that the efficiency value was slightly lower for the GCPLAP 1:10 systems. Pickering emulsions produced with the same copolymers in previous work have a much

lower encapsulation efficiency (21 to 47 % respectively for Pickering emulsion stabilized with CGPLAP 1:1 and 1:10) compared with self-assembling nanoparticles [36].

Other authors also reported high values for encapsulation efficiency, confirming that amphiphilic systems are efficient in incorporating amphotericin B. Polycarbonate micelles obtained LE values (%) ranging from 66.4 to 76.4 % [44]. AMB-containing PLA-PEG nanoparticles were successfully obtained by the emulsion-solvent evaporation method. The mean diameter in the NPs was 223 ± 25 nm and the encapsulation efficiency were 68.9% [64]. Micelles of linolenic acid-modified PEG-oligochitosan conjugates prepared by dialysis method has 82.27 ± 1.96 % of drug encapsulation efficiency [65]. Nanoparticles of PLGA has efficiency loading approximately 80 %[66]. So our samples were very efficiently in the encapsulation of AMB in comparison with other systems.

AMB tends to aggregate in commercial formulations, leading to an increase of toxicity. The UV spectroscopy was used to determinate the aggregation states of AMB when encapsulated in nanoparticles. The UV-vis for AMBin DMSO and a commercial solution of AMBfrom Sigma Aldrich ($2x10^{-5}$ $mol.L^{-1}$) in water are show in Fig. 5a. Bands located at 407-419 nm (Band IV) is the characteristic peak for monomeric form of AMB, while Band I, at approximately 330-340 nm, represents the aggregated form [45]. The spectrum for AMB in DMSO showed high intensity peaks at 370, 391 and 415 nm, representing the monomeric form and a low intensity peak at 353 nm. In contrast, the absorbance spectrum for the commercial AMB solution in water showed a high intensity peak at 327 nm, which represent the aggregate state of AMB and peaks of low intensity at 362, 385 and 408 nm.

The UV-vis for the CGPLAP nanoparticles can be seenin Fig 5 band showed a broad peak of high intensity at 332 nm for both copolymers (GCPLAP 1:1 and 1:10). Lower intense peaks were observed at 362, 388 and 419 nm. Spectra with similar absorption profiles were found in polycarbonate micelles [44],cluster dextrin [67], linolenic acid-modified and oligochitosan conjugates micelles [65] and PLGA nanoparticles [66].

In general, the spectral changes induced by the aggregation of AMB may be represented as the value of the ratio of the intensities of the major absorption bands at λ = 348 and 409 nm (i.e., $\sim A_{348}/\sim A_{409}$ ratio). This ratio assumes a value ≥ 2.0 for AMB aggregated species, and of ~ 0.25 for the monomeric form [68].

In commercial formulations of AMB (e.g., Amphocil®, Fungizone®, Abelcet® and AMBisone®) A_{348}/A_{409} ratios were reported as9.1, 4.8, 1.3 and 2.9, respectively [69] thus reflecting that AMB occurs in the aggregated form in such all cases, been Abelcet® and AMBisone® in less aggregated form. The A_{332}/A_{419} ratio values for the CGPLAP 1:1 (A_{332}/A_{419} ratio 3.18) and 1:10(A_{332}/A_{419} ratio 3.28) nanoparticles indicate that the AMB loaded in CGPLAP nanoparticles are in aggregate form, however they are in a less aggregated state than the observed for Amphocil®, Fungizone® [69]. If compared with commercial AMB solution from Sigma-Aldrich (A_{332}/A_{419} ratio 6.12) values obtained for the CGPLAP copolymers are almost half of the value.

CGPLAP Pickering emulsion was more effective in loaded AMB without aggregation than the nanoparticles produced by self-assembling [36]. The values of A_{348}/A_{409} was 1.7 and 1.6 respectively for CGPLAP 1:1 and 1:10 copolymers Pickering emulsion [36].

The size, PDI and zeta potential for the CGPLAP nanoparticles unload and load with AMB and for commercial AMB solution (Sigma-Aldrich) is shown in Fig 6. It was possible to observe an expressive increase in the particle size with AMB encapsulation for both systems, with values of 230.4 ± 7.7 and 243.6 ± 10.7 nm for unloaded and 1192 ± 23.6 and 1025 ± 143 nm for loaded AMB respectively for CGPLAP 1:1 and 1:10 nanoparticle. This occurs, probably due to electrostatic interferences of the drug with the polymer chain during the formation of the nanoparticles [14]. The commercial AMB solution appeared almost four times larger than the two nanoparticle systems, with a value of 3682 ± 231 nm.

When evaluating the PDI for the nanoparticles, there is a decrease in their values for both, from 0.277 to 0.096 for the nanoparticle CGPLAP 1: 1 and from 0.307 to 0.254 for the nanoparticle CGPLAP 1:10. This indicates that the AMB load into the nanoparticles causes the particle size to become more homogeneous. The commercial AMB solution has a higher PDI value (0.413 ± 0.06) than the CGPLAP nanoparticles.

The zeta potential is another important parameter because indicates the stability of nanoparticles and provides the aggregation. For unload CGPLAP 1:1 and 1:10 nanoparticles, has a zeta potential of -26.1 ± 1.9 and -24.3 ± 2.3 mV, respectively. For the load AMB nanoparticles has a decrease in zeta potential value of -10.3 ± 0.2 and -12.7 ± 0.9 mV for GCPLAP 1:1 and 1:10, respectively. The zeta potential for

commercial AMB is close to that of nanoparticles without AMB, with a value of -22.4 ± 0.9 mV.

3.7Biocompatibility assessment

The extent of hemolysis caused after incubation with RBCs is show in Fig 7 for CGPLAP 1:1, CGPLAP 1:10 blank and AMB loaded nanoparticles and commercial AMB. Incubation of RBCs with triton X (0.1%), as a positive control, led to lysis in red cells instantly, causing 100% hemolysis and releasing hemoglobin, which causes the solution to become cloudy after centrifugation, saline solution (0.9%) was used as negative control. Commercial AMB solution shows a large extent of erythrocyte damage.

It can be observed for blank nanoparticles (in this case concentration is the copolymer concentration), in the all concentrations studied, there is no statistical difference between CGPLAP 1:1 and CGPLAP 1:10 nanoparticles, with hemolysis extended less than 5%, indicating excellent biocompatibility for nanoparticles matrix.

For AMB loaded copolymers the concentration in Fig. 7 refers to AMB concentration. Statistical analysis using Tukey test revealed that the differences between CGPLAP 1:1 + AMB and CGPLAP 1:10 + AMB nanoparticles were significant ($P < 0.05$) when the AMB concentrations were 250 and 125 $\mu g.mL^{-1}$.

CGPLAP 1:1 + AMB has a lower percentage of hemolysis (47.0 ± 3.7 and 2.7 ± 1.4 % respectively for 250 and 125 $\mu g.mL^{-1}$ AMB concentrations) than CGPLAP 1:10 + AMB (85.9 ± 4.2 and 20.5 ± 5.3 % respectively for 250 and 125 $\mu g.mL^{-1}$ AMB concentrations). Despite the high hemolysis for CGPLAP 1:10 + AMB (85.9 ± 4.2 % at AMB concentration of 250 $\mu g.mL^{-1}$), the damage is lower than for commercial AMB (109.8 ± 2.2 %) at the same concentration (250 $\mu g.mL^{-1}$).

When the AMB concentration was decrease to 62.5 $\mu g.mL^{-1}$, no difference statistical was observed for CGPLAP 1:1 + AMB and CGPLAP 1:10 + AMB, with a hemolysis extended less than 5 %. CGPLAP 1:1 + AMB exert a stabilizing and protective effect on the cells against hemolysis even at higher AMB concentration than the previously reported systems.

3.8 Drug release

To investigate the potential CGPLAP nanoparticles as AMB drug delivery devices, an *in vitro* release experiment was carried out using PBS buffer with 0.25 % sodium lauryl sulfate as release medium (Fig 8).

The release profile shows a sustained drug delivery, with maximal release percentage of 42.6 ± 4.5 and 52.2 ± 3.9 % after 168 h, for CGPLAP 1:1 and CGPLAP 1:10, respectively. The highest release percentage was obtained for CGPLAP 1:10 nanoparticles, probably due to its lower DS (1.10), which promotes less interaction with AMB, releasing a higher amount of drug.

Similar release profile is found for chitosan and AMB complex, releasing approximately 21% in the first 24 h, followed by 44.5% release after 20 days of experiment [70].

3.9*In vitro* antifungal test

Unloaded CGPLAP nanoparticles showed inhibition activity against all *C. albican* strains (Table 3). The minimum inhibitory concentration (MIC) values for CGPLAP 1:1 was similar to that of commercial amphotericin B. As far as we concern no previous report on polylactideantifungal activity has been reported MIC values of AMB-loaded CGPLAP 1:1 and 1 : 10 (Table 3) are half the figure observed for commercial AMB solution, a fact that may be due to a synergistic effect of copolymer which also exhibits antifungal activity. These results indicate that CGPLAP nanoparticles have great potential as nanocarrier delivery system.

4. Conclusions

Copolymers of cashew gum and L-Lactide were synthesized using two CG:PLA molar ratios. Nanoparticles were produced through equilibrium dialysis using DMSO as a solvent. The copolymer nanoparticles have similar particle size and zeta potential. The CGPLAP 1:10 shows better stability, but CGPLAP 1:1exhibits a narrower particles size

distribution. The potential use of CGPLAP nanoparticles as drug delivery device was tested using AMB as a proof-of concept. A high efficiency of encapsulation was obtained if compared with CGPLAP Pickering emulsion system previously reported, however AMB was more aggregated in the self-assembling nanoparticle than in Pickering emulsion, but less than in Fungizone®. Unloaded CGPLAP nanoparticles showed inhibition activity against all *C. albican* strains. MIC values of AMB-loaded CGPLA are smaller than that observed for commercial AMB solution, a fact that may be due to a copolymer synergistic effect, indicating that the system has a good potential as AMB nanocarrier delivery.

Acknowledgement

This study was partially supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)-Finance code 001, by CNPq (Brazil), INOMAT/INCT (Brazil) and FUNCAP (Brazil). The authors are grateful to the Central Analítica-UFC/CT-INFRA/MCTI-SISANO/Pró-Equipamentos CAPES for the support with SEM imaging and to CENAURENM for NMR analysis .

References

[1] I. Katouzian, S.M. Jafari, Nano-encapsulation as a promising approach for targeted delivery and controlled release of vitamins, Trends Food Sci. Technol. 53 (2016) 34-48. https://doi.org/10.1016/j.tifs.2016.05.002

[2] Y. Wang, P. Li, T.T.D. Tran, J. Zhang, L. Kong, Manufacturing techniques and surface engineering of polymer based nanoparticles for targeted drug delivery to cancer, Nanomaterials. 6 (2016) 26. https://doi.org/10.3390/nano6020026

[3] S. Gaurava, M. Ankita, S. Pradeep, Characterization and in vitro degradation studies of synthesized polylactide (PLA),Res. J. Chem. Environ. 16 (2012), 14-21.ISSN: 0972-0626

[4] R.P. John, K.M.Nampoothiri, A.S. Nair, A Pandey,L (+)-lactic acid production using *Lactobacillus casei* in solid-state fermentation, Biotechnol. Lett. 27 (2005), 1685-1688.https://doi.org/10.1007/s10529-005-2731-8

[5] Y. Guo, X. Wang, X. Shu, Z. Shen, R. Sun,Self-assembly and paclitaxel capacity of cellulose-graft-poly (lactide) nanomicelles, J. Agr. Food. Chen. 60 (2012) 3900-3908.https://doi.org/10.1021/jf3001873

[6] H. Feng, C. Dong, Preparation, characterization and self-assembled properties of biodegradable chitosan-poly (L-lactide) hybrid amphiphiles,Biomacromolecules 7 (2006) 3069-3075. https://doi.org/10.1021/bm060568l

[7] H. Yan, J. Wu, J. Zhang, J. He, J. Zhang,Hydrolytic degradation of cellulose-graft-poly(L-lactide) copolymers,Polym.Degrad. Stabil. 118 (2015) 130-136.http://dx.doi.org/10.1016/j.polymdegradstab.2015.04.019

[8] W. Ge, Y. Guo, H. Zhong, X. Wang, R. Sun, Synthesis, characterization, and micellar behaviors of hydroxyethyl cellulose-graft-poly(lactide/ε-caprolactone/*p*-dioxanone),Cellulose 22 (2015) 2365-2374. https://doi.org/10.1007/s10570-015-0663-6

[9] R. Lipsa, N. Tudorachi, C. Vasile, A. Chiriac, A. Grigoras, Novel environmentally friendly copolymers carboxymethyl starch grafted poly(lactic acid),J. Polym. Environ. 21 (2013) 461-471.https://doi.org/10.1007/s10924-012-0470-1

[10] H. Lu, H. He, B. Zhang, G. Liu, M. Li, Q.Nie, Facile synthesis of the amphiphilic chitosan-g-poly(lactic acid) derivatives and the study of their controlled drug release,J. Appl. Polym. Sci. 130 (2013), 908-915.https://doi.org/10.1002/app.39205

[11] X. Tang, J. Huang, L. Xu, Y. Li, J. Song, Y. Ma, L. Yang, D. Yuan, H. Wu,Microwave-assisted rapid synthesis, characterization and application of poly (D,L-lactide)-graft-pullulan,Carbohyd. Polym. 107 (2014) 7-15.http://dx.doi.org/doi:10.1016/j.carbpol.2014.02.038

[12] J. Cho, W. Park, K. Na,Self-organized nanogels from pullulan-g-poly(L lactide) synthesized by one-pot method: Physicochemical characterization and in vitro doxorubicin release, J. Appl. Polym. Sci, 113 (2009) 2209-2216.https://doi.org/10.1002/app.30049

[13] S. Seo, C. Lee, Y. Jung, K. Na, Thermo-sensitivity and triggered drug release of polysaccharide nanogels derived from pullulan-g-poly (L-lactide) copolymers,Carbohyd.Polym. 87 (2012) 1105-1111. https://doi.org/10.1016/j.carbpol.2011.08.061

[14] A.D. Martino, V. Sedlarik, Amphiphilic chitosan-grafted-functionalized polylactide acid based nanoparticles as a delivery system for doxorrubicin and temozolomide co-therapy,Int. J. Pharm.474 (2014) 134-145.https://doi.org/10.1016/j.ijpharm.2014.08.014

[15]T.S Demina, T.A.Akopova, L.V.Vladimirov, A.N.Zelenetskii, E.A.Markivicheva, C. Grandgils**,**Polylactide-based microspheres prepared using solid-state copolymerized chitosan and D,L-lactide,Mat. Sci. Eng. C, 59 (2016) 333-338.https://doi.org/10.1016/j.msec.2015.09.094

[16]Li, J., Kong, M., Cheng, X.J., Dang, Q.F., Zhou, X., Wei, Y.N. & Chen, X.G. (2012). Preparation of biocompatible chitosan grafted poly(lactic acid) nanoparticles. Int. J. Biol. Macromol.51, 221-227. http://dx.doi.org/10.1016/j.ijbiomac.2012.05.011

[17] P.L.R. Cunha, R.C.M. de Paula, J.P.A. Feitosa, Polissacarídeos da biodiversidade brasileira: uma oportunidade de transformar conhecimento em valor econômico, Quím. Nova, 32 (2009) 649-660. http://dx.doi.org/10.1590/S0100-40422009000300009

[18] R.C.M. de Paula, J.F. Rodrigues, Composition and rheological properties of cashew tree gum, the exudate polysaccharide from *Anacardium occidentale* L. Carbohyd. Polym. 26 (1995), 177-181.https://doi.org/10.1016/0144-8617(95)00006-S

[19] R.C.M. de Paula, F. Heatley, P.M. Budd, Characterization of *Anacardium occidentale*exsudate polysaccharide, Polym. Int. 45 (1998) 27- 35. https://doi.org/10.1002/(SICI)1097-0126(199801)45:1<27::AID-PI900>3.0.CO;2-9

[20] M.R. Guilherme, G.M. Campese, E. Radovanovic, A.F. Rubira, J.P.A. Feitosa, E.C. Muniz, Morphology and water affinity of superabsorbent hydrogels composed of methacrylated cashew gum and acrylamide with good mechanical properties, Polym. 46(2005a) 7867-7873.https://doi.org/10.1016/j.polymer.2005.06.068

[21] M.R. Guilherme, A.V. Reis, A.H. Takahashi, A.F. Rubira, J.P.A. Feitosa, E.C. Muniz, Synthesis of a novel superabsorbent hydrogel by copolymerization of acrylamide and cashew gum modified with glycidyl methacrylate, Carbohyd. Polym. 61 (2005b) 464-471. https://doi.org/10.1016/j.carbpol.2005.06.017

[22] D.A. da Silva, R.C.M. de Paula, J.P.A. Feitosa,Graftcopolymerizationofacrylamideontocashewgum,Eur. Polym. J. 43 (2007) 2620-2629.https://doi.org/10.1016/j.eurpolymj.2007.03.041

[23] J.M. Klein, V.S. de Lima, J.M.C. da Feira, R.N. Brandalise, M.M.C. Forte, Chemical modification of cashew gum with acrylamide using an ultrasound-assisted method,J.Appl. Polym. Sci. 133 (2016) 43634-43645.https://doi.org/10.1002/app.43634

[24] M.T. Ramesan, K. Surya, Synthesis, characterization and properties of cashew gum graft poly(acrylamide)/magnetite nanocomposites,J. Appl. Polym. Sci. 133 (2016) 43496-43504. https://doi.org/10.1002/app.43496

[25] D.A. da Silva, J.P.A. Feitosa, H.C.B. Paula, R.C.M. de Paula, Synthesis and characterization of cashew gum/acrylic acid nanoparticles, Mat. Sci. Eng. C 29 (2009) 437-441. https://doi.org/10.1016/j.msec.2008.08.029

[26] C.M.W. Abreu, H.C.B. Paula, V. Seabra, J.P.A. Feitosa, B. Sarmento, R.C.M. de Paula, Synthesis and characterization of non-toxic and thermo-sensitive poly(N-isopropylacrylamide)-grafted cashew gum nanoparticles as a potential epirubicin delivery matrix, Carbohyd. Polym. 154 (2016) 77-85.https://doi.org/10.1016/j.carbpol.2016.08.031

[27] J. Gomes-Estaca, T.A. Comunian, P. Montero, R. Ferro-Furtado, C.S. Favaro-Trindade, Encapsulation of an astaxanthin-containing lipid extract from shrimp waste by complex coacervation using a novel gelatin–cashew gum complex,Food Hydrocolloid. 61 (2016) 155-162.https://doi.org/10.1016/j.foodhyd.2016.05.005

[28] S.F.L. Dias, S.S. Nogueira, F.F. Dourado, M.A. Guimarães, N.A.O. Pitombeira, G.G. Gobbo, F.L. Primo, R.C.M. de Paula, J.P.A. Feitosa, A.C. Tedesco, L.C.C. Nunes, J.R.S.A. Leite, D.A. da Silva, Acetylated cashew gum-based nanoparticles for transdermal delivery of diclofenac diethyl amine, Carbohyd. Polym. 143 (2016) 254-261.https://doi.org/10.1016/j.carbpol.2016.02.004

[29] N.A.O. Pitombeira, J.G. VerasNeto, D.A. Silva, J.P.A. Feitosa, H.C.B. Paula, R.C.M. Paula, Self-assembled nanoparticles of acetylated cashew gum: Characterization and evaluation as potential drug carrier, Carbohyd. Polym. 117 (2015) 610-615. https://doi.org/10.1016/j.carbpol.2014.09.087

[30] M.R.L. Cardial, H.C.B. Paula, R.B.C. da Silva, J.F.S. Barros, A.R. Richter, F.M. Sombra, R.C.M. de Paula, Pickering emulsions stabilized with cashew gum nanoparticles as indomethacin carrier, Int. J. Biol. Macromol. 132 (2019) 534-540. https://doi.org/ 10.1016/j.ijbiomac.2019.03.198.

[31]G.A. Magalhães Jr., C.M.W. Santos, D.A. Silva, J.S. Maciel, J.P.A. Feitosa, H.C.B. Paula, R.C.M. de Paula, Microspheres of chitosan/carboxymethyl cashew gum (CH/CMCG): Effect of chitosan molar mass and CMCG degree of substitution on the swelling and BSA release, Carbohyd. Polym. 77 (2009) 217-222.https://doi.org/10.1016/j.carbpol.2008.12.037

[32] J.S. Maciel, H.C.B. Paula, M.A. Miranda, J.M. Sasaki, R.C.M. de Paula, Reacetylated chitosan/cashew gum gel: Preliminary study for potential utilization as drug release matrix,J. Appl.Polym. Sci.99 (2006) 326-334.https://doi.org/10.1002/app.22099

[33] B. Das, S. Dutta, A.K. Nayak, U. Nanda, Zinc alginate-carboxymethyl cashew gum microbeads for prolonged drug release: development and optimization,Int. J. Biol.Macromol.70 (2014) 506-515. https://doi.org/10.1016/j.ijbiomac.2014.07.030

[34] J.A. Rodrigues, A.R. de Araújo, N.A.O. Pitombeira, A. Plácido, M.P. de Almeida, L.C.M. Veras, C. Matos-Delerue, F.C.D.A. Lima, A.B. Neto, R.C.M. de Paula, J.P.A. Feitosa, P. Eaton, J.R.S. Leite, D.A. da Silva, Acetylated cashew gum-based nanoparticles for the incorporation of alkaloid epiisopiloturine, Int. J. Biol. Macromol.128 (2019) 965-972.https://doi.org/10.1016/j.ijbiomac.2019.01.206

[35] E.L.V. Silva, A.C.J. Oliveira, Y.B.G. Patriota, A.J. Ribeiro, F. Veiga, E.C.S.F. Hallwass, D.A. da Silva, M.F.R. Soares, A.G. Wanderley, J.L. Soares-Sobrinho, Solvent-free synthesis of acetylated cashew gum for oral delivery system of insulin, Carbohyd. Polym. 207 (2019) 601-608. https://doi.org/10.1016/j.carbpol.2018.11.071

[36] A.R. Richter, J.P.A. Feitosa, H.C.B. Paula, F.M. Goycoolea, R.C.M. de Paula,Pickering emulsion stabilized by cashew gum- poly-L-lactide copolymer nanoparticles: Synthesis, characterization and amphotericin B encapsulation, Colloid. Surfaces B 164 (2018) 201–209.https://doi.org/10.1016/j.colsurfb.2018.01.023

[37] N. Gold, H.A. Stout, J.F. Pagano, R. Donovick, Amphotericin A and B, antifugal antibiotics produced by a streptomycete I. *in vivo* studies, Antibiotic Annual 3 (1955-1956) 579-586

[38] R.J. Hamill, Amphotericin B formulations: A comparative review of efficacy and toxicity, Drugs 73 (2013) 919.https://doi.org.10.1007/s40265-013-0069-4.

[39] B. Kruijff, R.A. Demel, Polyene antibiotic-sterol interactions in membranes of Acholeplasmalaidlawii cells and lecithin liposomes. III. Molecular structure of the polyene antibiotic-cholesterol complexes, Biochim. Biophys. Acta. 339 (1974) 57-70. https://doi.org/10.1016/0005-2736(74)90332-0

[40] T. Jabri, M. Imran, Shafiullah, K. Rao, I. Ali, M. Arfan, M.R. Shah, Fabrication of lecithin-gum tragacanth muco-adhesive hybrid nano-carrier system for in-vivo performance of Amphotericin B, Carbohyd. Polym. 194 (2018) 89–96. https://doi.org/10.1016/j.carbpol.2018.04.013

[41] R.G.J.V. Marcano, T.T. Tominaga, N.M. Khalil, L.S. Pedroso, R.M. Mainardes, Chitosan functionalized poly(ε-caprolactone) nanoparticles for amphotericin B delivery, Carbohyd. Polym. 202 (2018) 345–354. https://doi.org/10.1016/j.carbpol.2018.08.142

[42] M. R. Lima, H.C.B. Paula, F.O.M.S. Abreu, R.B.C. da Silva, F.M. Sombra, R.C.M. de Paula, Hydrophobization of cashew gum by acetylation mechanism and amphotericin B encapsulation, Int. J. Biol. Macromol. 108 (2018) 523-530. https://doi.org/10.1016/j.ijbiomac.2017.12.047

[43] V. Aparna, A.R. Melge, V.K. Rajan, R. Biswas, R. Jayakumar, C.G. Mohan, Carboxymethylated □-carrageenan conjugated amphotericin B loaded gelatin nanoparticles for treating intracellular *Candida glabrata* infections. Int. J. Biol. Macromol. 110 (2018) 140–149.https://doi.org/10.1016/j.ijbiomac.2017.11.126

[44] Y. Wang, X.Ke, Z.X.Voo, S.S.L. Yap, C. Yang, S. Gao, S. Liu, S. Venkataraman, S.A.O.Obuobi, J.S.Khara, Y.Y. Yang, Biodegradable functional polycarbonate micelles for controlled release of amphotericin B,Acta Biomater.46 (2016) 211-220.https://doi.org/10.1016/j.actbio.2016.09.036

[45] K. Gilani, E. Moazeni, T. Ramezanli, M. Amini, M.R. Fazeli, H. Jamalifar, Development of respirable nanomicelle carriers for delivery of amphotericin B by jet nebulization, J. Pharm. Sci. 100 (2011) 252–259. https://doi.org/10.1002/jps.22274

[46] K.K. Nishi, M. Antony, P.V. Mohanan, T.V. Anilkumar, P.M. Loiseau, A. Jayakrishnan, Amphotericin B-gum arabic conjugates: Synthesis, toxicity, bioavailability, and activities against Leishmania and fungi. Pharm. Res. 24 (2007) 971–980. https://doi.org/10.1007/s11095-006-9222-z

[47] Z. Ishaq, N. Ahmed, M.N. Anwar, I. Haq, T. Rehman, N.M. Ahmad, A. Elaissari, Development and in vitro evaluation of cost effective amphotericin B polymeric emulsion,J.Drug Deliv. Sci. Tec. 46 (2018) 66–73.https://doi.org/10.1016/j.jddst.2018.05.001

[48] L. Sosa, B. Clares, H.L. Alvarado, N. Boza, O. Domenech,A.C. Calpena, Amphotericin B releasing topical nanoemulsion for the treatment of candidiasis and aspergillosis. Nanomed-Nanotechnol. 13 (2017) 2303–2312. https://doi.org/10.1016/j.nano.2017.06.021

[49] D.H. Donabedian, S.P. McCarthy, Acylation of pullulan by ring-opening of lactones,Macromol. 31 (1998) 1032-1039.https://doi.org/10.1021/ma961741g

[50] Y. Teramoto, Y. Nishio, Cellulose diacetate-graft-poly (lactic acid)s: Synthesis of wide-ranging compositions and their thermal and mechanical properties,Polym. 44 (2003) 2701-2709.https://doi.org/10.1016/S0032-3861(03)00190-3

[51]Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast*. Third Edition: Approved Standard M27-A3. CLSI: Wayne, PA, USA, 2008.

[52] L. Dai, D. Li, J. He, Degradation of grafted polymer and blend based on cellulose and poly (L-lactide), J. Appl. Polym. Sci. 130 (2013) 2257-2264. https://doi.org.10.1002/app.39451

[53] R. Liu, L. Dai, Z. Zhou, C. Si, Drug-loaded poly(L-lactide)/lignin stereocomplex film for enhancing stability and sustained release of trans-resveratrol, Int. J. Biol. Macromol. 119 (2018) 1129-1136.https://doi.org/10.1016/j.ijbiomac.2018.08.040

[54] A.H. Mijinyawa,G. Durga, A. Mishra, Isolation, characterization and microwave surface modification of *Colocasiasculenta*(L.) Schott mucilage by grafting polylactide,Int. J. Biol.Macromol. 119 (2018) 1090-1097.https://doi.org/10.1016/j.ijbiomac.2018.08.045

[55] C. Lee, C. Huang, Y. Lee, Preparation of amphiphilic poly (L-lactide)-graft chondroitin sulfate copolymer self-aggregates and its aggregation behavior,Biomacromol. 7 (2006) 1179-1186. https://doi.org/10.1021/bm050995j

[56] T.A. Dick, J. Couve, O. Gimello, A. Mas, J.J. Robin, Chemical modification and plasm-induced grafting of pyrolytic lignin. Evaluation of the reinforcing effect on lignin/poly(L-lactide),Polym.118 (2017) 280-296.http://dx.doi.org/10.1016/j.polymer.2017.04.036

[57] P.L.R. Cunha, J.S. Maciel, M.R. Sierakowski, R.C.M. de Paula, J.P.A. Feitosa, Oxidation of cashew tree gum exudate polysaccharide with TEMPO reagent, J. Brazil. Chem. Soc. 18 (2007) 85-92. http://dx.doi.org/10.1590/S0103-50532007000100009

[58] H.C.B. Paula, F.J.S. Gomes, R.C.M. de Paula, Swelling studies of chitosan/cashew nut gum physical gels, Carbohyd. Polym.48 (2002) 313-318. https://doi.org/10.1016/S0144-8617(01)00264-8

[59] D.A. Silva, R.C.M. de Paula, J.P.A. Feitosa, A.C.F. de Brito, J.S. Maciel, H.C.B. Paula, Carboxymethylation of cashew tree exudate polysaccharide, Carbohyd. Polym. 58 (2004) 163-171. https://doi.org/10.1016/j.carbpol.2004.06.034

[60] E. Moura-Neto, J.S.M. Maciel, P.L.R. Cunha, R.C.M. de Paula, J.P.A. Feitosa, Preparation and characterization of a chemically sulfated cashew gum polysaccharide, J. Brazil. Chem. Soc. 22 (2011) 1953-1960. http://dx.doi.org/10.1590/S0103-50532011001000017

[61] E.L. de Paula, F. Roig, A. Mas, J.P. Habas, V. Mano, F.V. Pereira, J.J. Robin, Effect of surface-grafted celulose nanocrystals on the thermal and mechanical properties of PLLA based nanocomposites, Eur.Polym. J. 84 (2016). 173-187. https://doi.org/10.1016/j.eurpolymj.2016.09.019

[62] C. Lee, C. Huang, Y. Lee, Synthesis and characterization of amphiphilic poly (L-lactide)-graft-chondroitin sulfate copolymer and its application as drug carrier,Biomol. Eng. 24 (2007) 131-139. https://doi.org/10.1016/j.bioeng.2006.05.010

[63] C.R. Müller, S.A. Hass, V.L. Bassani, S.S. Guterres, H. Fessi, M.C.R. Peralba, A.R. Pohlmann, Degradação e estabilização do diclofenaco em nanocápsulaspoliméricas. Quim. Nova, 27 (2004) 555–560. http://dx.doi.org/10.1590/S0100-40422004000400008

[64] C.D. Rodrigues, K.N. Maissar, R.M. Mainardes, Determinationofamphotericin B in PLA-PEG blendnanoparticlesby HPLC-PDA, Braz. J. Pharm. Sci. 50 (2014) 859-868. http://dx.doi.org/10.1590/S1984-82502014000400021

[65] Z. Song, Y. Wen, P. Deng, F. Teng, F. Zhou, H. Xu, S. Feng, L. Zhu, R. Feng, Linolenic acid-modified methoxy poly (ethylene glycol)-oligochitosan conjugate micelles for encapsulation of amphotericin B,Carbohyd. Polym. 205 (2019) 571-580.https://doi.org/10.1016/j.carbpol.2018.10.086

[66] A.A. Ammar, A.Nasereddin, S.Ereqat, M. Dan-Goor, C.L. Jaffe, E.Zussman, Z.Abdeen, Amphotericin B-loaded nanoparticles for local treatment of cutaneous leishmaniasis,Drug Deliv. Transl. Re. 9 (2018) 76-84.https://doi.org/10.1007/s13346-018-00603-0

[67] Y. Kaneo, K. Taguchi, T. Tanaka, S. Yamamoto, Nanoparticles of hydrophobized cluster dextrin as biodegradable drug carriers: solubilization and encapsulation of amphotericin B, J. Drug Deliv. Sci.Techn.24 (2014) 344-351.https://doi.org/10.1016/S1773-2247(14)50072-X

[68] J. Barwicz, S. Christian, I. Gruda, Effects of the aggregation state of amphotericin B on its toxicity to mice,Antimicrob. Agents Ch. 36 (1992) 2310–2315.https://doi.org/10.1128/aac.36.10.2310

[69] A.B. Mullen, K.C. Carter, A.J. Baillie, Comparison of the efficacies of various formulations of amphotericin B against murine visceral leishmaniasis, Antimicrob. Agents Ch. 41 (1997) 2089–2092.https://doi.org/10.1128/AAC.41.10.2089

[70] R.A. Krishnan, T. Pant, S. Sankaranarayan, J. Sternberg, R. Jain, P. Dandekar, Protective nature of low molecular weight chitosan in a chitosan–Amphotericin B nanocomplex – A physicochemical study, Mat. Sci. Eng. C 93 (2018) 472–482.https://doi.org/10.1016/j.msec.2018.08.016.

Legend list

Fig. 1. ^{1}H NMR spectra for CGPLAP 1:10 and CGPLAP 1:1 in DMSO-d_6

Fig. 2. Stability of copolymers and particle size distributionover time (a), particle size distribution of CGPLAP 1:1 (b) and CGPLAP 1:10 (c) nanoparticles in phosphate buffer 7.4 at 37 °C.

Fig 3. SEM micrographs of CGPLAP 1:1 (a) and CGPLAP 1:10 (b). AFM measurements of blank nanoparticles.AFM micrograph height images of CGPLAP 1:1(c) andCGPLAP 1:10 (d) and AFM histograms graphics of CGPLAP 1:1(e) and CGPLAP 1:10(f) blank nanoparticles.

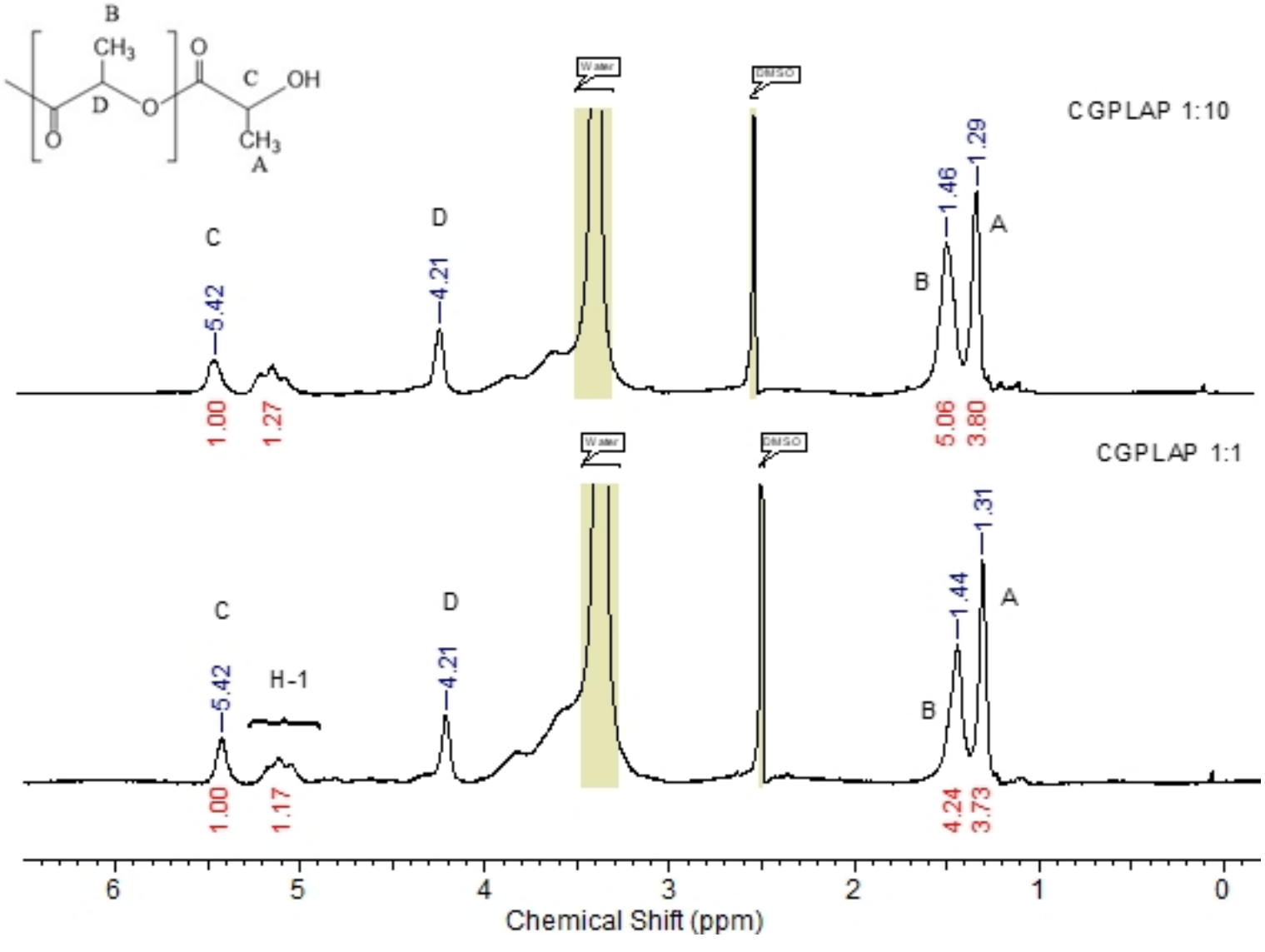
Fig. 4 AFM images of amplitude for (a) CGPLAP 1:1 and (b) CGPLAP 1:10 and phase images of (c) CGPLAP 1:1 and (d) CGPLAP 1:10 blank nanoparticles.

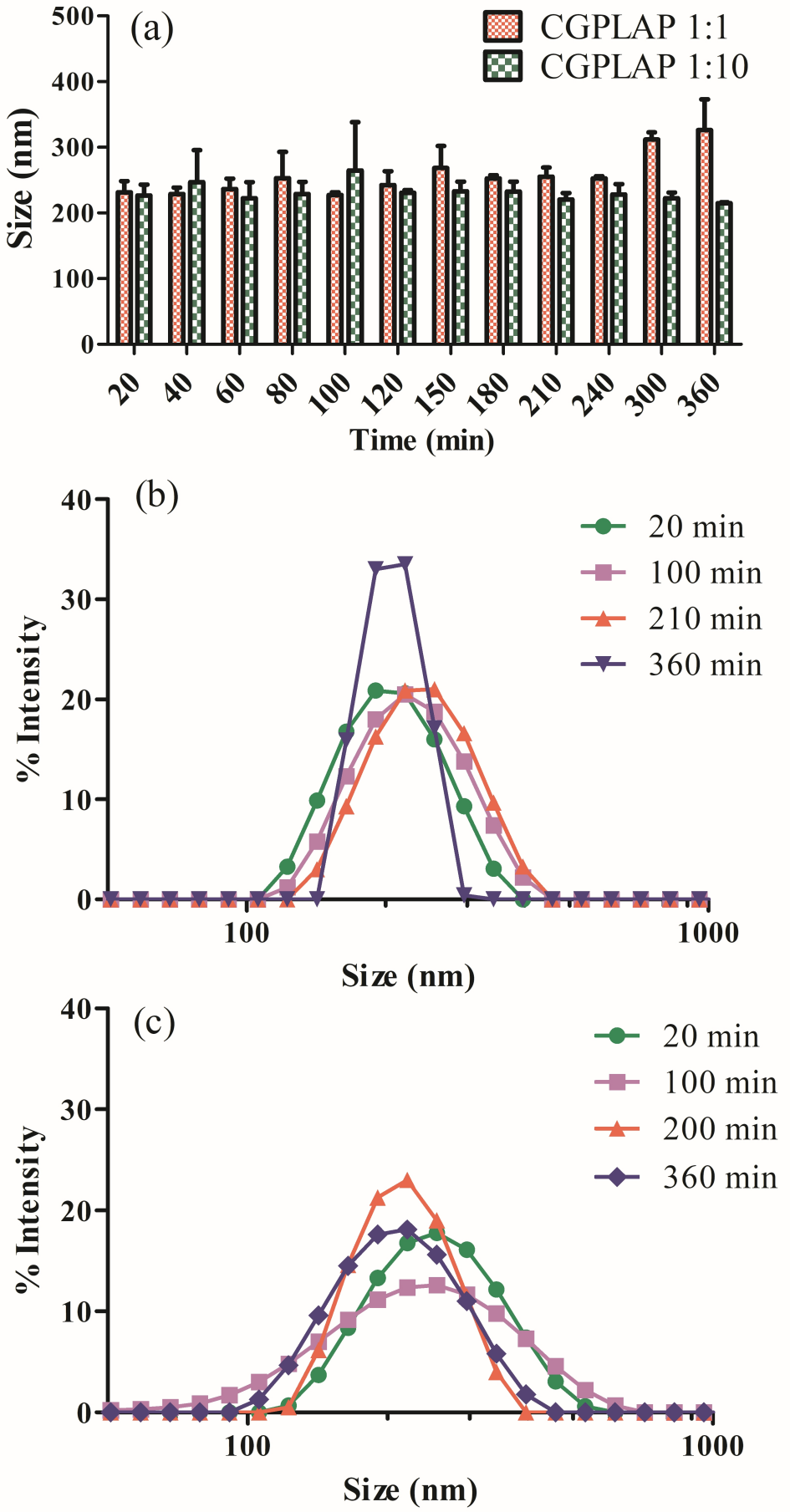
Fig. 5UV-Vis spectra ofAMB in DMSO and a commercial AMB aqueous solution(Sigma-Aldrich) (a) and AMB loaded CGPLAP nanoparticles aqueous solution (b).

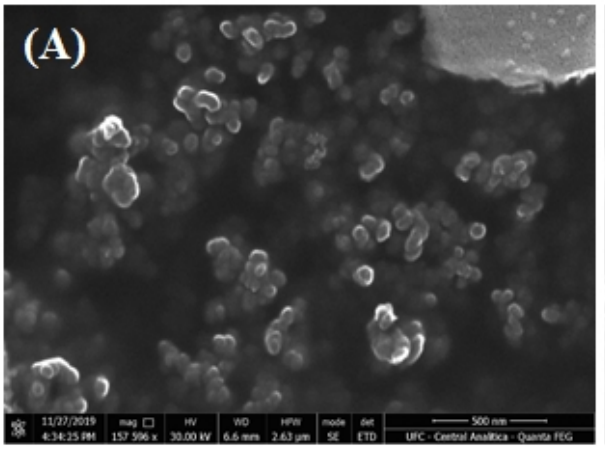
Fig 6. Effect of AMB load on size, PDI and zeta potential for the CGPLAP nanoparticles.

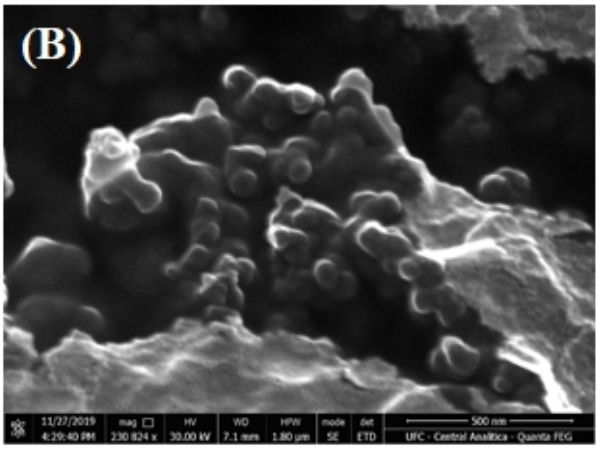
Fig. 7. Hemolysis percentage as a function of concentration for CGPLAP 1:1 and CGPLAP 1:10 nanoparticles commercial AMB. Triton was used as a positive control.

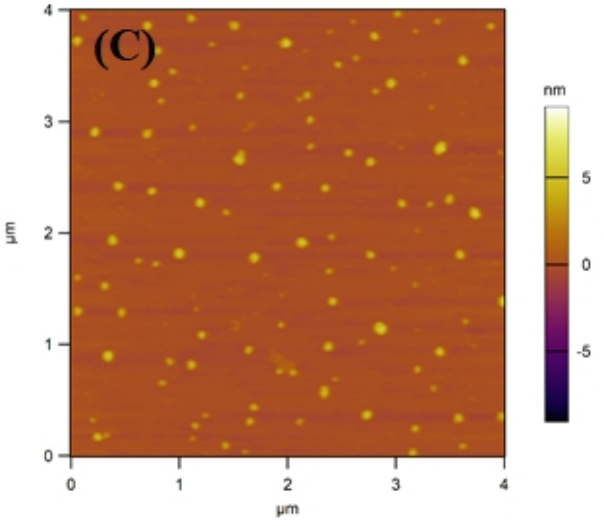
Fig 8. *In vitro*AMB release profile for CGPLA nanoparticlesnanocapsule in PBS buffer solution containing 0.25% sodium lauryl sulfate, at pH 7.4, and 37 °C

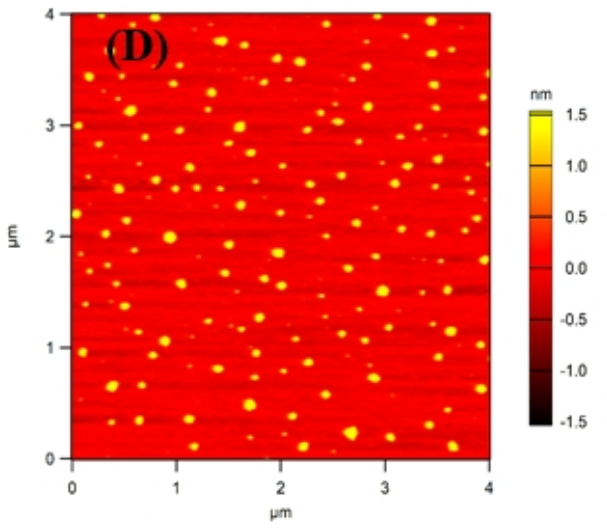

B
CH3
O
C
OH
D
O
O
CH3
A
Water
DMSO
CGPLAP 1:10
C
5.42
D
4.21
B
1.46
1.29
A
1.00
1.27
5.06
3.80
Water
DMSO
CGPLAP 1:1
C
5.42
H-1
D
4.21
B
1.44
1.31
A
1.00
1.17
4.24
3.73
6
5
4
3
2
1
0
Chemical Shift (ppm)

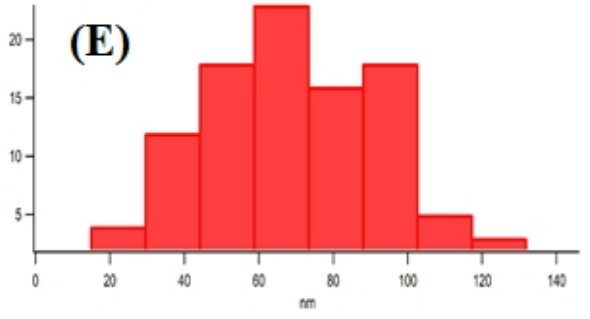

(a)
CGPLAP 1:1
CGPLAP 1:10
Size (nm)
500
400
300
200
100
0
20
40
60
80
100
120
150
180
210
240
300
360
Time (min)
(b)
20 min
100 min
210 min
360 min
% Intensity
40
30
20
10
0
100
1000
Size (nm)
(c)
20 min
100 min
200 min
360 min
% Intensity
40
30
20
10
0
100
1000
Size (nm)

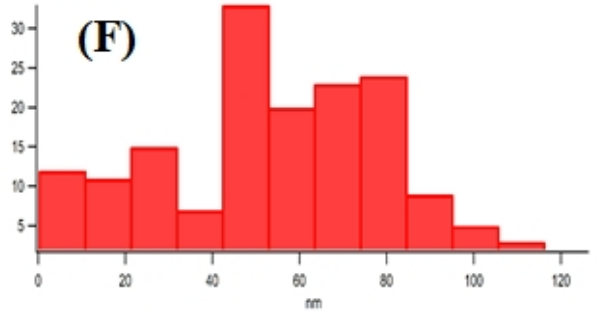

(A)
11/27/2019
4:34:25 PM
mag
157 596 x
HV
30.00 kV
WD
6.6 mm
HFW
2.63 µm
mode
SE
det
ETD
500 nm
UFC - Central Analítica - Quanta FEG

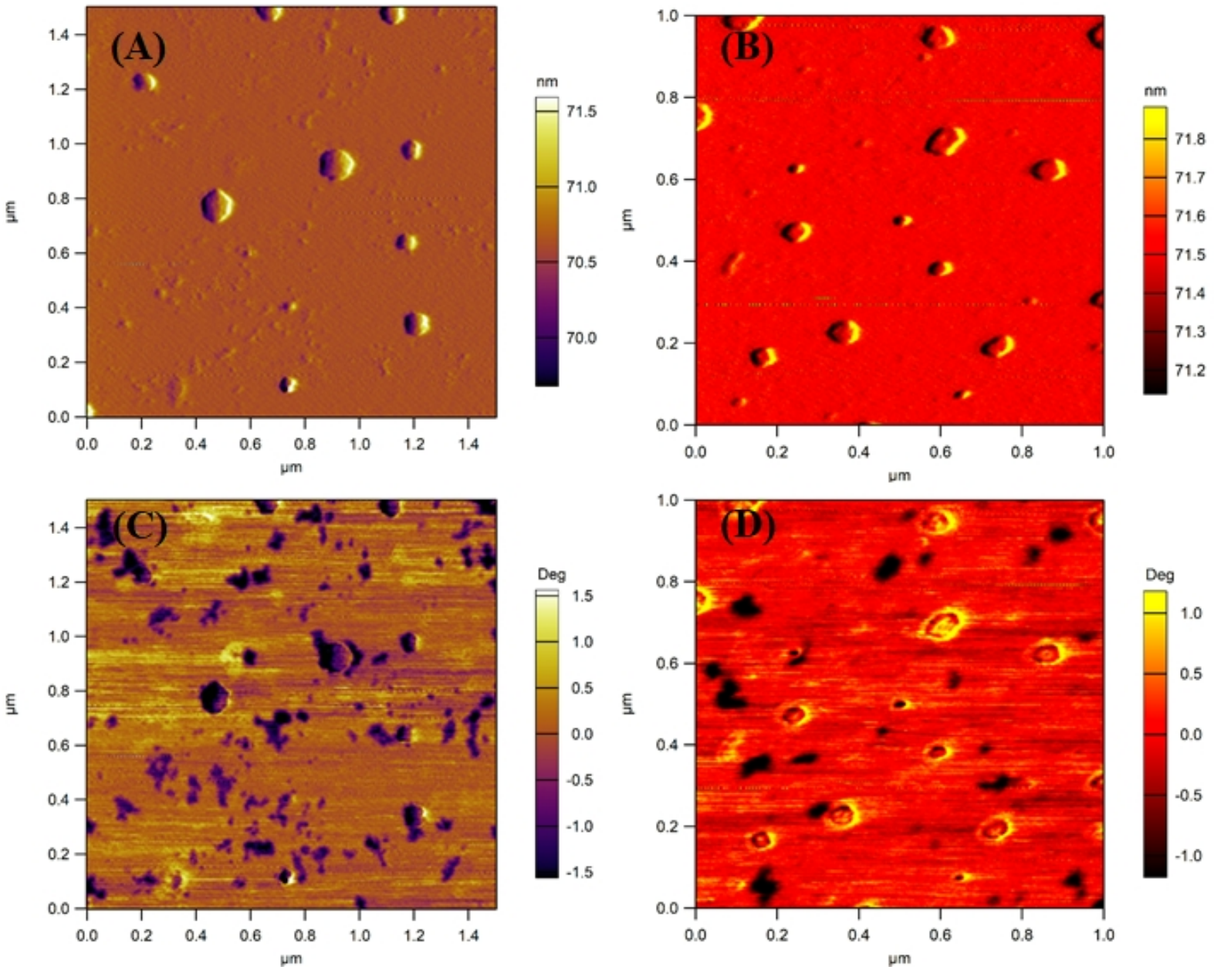

(B)
11/27/2019
4:29:40 PM
mag
230 824 x
HV
30.00 kV
WD
7.1 mm
HFW
1.80 µm
mode
SE
det
ETD
500 nm
UFC - Central Analítica - Quanta FEG

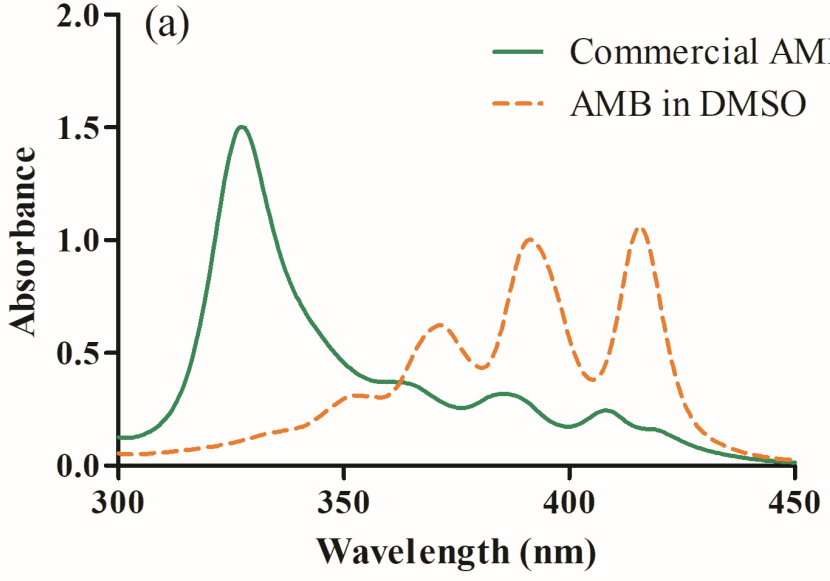

(C)
nm
5
0
-5
µm
0
1
2
3
4

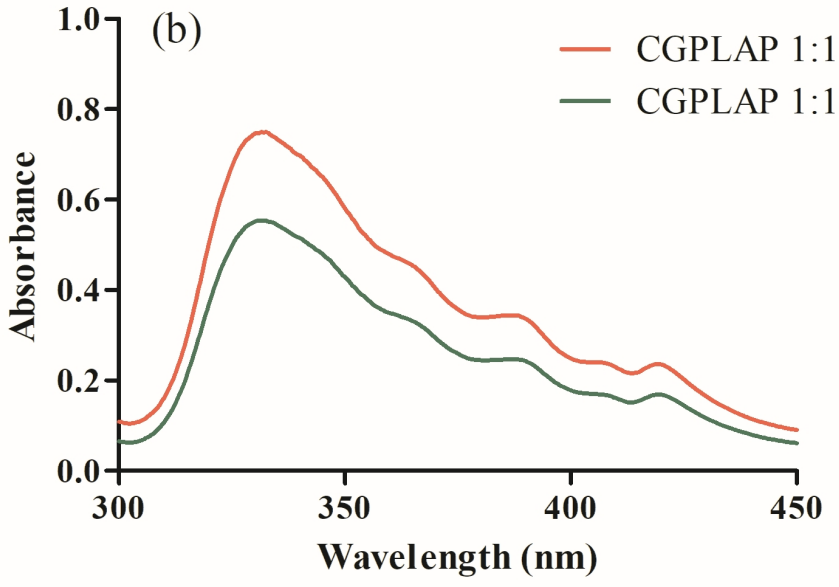

(D)
nm
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
µm
0
1
2
3
4

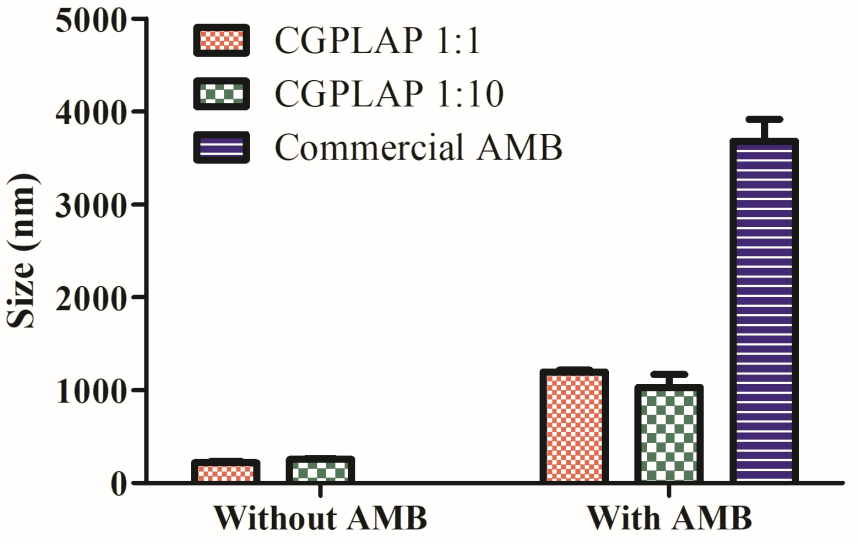

(E)
5
10
15
20
0
20
40
60
80
100
120
140
nm

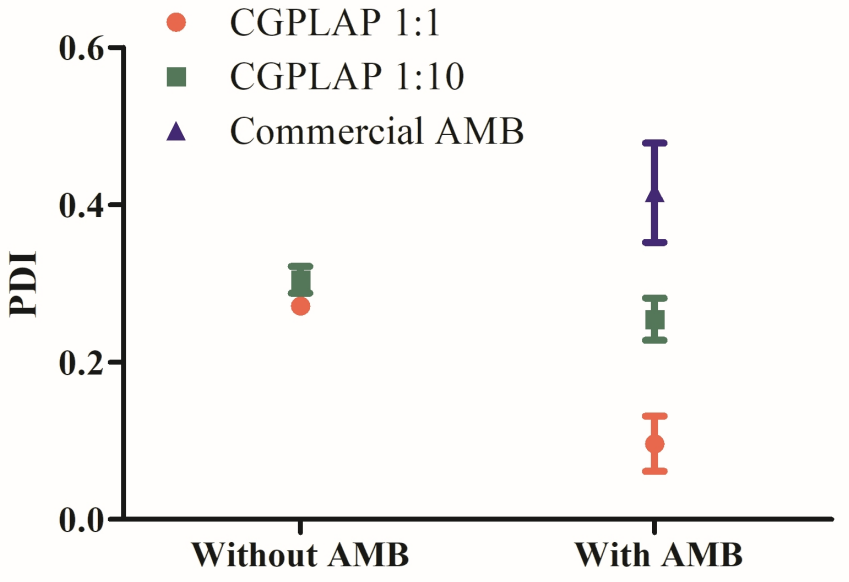

(F)
5
10
15
20
25
30
0
20
40
60
80
100
120
nm

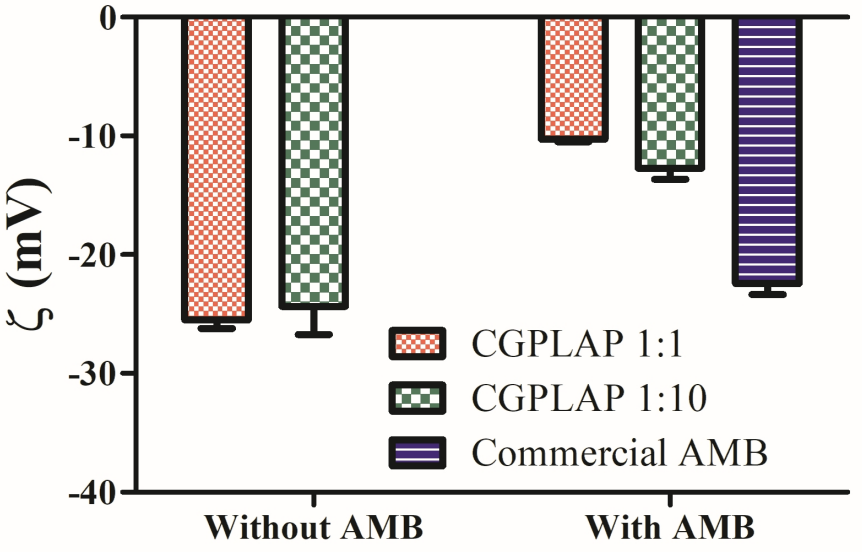

(A)
(B)
(C)
(D)
µm
nm
Deg
71.5
71.0
70.5
70.0
71.8
71.7
71.6
71.5
71.4
71.3
71.2
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5

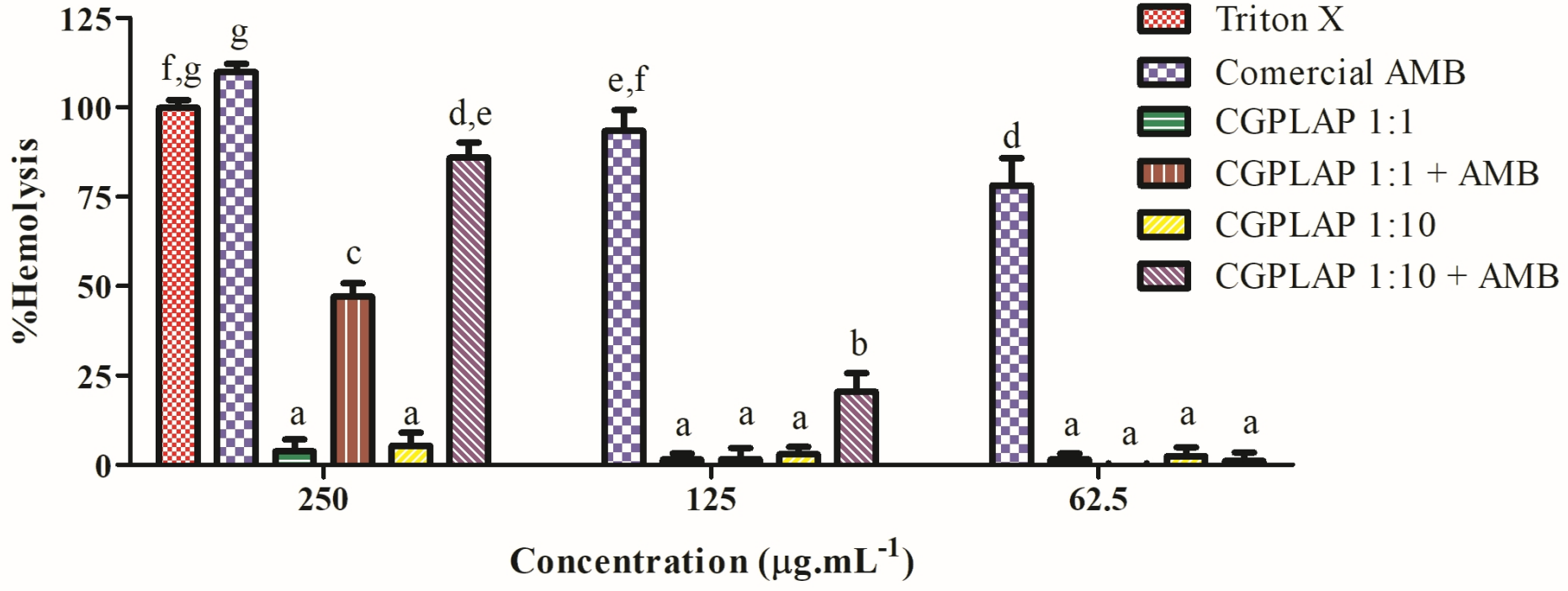

(a)
Commercial AMB
AMB in DMSO
Absorbance
2.0
1.5
1.0
0.5
0.0
300
350
400
450
Wavelength (nm)

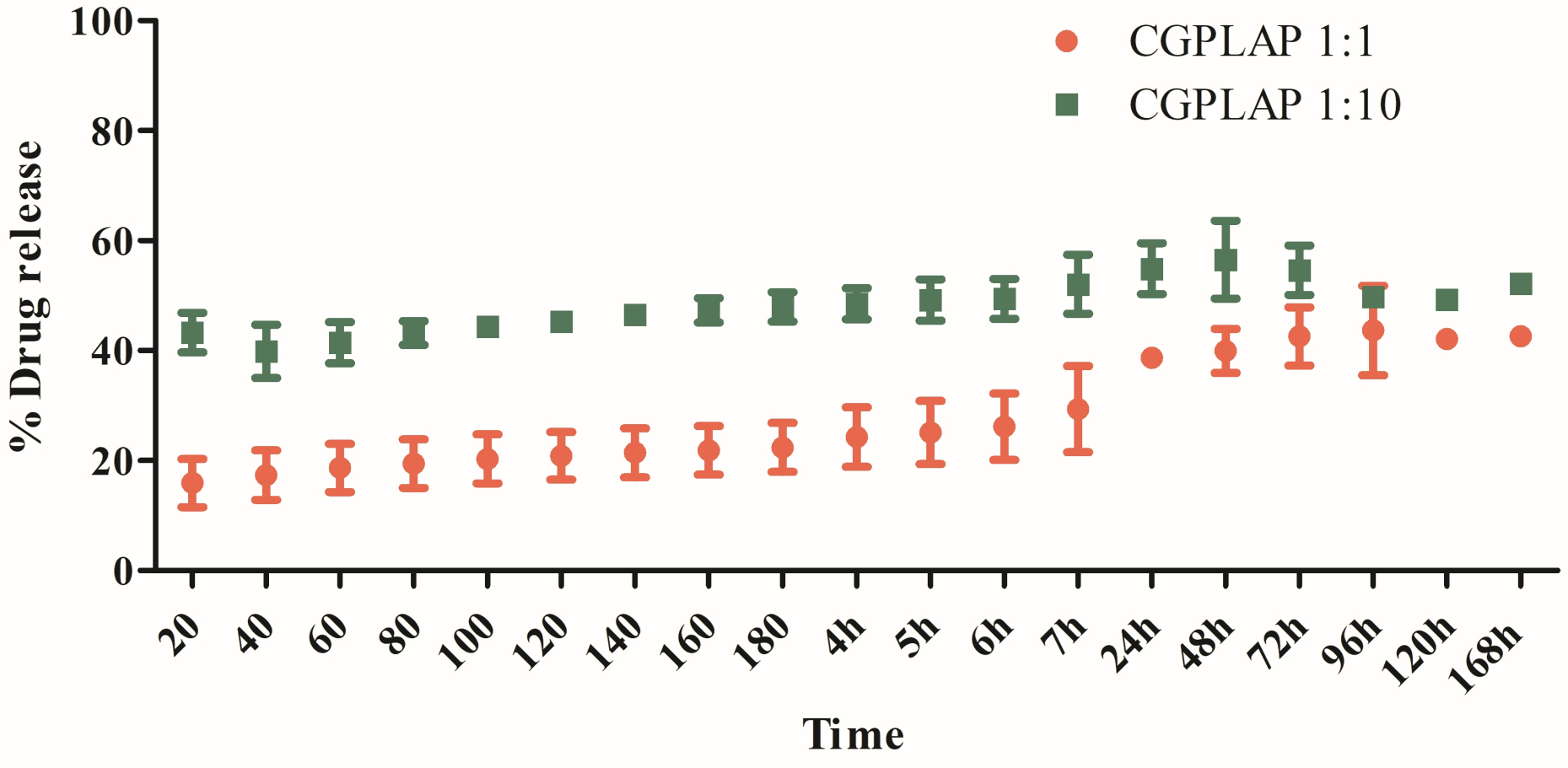

(b)
CGPLAP 1:1
CGPLAP 1:10
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
300
350
400
450
Wavelength (nm)

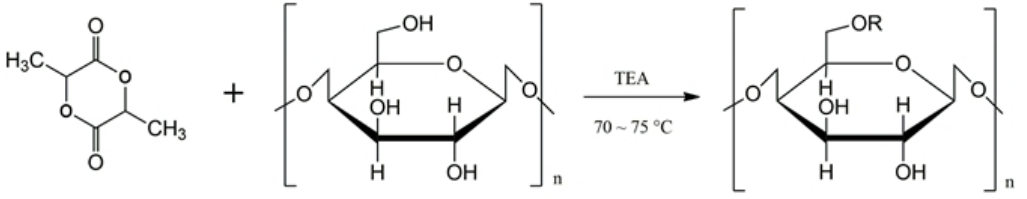

5000
4000
3000
2000
1000
0
Size (nm)
CGPLAP 1:1
CGPLAP 1:10
Commercial AMB
Without AMB
With AMB


0.6
0.4
0.2
0.0
PDI
CGPLAP 1:1
CGPLAP 1:10
Commercial AMB
Without AMB
With AMB


0
-10
-20
-30
-40
ζ (mV)
CGPLAP 1:1
CGPLAP 1:10
Commercial AMB
Without AMB
With AMB


125
100
75
50
25
0
%Hemolysis
f,g
g
a
c
a
d,e
e,f
a
a
a
b
d
a
a
a
a
250
125
62.5
Concentration (µg.mL-1)
Triton X
Comercial AMB
CGPLAP 1:1
CGPLAP 1:1 + AMB
CGPLAP 1:10
CGPLAP 1:10 + AMB

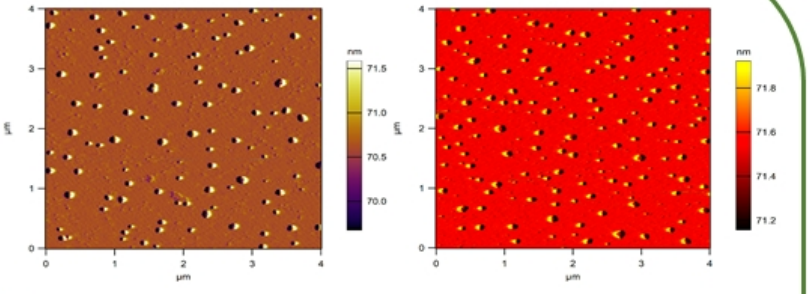

CGPLAP 1:1
CGPLAP 1:10
% Drug release
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
4h
5h
6h
7h
24h
48h
72h
96h
120h
168h
Time



L-lactide

Cashew Gum

R=



self-assembled CGPLAP nanoparticles

	Size (nm)	PDI	Zeta potential
CGPLAP 1:1	230.4 ± 7.7	0.272 ± 0.002	-26.1 ± 1.9
CGPLAP 1:10	243.6 ± 10.7	0.307 ± 0.018	-24.3 ± 2.3

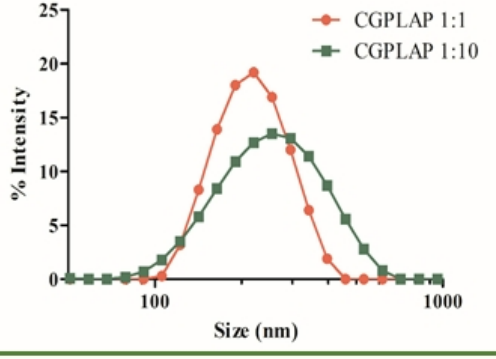


Table 1 - Amount of reagents and solvent used for the synthesis of CGPLAP copolymers

gum:L-lactide molar ratio[a]	DMSO (mL)	L-Lactide (g)	TEA (mL)
1:1	8.89	0.889	0.178
1:10	88.9	8.89	1.78

[a]Amount of cashew gum was maintained at 1.0 g

Table 2- Yield and characteristics of copolymers obtained with different CG:PLA ratios.

Parameter	CG:PLA ratio	
	1:1	1:10
Reaction yield (%)[a]	66.5 ± 1.2	14.8 ± 0.7
Copolymer yield (%)[b]	98.2± 1.4	87.7 ± 5.4
Homopolymer yield (%)[b]	1.8 ± 1.4	12.3 ±5.4
Grafting %[c]	18.7± 1.9	23.3 ± 7.7
MS	2.27	2.33
DS	1.06	1.00
DP	2.14	2.33

[a] In relation to initial weight of CG+PLA; [b] in relation to unpurified copolymer; [c]mass of copolymer-mass of CG/mass of purified copolymer. MS, DS, and DP were calculated as described in equations 1 to 3.

Table 3. Minimum inhibitory concentration (MIC) against *Candida albicans* strains.

Samples	MIC ($\mu g.mL^{-1}$)			
	C. albicans			
	ATCC 90028	LABMIC 0101	LABMIC 0102	LABMIC 0104
CGPLAP 1:1blank[a]	2	2	2	2
CGPLAP 1:10 blank[a]	1	1	1	1
AMB loaded CGPLAP 1:1	0.25	0.5	0.5	0.5
AMB loaded CGPLAP 1:1	0.5	0.5	0.5	0.5
AMB (Sigma)	1	1	1	1

[a] Copolymer concentration

AUTHOR STATEMENT

Ana Rosa Richter- Investigation (synthesis, Physicochemical analysis, design methodology), analysis and discussion of results, writing original draft, visualization

Maria J. Carneiro- hemolysis experiments

Nayara A. de Sousa - hemolysis experiments

Vicente P. T. Pinto - hemolysis experiments

Rosimeyre S. Freire, R- *Nanoparticles Morphology analysis*

Jeanlex S. de Sousa *Nanoparticles Morphology analysis*

Josilayne de Fátima Souza Mendes antifungal activity

Raquel Oliveira dos Santos Fontenelle antifungal activity

Judith P. A.Feitosa - writing review analysis and discussion of results

Haroldo C. B. Paula- writing review analysis and discussion of results

Francisco M. Goycoolea- writing review analysis and discussion of results

Regina C. M. de Paula-funding acquisition, supervision, ideas, writing review, analysis and discussion of results