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Original Article

Effect of cationic surfactant on the physicochemical and antibacterial properties of colloidal systems (emulsions and microemulsions)

Efeito do surfactante catiônico nas propriedades físico-químicas e antibacteriana de sistemas coloidais (emulsões e microemulsões)

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Abstract

Colloidal systems have been used to encapsulate, protect and release essential oils in mouthwashes. In this study, we investigated the effect of cetylpyridinium chloride (CPC) on the physicochemical properties and antimicrobial activity of oil-in-water colloidal systems containing tea tree oil (TTO) and the nonionic surfactant polysorbate 80. Our main aim was to evaluate whether CPC could improve the antimicrobial activity of TTO, since this activity is impaired when this essential oil is encapsulated with polysorbate 80. These systems were prepared with different amounts of TTO (0-0.5% w/w) and CPC (0-0.5% w/w), at a final concentration of 2% (w/w) polysorbate 80. Dynamic light scattering (DLS) results revealed the formation of oil-swollen micelles and oil droplets as a function of TTO concentration. Increases in CPC concentrations led to a reduction of around 88% in the mean diameter of oil-swollen micelles. Although this variation was of only 20% for the oil droplets, the samples appearance changed from turbid to transparent. The surface charge of colloidal structures was also markedly affected by the CPC as demonstrated by the transition in zeta potential from slightly negative to highly positive values. Electron paramagnetic resonance (EPR) studies showed that this transition is followed by significant increases in the fluidity of surfactant monolayer of both colloidal structures. The antimicrobial activity of colloidal systems was tested against a Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureaus*) bacteria. Our results revealed that the inhibition of bacterial growth is observed for the same CPC concentration (0.05% w/w for *E. coli* and 0.3% w/w for *S. aureus*) regardless of TTO content. These findings suggest that TTO may not act as an active ingredient in polysorbate 80 containing mouthwashes.

Keywords: cetylpyridinium chloride, polysorbate 80, microemulsion, tea tree oil, antibacterial activity.

Resumo

Sistemas coloidais têm sido usados para encapsular, proteger e liberar óleos essenciais em enxagues bucais. Neste estudo, investigamos o efeito do cloreto de cetilpiridínio (CPC) nas propriedades físico-químicas e na atividade antimicrobiana de sistemas coloidais do tipo óleo-em-água contendo óleo de melaleuca (TTO) e o surfactante não iônico polissorbato 80. Nosso principal objetivo foi avaliar se o CPC poderia melhorar a atividade antimicrobiana do TTO, uma vez que ela é prejudicada quando este óleo essencial é encapsulado com polisorbato 80. Estes sistemas foram preparados com diferentes quantidades de TTO (0-0,5% m/m) e CPC (0-0,5% m/m) para uma concentração final de polissorbato 80 de 2% (m/m). Os resultados de espalhamento de luz dinâmico (DLS) revelaram a formação de micelas e gotículas inchadas de óleo em função da concentração de TTO. Aumentos nas concentrações de CPC resultaram numa redução de cerca de 88% no diâmetro médio das micelas inchadas com óleo. Embora esta variação tenha sido de apenas 20% para as gotículas de óleo, a aparência das amostras mudou de turva para transparente. A carga superficial das estruturas coloidais também foi significativamente afetada pelo CPC, como demonstrado pela transição no potencial zeta de valores levemente negativos para valores muito positivos. Estudos de ressonância paramagnética eletrônica (EPR) mostraram que esta transição é seguida por aumentos significativos na fluidez da monocamada de surfactante em ambas as estruturas coloidais. A atividade antimicrobiana dos sistemas coloidais foi testada contra bactérias Gram-negativas (*Escherichia coli*) e Gram-positivas (*Staphylococcus aureaus*). Nossos resultados revelaram que a inibição do crescimento bacteriano é observada para a mesma concentração de CPC (0,05% m/m para *E. coli* e 0,3% m/m para *S. aureus*), independentemente do conteúdo de TTO. Estas descobertas sugerem que o TTO não deve atuar como ingrediente ativo em enxagues bucais contendo polissorbato 80.

Palavras-chave: cloreto de cetilpiridínio, polissorbato 80, microemulsão, óleo de melaleuca, atividade antibacteriana.

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1. Introduction

Tea tree oil (TTO) is the essential oil steam-distilled from the Australian native plant, Melaleuca alternifolia. Comprised mostly of monoterpenes, sesquiterpenes, and their associated alcohols, the use of TTO is gaining increasing acceptance throughout the world mainly due to its antimicrobial and anti-inflammatory activities (Brand et al., 2001; Carson et al., 2006; Swords and Hunter 1978; Kairey et al., 2023). Topical formulations containing TTO such as creams and gels can be found in the market and compounding pharmacies of many countries for the treatment of skin infections and inflammations. More recently, TTO has also been used in mouthwashes for the elimination of odor-causing bacteria and controlling gingivitis. Although in vitro studies have confirmed the effectiveness of this essential oil (in the free form) against oral microorganism (Forrer et al., 2013; Ramage et al., 2012), there is no much information on its activity after having been incorporated into the mouthwashes. For this particular application, because essential oils are insoluble or sparingly soluble in water, two different approaches have been taken to ensure their homogeneous dispersion throughout the product. The first is based on the use of organic solvents such as ethyl alcohol, while the second involves the encapsulation of essential oil in colloidal systems. However, due to the potential association between use of alcohol-containing mouthwashes and an increased risk of mouth cancer (Winn et al., 1991; Blot et al., 1983), there has been an ongoing rise in the demand for alcohol-free mouthwash in the market. As a result, many oral health care companies are focusing on the development of alcohol-free mouthwashes. Such formulations are usually designed as oil-in-water microemulsions, which correspond to thermodynamically stable, low viscosity and isotropic liquid mixtures of oil and water stabilized by an interfacial film of surfactant molecules (Danielsson and Lindman, 1981). Besides contributing to the TTO solubilization, these colloidal systems (microemulsions) are also known to protect the essential oil against possible thermal or photo degradation, ensuring increased stability, flavor and activity, thereby extending the final product shelf life (Baser and Buchbauer, 2010; McClements, 2012).

Optically transparent microemulsions are usually formed using relatively high surfactant concentrations and, therefore, the choice of chemically stable and less toxic surfactants is needed. As there is a consensus that nonionic surfactants tend to be less toxic than ionic surfactants, they have been widely used not only in mouthwashes but also in a number of pharmaceutical formulations and as food additive (American College of Toxicology, 1984; Kerwin, 2008). The two main types of nonionic surfactants currently used in the production of TTO containing mouthwashes are the polyoxyethylene derivatives of castor oil and polyoxyethylene sorbitan fatty acid esters (also known as polysorbates or tweens). To understand the challenges involved in the production of these aqueous formulations, recently we investigated the formation, physicochemical and antimicrobial properties of TTO containing colloidal systems (microemulsions and emulsions) stabilized by the polysorbate 80 (Lins et al., 2016). In addition to stablishing the experimental conditions for which clear formulations are obtained, these investigations also showed that the encapsulation of TTO with this nonionic surfactant led to a loss of biocidal activity. The deactivation of essential oils and phenolic compounds by polysorbates has also been reported by other studies (Terjung et al., 2012). In general, this effect has been attributed to the strong interactions of antibacterial agents either with the polar head groups (via hydrogen bounds) or hydrocarbon tails of polysorbate molecules (via hydrophobic interactions), which could preclude their binding to the microorganisms. Additionally, since the colloidal systems stabilized with polysorbates generally exhibit a slight negative zeta potential, we believe this lack of bacterial activity could also be caused by ionic repulsion forces resulting from identical charges on the surface of the dispersed structures and bacteria. In fact, this is supported by previous data in the literature that demonstrated the importance of the electrostatic interactions for the binding of nanoparticles to bacterial membranes (Dong et al., 2015; Fang et al., 2015). The higher affinity of cationic liposomes and surfactant lipid preparations to bacterial suspensions has also been confirmed based on their biocidal activity (Hamounda and Baker Junior, 2000). Thus, according to these findings, it is likely that changes on the surface charge of colloidal structures containing TTO and polysorbate 80 from negative to positive may improve their affinity to bacterial membrane.

In the present study, the TTO containing colloidal systems stabilized with polysorbate 80 were prepared with increasing concentrations of cetylpyridinium chloride (CPC). This cationic surfactant found in some market mouthwashes is used for treating and preventing plaque and gingivitis (Williams, 2011). The positively charged hydrophilic region of the CPC molecule plays a major role in its antimicrobial activity, imparting a high binding affinity for bacterial cells whose outermost surface carries a net negative charge. The strong positive charge and hydrophobic region of CPC enable the compound to interact with the microbial cell surface and integrate into the cytoplasmic membrane. As a result of this interaction, there is disruption of membrane integrity resulting in cytoplasmic component leakage, interference with cellular metabolism, cell growth inhibition, and cell death (Williams, 2011). The influence of CPC on the mean diameter of dispersed phases of microemulsions (oil-swollen micelles) and emulsions (oil droplets) was evaluated based on the scattering intensity distribution obtained from DLS studies. Zeta potential measurements also allowed to examine the changes in their surface charge due to increases in CPC concentration. This information was relevant to evaluate whether CPC can or not improve the antimicrobial activity of the TTO containing colloidal systems when their zeta potential change from negative to positive. Further information on the molecular dynamic within the surfactant monolayer of both colloidal systems was obtained from the electron paramagnetic resonance (EPR) studies. They were useful in proposing the probable mechanism by which CPC changes the size of both colloidal structures. Finally, the in vitro susceptibility of a gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria to the TTO

containing colloidal systems was evaluated to explore the question of whether or not the incorporation of CPC improves their antibacterial activity. We believe this study provides fundamental information on the physicochemical and antimicrobial properties of colloidal systems that can be useful for the rational design of alcohol-free formulations containing essential oils.

2. Experimental

2.1. Materials

Polysorbate 80 (CAS Number 9005-65-6), cetylpyridinium chloride (CPC), dimethyl sulfoxide (DMSO), resazurin and brain-heart infusion (BHI) broth were acquired from Sigma Chemical Co. (St. Louis, MO, USA). The essential oil of *M. alternifolia* (tea tree oil) was supplied by Ferquima Indústria e Comércio LTDA (Vargem Grande Paulista, SP, Brazil). The main components of this essential oil are: terpinen-4-ol (42%), γ-terpinene (22%), α-terpinene (10%), and 1,8-cineole (1.5%). The spin probes 5- and 16-doxyl stearic acid (5- and 16-DSA, respectively) were purchased from Avanti Polar Lipids (Alabaster, Al, USA). 5-DSA has the magnetic fragment (doxyl moiety) attached to the 5th carbon of the acyl chain, whereas for 16-DSA this fragment is attached to the 16th carbon.

2.2. Sample preparation

Microemulsions and emulsions were formed based on the spontaneous emulsification process described in previous studies (Lins et al., 2016; Sabari et al., 2013a, b). In brief, spontaneous emulsification was performed by adding small aliquots of distillated water (aqueous phase) to a mixture of surfactant and essential oil (organic phase) while magnetically stirring (600 rpm) the system at 25 °C. The organic phase consisted of different amounts of TTO (0%, 0.25%, 0.5%, 0.75% and 1% w/w) and polysorbate 80 (4% w/w). Each formulation was divided into five aliquots followed by a 1:1 dilution with suitable volumes of a CPC stock solution (100 mg/mL) and distillated water to produce samples containing 0%, 0.05%, 0.1%, 0.3% and 0.5% w/w CPC. The final concentrations of TTO and polysorbate 80 were reduced to half.

2.3. Preparation of spin probe samples

5- and 16-DSA spin probes were used to explore the molecular dynamic of colloidal systems. A small aliquot of spin probes dissolved in ethanol (10^{-2} M) was placed in a polypropylene microtube and the solvent was evaporated under a stream of nitrogen gas. Then 1 mL samples were added to the microtube to reach a final concentration of spin labels of 10^{-4} M. After labeling under gentle stirring, the samples were introduced into a capillary tube for EPR measurements.

2.4. Dynamic light scattering (DLS) and zeta potential measurements

DLS and zeta potential measurements were carried out in a Zetasizer Nano ZS, Malvern Instruments Ltd (London, U.K.), at 25 °C. DLS measurements were carried out using the general purpose analysis model. The size of dispersed structures was calculated as an average over twelve independent measurements, where the number of subruns for each measurement was automatically optimized by the instrument. The zeta potential, ξ, is calculated by determining the electrophoretic mobility, μ_e, and applying the Henry equation. This calculation was performed using the Smoluchowski approximation. The external cell voltage was fixed at 40 V. The average ξ values were calculated using a general purpose model over twelve independent measurements. The measurement duration was also optimized by the instrument. Before each zeta potential and DLS measurement, the samples were equilibrated for 5 minutes at 25 °C.

2.5. Electron paramagnetic resonance (EPR) measurements

A Bruker EPR 300 spectrometer equipped with the ER 4102 ST resonator and operating at X-band (9.4 GHz) was utilized in our investigations (Rheinstetten, Germany). EPR spectra were recorded at 25 °C with the following set parameters: microwave power, 20 mW; modulation frequency, 100 KHz; modulation amplitude, 1.024 G; magnetic field scan, 100 G; sweep time, 168 s and detector time constant, 41 ms. Spectral analysis were performed by using the nonlinear least-squares (NLLS) fitting program (Schneider and Freed 1989; Budil et al., 1996). This program allows to deconvolute overlapped EPR spectra. The parameters used in the fitting model were the rotational diffusion constant (R), the order parameter (S_0), the lorentzian inhomogeneous broadening (lib), and the components of the magnetic tensors g and A. The dynamic of the spin probes is characterized by R, which is calculated based on the rotational diffusion rates of the nitroxide radical around the axes perpendicular and parallel to the mean symmetry axis for the rotation. This symmetry axis is also the direction of preferential orientation of the spin probe moiety, which is measured with respect to the surface normal of colloidal particles (S_0 parameter). In general, the higher the S_0 values the more restricted is the range in motion relative to the surface normal. The magnetic tensor components were in the spectrum fittings were: $g_{xx}(1) = 2.0086$, $g_{yy}(1) = 2.0051$, $g_{zz}(1) = 2.0019$, $g_{xx}(1) = 2.0086$, $g_{yy}(1) = 2.0051$, $g_{zz}(1) = 2.0020$, $a_{xx}(1) = 4.5$, $a_{yy}(1) = 4.5$, $a_{zz}(1) = 35.3$, $a_{xx}(2) = 4.5$, $a_{yy}(2) = 4.5$, $a_{zz}(2) = 35.8$. They were kept fixed during spectral analysis.

2.6. In vitro antibacterial activity of colloidal systems

E. coli (ATCC 25922) and *S. aureus* (ATCC 25923) were grown in BHI broth at 37 °C for 18 h and then adjusted nephelometrically to 3.5 x 10^8 CFU/mL based on 0.5 McFarland standards. The minimal inhibitory concentration (MIC) tests were performed by incubating 100 µL of colloidal systems containing different amounts of CPC with 100 µL of bacterial suspension in 96-well microplates. The microplates were sealed with parafilm and placed in an oven at 37 °C for 18h. After this period, 15 µL of 0.02% resazurin aqueous solution was poured into each microplate reservoir to indicate microorganism viability.

3. Results and Discussion

3.1. Physicochemical properties of colloidal systems

The influence of CPC on the size of dispersed phases containing different amounts of TTO was investigated by DLS. These systems were prepared by spontaneous emulsification process using the polysorbate 80 as emulsifier. For each amount of TTO the CPC concentration was varied from 0% to 0.5% w/w. The effect of such variations on the scattering intensity distributions of samples containing 0%, 0.25% and 0.5% TTO is shown in Figure 1. The results obtained with 0.125% and 0.375% TTO have been included in the appendix (Figure S1). Figure 1A (0% TTO) shows that the peak 1 assigned to the polysorbate 80 micelles undergo significant shifts towards lower size values as a function of CPC concentration.

These changes are summarized in Figure 2A, from which is possible to observe a significant decrease in the mean intensity diameter of surfactant micelles from 14.0 ± 1.5 (0% CPC) to 1.7 ± 0.8 nm (0.5% CPC). This result indicates that the CPC incorporation into the micellar structures result in a greater curvature of the surfactant monolayer, possibly due to an increase in the mean spacing between the surfactant head groups. The extremely small diameter of polysorbate 80 micelles containing 0.5% CPC was found to be very close to that calculated for pure CPC micelles (~1.0 nm). The distribution data of pure CPC micelles is given in Figure S2 (see Supplementary Material). Still concerning the Figure 1A, lower intensity peaks were also detected at higher CPC concentrations (see arrows). At first sight, these peaks could be attributed to the formation of large micellar aggregates. However, as it will be shown later in this paper, this hypothesis can be ruled out since the samples with 0% TTO remained optically transparent over the whole CPC concentration. Thus, these additional peaks may arise from impurities or artifacts within the sample, where their signal would become noticeable when the light scattered by micelles are very weak in intensity. Indeed, as there is a sixth-power dependence of the intensity of light scattered with respect to particle diameter in the intensity distributions, it is possible to estimate that the polysorbate 80 micelles with 1.7 nm scatter around three hundred thousand times less light than those with 14 nm.

Similar trends were also observed for the sample with 0.125% TTO (Figure S1). Since the peak 2 attributed to the oil-swollen micelles was significantly shifted towards lower size values with increasing CPC concentrations. These changes led to a reduction of around 88% in the mean intensity diameter of oil-swollen micelles between 0% and 0.5% CPC (Figure 2A). As the magnitude of this effect was very similar to that of pure polysorbate 80 micelles, it can be stated that the changes in the curvature of surfactant monolayer caused by CPC are indifferent to the presence or absence of TTO molecules into the hydrophobic core of oil-swollen micelles. The influence of CPC on the size of microemulsion structures containing carvacrol has also been reported in a previous study (Shaaban and Edris, 2015). DLS results revealed, for instance, that the dispersed phase of the CPC-formulated carvacrol microemulsions is much smaller in size than those stabilized with nonionic surfactants. At 0.25% TTO (Figure 1B), in addition to the peak of oil-swollen micelles (peak 2), the presence of a second peak (peak 3) indicates that oil droplets have also been formed. The formation of both types of colloidal structures (oil-swollen micelles and oil droplets) has also been noticed in previous DLS studies with oil-in-water emulsions containing the lemon essential oil (Ziani et al., 2012a, b). According to the authors, the appearance of oil droplets often occurs when the saturation limit of the micelles is exceeded. Thus, it can be concluded that this saturation seems to occur at 0,25% TTO for oil-swollen micelles stabilized 2% w/w polysorbate 80. Figure 2B shows that the size of oil droplets is less affected by CPC as compared to the oil-swollen micelles (Figure 2A), since a reduction of only 20% in their mean intensity diameters was estimated from 0% (191 nm) to 0.5% CPC (154 nm).

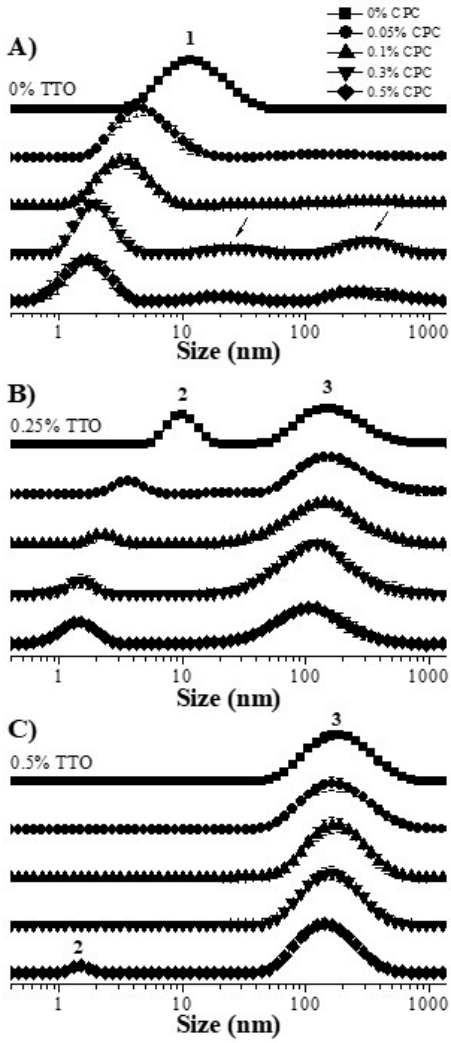

Figure 1. Effect of CPC on the scattering intensity distributions of colloidal systems formed with (A) 0%, (B) 0.25% and (C) 0.5% TTO. The numbers 1-3 refers to the types of colloidal structures formed, while the arrows indicate the presence of impurities or artifacts within the samples.

These small variations in size were also observed for the colloidal systems containing 0.375% and 0.5% TTO (Figure 2B). With regard to the intensity distribution of sample with 0.5% TTO (Figure 1C), while the peak 3 is present over the whole CPC concentration range, the peak 2 is only observed at 0.5% CPC. This result indicates that CPC not only change the size of the oil droplets but also promotes their conversion into oil-swollen micelles. This effect has also been confirmed by the particle number distributions (Figure 3), where the peak assigned to the oil droplets in the sample with 0.5% TTO (Figure 3C) is gradually converted to that of oil-swollen micelles at higher CPC concentrations. Thus, due to the distribution of CPC molecules between both colloidal structures, the CPC concentration in the oil droplets are not enough to produce significant decreases in size such as those observed in Figure 2A. Based on the changes in Figure 2B, the conversion from oil droplets to oil-swollen micelles seems to occur regardless of the TTO concentration.

It is worth mentioning that the number distributions are less sensitive to large particles as compared to the intensity distributions since a simple first-powder relationship between the intensity of light scattered and the particle diameter is involved. This helps explain the significant differences between the Figure 1 and 3. At 0.25% TTO, for instance, while the intensity distributions revealed the coexistence of two colloidal structures, only a single peak attributed to the oil-swollen micelles is present in the number distributions. Despite these differences, a similar behavior in relation to the effect of CPC on the size of scattered particles was evidenced in both distributions.

Figure 4 shows the effect of CPC on the zeta potential (ζ value) of dispersed phases. In the absence of CPC, the ζ values varied between -8 and -12 mV for all colloidal systems with and without TTO. A possible reason for this might be associated to the presence of anionic impurities such as free fatty acids. Accordingly to previous HPLC studies (Hu et al., 2003; Adamo et al., 2010), traces of oleic acids are found even in high purity polysorbate 80 standards. This could explain the fact that colloidal systems stabilized with polysorbates generally exhibit negative zeta potential. Additionally, the OH^- ions ability to interact via hydrogen bond with the numerous ethylene oxide groups at the surfactant head groups could also contribute for reducing the zeta potential values (McClements, 2004; Jyh-Ping and Nacu, 2003). This assumption is supported by a study performed with thin films of pentaethylene glycol monododecyl ether (nonionic surfactant), where the dependence of zeta potential on pH was caused by the "unbalance" of H^+ and OH^- ions at the surfactant-water interface (Mane and Pugh, 1991). As an excess of OH^- ions was observed even in slightly acidic media, which resulted in negative zeta potentials, it is likely that a similar effect may also occur with the colloidal systems stabilized with polysorbate 80. As shown in Figure 4, the addition of CPC resulted in significant increases in the ζ values. These changes were more abrupt for the samples containing 0.25%-0.5% TTO, for which an increase of around 47 mV in the ζ values was observed from 0% to 0.1% CPC. This increase was 34 mV and 19 mV for the samples containing 0.125% and 0% TTO, respectively (see Figure 4). Such differences suggest that the conformation of CPC head groups (and consequently the dipole moment of the pyridinium group) varies on going from lower to higher TTO concentration. Thus, the magnitude of the interactions between the dipole moments and electric field would affect the electrophoretic mobility of dispersed phases, thereby leading to different ζ values. Despite the differences mentioned above, the ζ values were similar for all colloidal systems at 0.5% CPC.

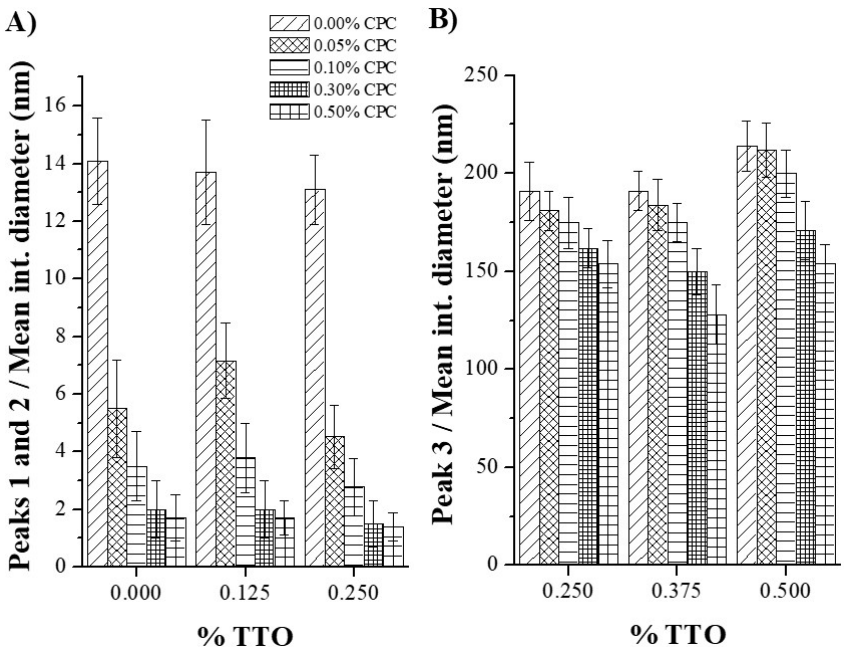

Figure 2. Influence of CPC on the mean intensity diameters of colloidal structures containing 0-0.5% TTO. This parameter was calculated from the intensity distributions for (A) the peaks 1 (surfactant micelles) and 2 (oil-swollen micelles) as well as for (B) the peak 3 (oil droplets).

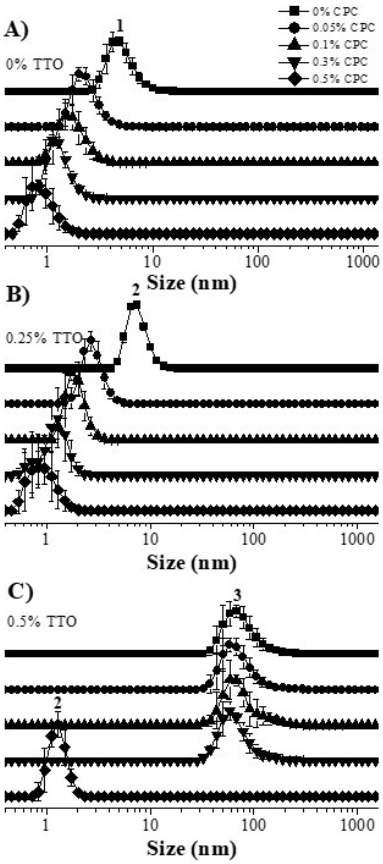

Figure 3. Effect of CPC on the particle number distributions of colloidal systems formed with (A) 0%, (B) 0.25% and (C) 0.5% TTO. The numbers 1-3 refers to the types of colloidal structures formed.

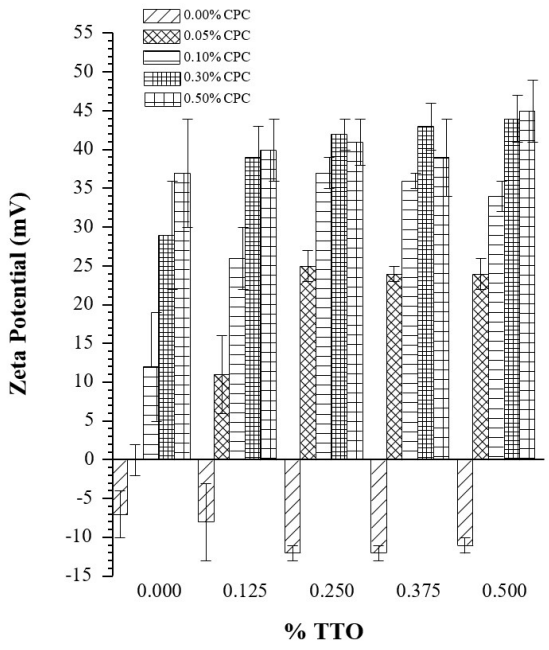

Figure 4. Influence of CPC on the zeta potential of dispersed structures containing 0-0.5% TTO.

The effect of CPC on the molecular dynamic and ordering within the hydrophobic core of dispersed structures was assessed by electron paramagnetic resona nce (EPR) using the 5-DSA as probe. Figure 5 shows the experimental and theoretical EPR spectra of 5-DSA incorporated to the dispersed phases formed with 0.5% TTO at increasing amounts of CPC. As it can be seen, the EPR spectra remained practically unchanged up to 0.05% CPC, whereas further addition of surfactant led to significant changes in line shape. Accordingly to the fitting results, these changes can be attributed to two phenomena: (1) the formation of different spectral components and (2) a higher dynamic of the spin labels in the surfactant monolayer. At 0% and 0.05% CPC (see Figure 5), for instance, the best-fit EPR spectra were obtained by using a single-component fitting model. As discussed in a previous study, this component (referred to as component 1) has been attributed to the fraction of spin labels that interact with the polysorbate 80 molecules in the surfactant monolayer (Lins et al., 2016). Although the component 1 is present over the whole range of CPC, the fitting analysis also revealed the existence of a second component (component 2) above 0.05% CPC (see right side in Figure 5). The coexistence of both components indicates the partitioning of spin labels into two different environments in terms of fluidity and ordering. The changes in the rotational diffusion constant of spin labels that form the component 1 (see R_1 values in Figure 6A) indicate that their mobility is markedly increased as a function of CPC. This result clearly demonstrates the strong interaction of CPC molecules with the surfactant monolayer of colloidal structures. Such interactions possibly impair the formation of hydrogen bonds between the polar head groups of polysorbate molecules, resulting in a lower intermolecular coupling and thereby leading to a more fluid surfactant monolayer (higher R_1 values). This impairment could also contribute for increasing the mean spacing between the polar head groups, which has been proposed to occur due to the addition of CPC. In fact, this proposal is supported by

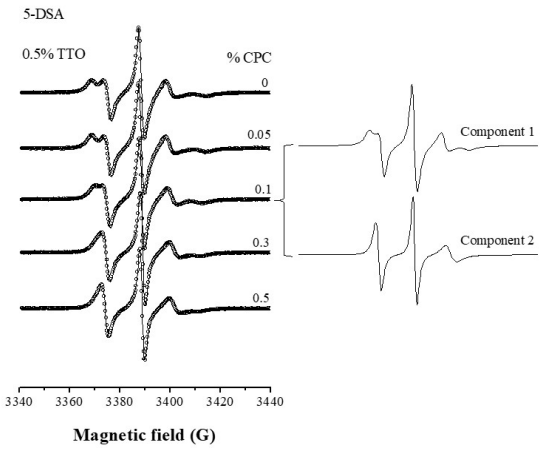

Figure 5. Experimental (empty cycles) and best-fit (solid lines) EPR spectra of 5-DSA (top two spectra) incorporated to the colloidal systems containing 0.5% TTO at different CPC concentrations. The components 1 and 2 obtained from the best-fit EPR spectra are also depicted.

the reductions in the mobility restriction of spin labels, as indicated by the slight decreases in S_0 values (Figure 6B). The small values of S_0 are consistent with the existence of very fluid domains that might be caused by the melted hydrocarbon chains of the polysorbate 80 molecules. According to the manufacturer's data sheet, around 70% of surfactant chains contain oleic acids, which are already in the liquid state at room temperature. In relation to the component 2, since its rotational diffusion constant remained practically unchanged as the increases in CPC, this parameter was fixed at $3.2x10^8$ s^{-1} during the spectral fittings. Moreover, the S_0 parameter from component 2 was neglected from the fittings since it did not significantly improved the fit quality. Thus, based on these findings, it has been proposed that the surfactant monolayer of the CPC containing samples is also formed by CPC-enriched domains (component 2). As shown in Figure 6C, the fraction of spin labels in the component 1 (N1 values) decreases as a function of CPC content, indicating that the CPC-enriched domains (N_2 values) are increased at the same rate.

The fitting data obtained for the other colloidal systems with lower amounts of TTO are also depicted in Figure 6. In general, similar trends were also observed in relation to the variations of R_1 and N_1 parameters as a function of CPC concentration. This result indicates that the magnitude of the interactions between the CPC molecules and the interfacial film of surfactant on the surface of colloidal structures does not depend on the amount of TTO. Moreover, since the changes in N_1 values were very similar in all studied samples, it is possible to conclude that the CPC-enriched domains are equally distributed in the micelles, oil-swollen micelles and oil droplets.

For applications in mouthwashes, it is desirable that the colloidal systems are transparent or slightly turbid. Thus, the optical properties of samples with different TTO content and increasing CPC concentrations were also investigated. Figure 7A shows that the turbidity of the samples with 0% and 0.125% TTO was 0 NTU over the whole CPC concentration range. This result demonstrates that polysorbate 80 and oil-swollen micelles are optically transparent, which is consistent with their small diameters given by the DLS studies. On the other hand, a marked increase in turbidity was observed at higher TTO content, which is attributed to the formation of oil droplets. Because their size is much larger than that of micellar systems (see Figure 2A and 2B), the oil droplets scatter the light more strongly. For the samples with 0.25-0.5% TTO, the addition of CPC resulted in significant decreases in turbidity (Figure 7A). At 0.5% TTO, for instance, a decrease of around 98% in turbidity was observed from 0% to 0.5% CPC. This result is consistent with the changes observed in the visual appearance of formulations, which undergo a transition from turbid to transparent within this CPC concentration range (Figure 7B). The changes in Figure 7A support the interpretation previously given on the conversion of oil droplets into oil-swollen micelles upon addition of CPC, since a reduction of 20% in the size of oil droplets (Figure 2B) would not be significant to reduce the turbidity in 97%. Interestingly, the variations in turbidity between 0% and 0.5% CPC were very similar for the samples containing 0.25-0.5% TTO, which corroborates with the assumption that the conversion from oil droplet to oil-swollen micelles does not depend on the TTO concentration.

3.2. Antibacterial activity of colloidal systems

The antibacterial activity of colloidal systems against the bacteria *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) was evaluated based on their ability to inhibit bacterial growth. Table 1 shows the results obtained from the resazurin assays after the treatment of both microorganisms with samples containing 0% and 0.5% TTO. As it can be noticed, the CPC concentration range for which there was inhibition of bacterial growth was the same for both formulations. *E. coli* was completely inhibited from 0.05% CPC, while this concentration was 6 times higher for *S. aureus*. This result indicates that the *E. coli* is much more susceptible to the CPC containing formulations than *S. aureus*, which is in agreement with a previous study. Such variation in sensitivity may be associated to the differences in cell wall structure between Gram-negative and Gram-positive bacteria. Furthermore, a comparison of these results with those of zeta potential also suggests that the inhibition of bacterial growth is not improved when the surface of colloidal structures carry higher positive charges.

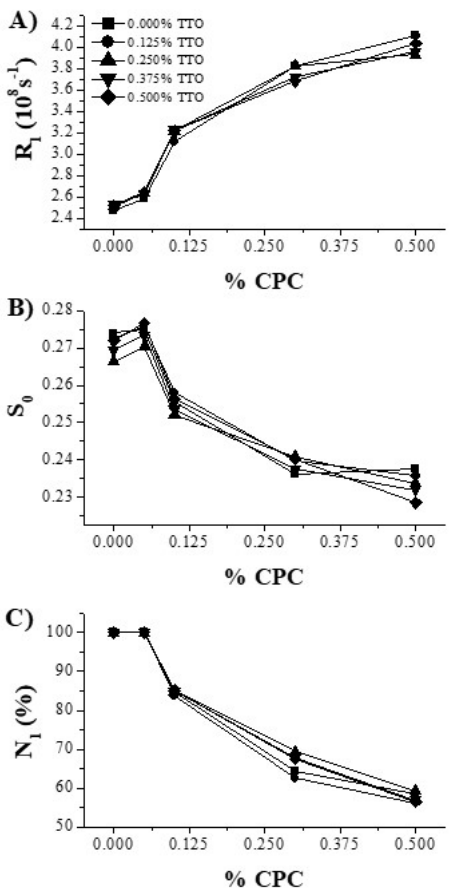

Figure 6. Influence of CPC on the (A) rotational diffusion constant (R_1 values), (B) order parameter (S_0 values) and (C) fraction of spin labels in the component 1 of EPR spectra of the colloidal systems containing 0-0.5% TTO.

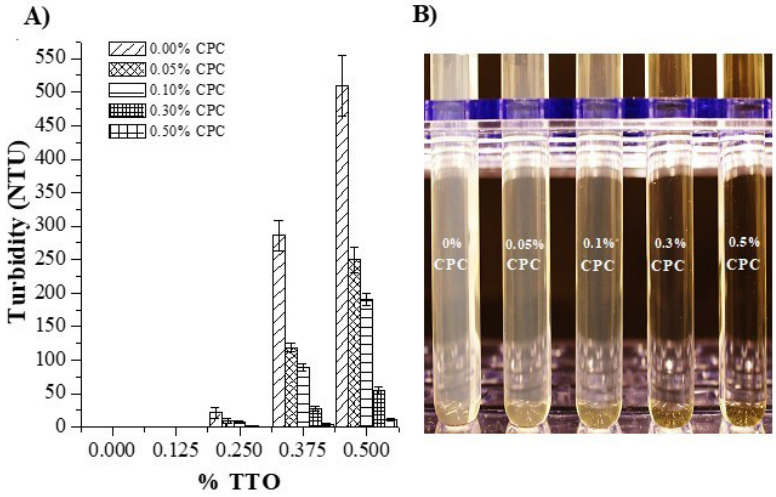

Figure 7. Influence of CPC on the (A) turbidity of colloidal systems with 0-0.5% TTO and (B) physical appearance of sample containing 0.5% TTO.

Table 1. Antibacterial activity of colloidal systems formed with 0% and 0.5% TTO at different CPC concentration against *E. coli* and *S. aureus*.

0% TTO				
% CPC	*E. coli*		*S. aureus*	
	BG[a]	BCC[b]	BG	BCC
0	+	>1.25x10^6	+	>6x10^5
0.05	-	0	+	>6x10^5
0.1	-	0	+	>6x10^5
0.3	-	0	-	0
0.5	-	0	-	0
Positive control	+	>1.25x10^6	+	>6x10^5
0.5% TTO				
% CPC	*E. coli*		*S. aureus*	
	BG	BCC	BG	BCC
0	+	>1.25x10^6	+	>6x10^5
0.05	-	0	+	>6x10^5
0.1	-	0	+	>6x10^5
0.3	-	0	-	0
0.5	-	0	-	0
Positive control	+	>1.25x10^6	+	>6x10^5

[a]BG = Bacterial growth; [b]BCC = Bacterial Colony Counting.

Accordingly to the Figure 3, the samples formed with 0.5% exhibited more positive zeta potentials as compared to the ones with 0% TTO over the whole CPC concentration. Based on the DLS results, it can be also stated that the bacterial growth inhibition is not affected by the type of colloidal structures. Thus, the accumulation of CPC molecules in both microorganisms seems to depend on their ability to migrate from the dispersed phase of formulations and bind via electrostatic interactions with the negatively charged lipopolysaccharides of Gram-negative cell surface, and also with the negatively charged teichonic acid, present in Gram-positive cells. The antibacterial activity of samples containing 0.125%, 0.25% and 0.375% TTO also reproduced the results given Table 1 (dada not shown).

In the absence of CPC, the Table 1 shows that the two samples with 0% and 0.5% TTO did not inhibit the *E. coli* and *S. aureus* growth. This lack of biocidal activity of TTO in colloidal systems (emulsion and microemulsion) has been discussed in our previous work (Lins et al., 2016).

For pure TTO dissolved in DMSO, we found MIC values of 0.31% w/w for *E. coli* and 0.62% w/w for *S. aureus*, which are in full agreement with other studies (Carson et al., 2002; Cox et al., 2000). However, the encapsulation of TTO with polysorbate 80 did not inhibit bacterial growth even at concentrations up to 1.24% w/w. Since both bacteria were exposed to the formulations for a relatively long period of time, it is likely that the TTO remains preferably solubilized in the hydrophobic core of colloidal structures, thereby reducing the accumulation of essential oil molecules in the bacterial membrane. Despite the lack of experimental data on the diffusion of essential oil molecules through the aqueous media, we believe their antimicrobial activity in the encapsulated form also depend on the molecules ability to diffuse from the dispersed phase to the bacterial membrane. The negative effect of polysorbate 80 (or Tween 80) on the antimicrobial activity of essential oils has been also reported in other studies (Qiumin et al., 2016; Hammer et al., 1999; Inouye et al., 2001; Remmal et al., 1992).

4. Conclusions

In this study, we investigated the influence of CPC on the physicochemical properties of the colloidal systems formed with different amounts of TTO. Our results showed, for instance, that the mean diameters of the surfactant micelles, oil-swollen micelles and oil droplets decrease gradually as a function of CPC concentration. Because this effect results in a higher curvature of surfactant monolayers, we have proposed that the changes in mean diameter are associated to increases in the area occupied by the surfactant head groups due to the CPC addition. Such interpretation is consistent with the EPR results, which revealed that CPC not only increase the mobility of spin labels in the surfactant monolayer but also decrease their local ordering. The surface charge of colloidal structures was also markedly affected by the addition of CPC, since their zeta potential ranged from slightly negative to highly positive values. In line with the changes in mean particle size, significant reductions in the turbidity of colloidal systems were observed as a rise in CPC concentration. A comparison of these findings with those obtained from DLS allowed us to conclude that CPC induce the conversion of oil droplets into oil-swollen micelles. This behavior was also observed upon sample storage, in which the transition from oil-droplets to oil-swollen micelles shown to be dependent on the TTO and CPC concentration (data not shown). In vitro experiments on the antibacterial properties of colloidal systems against the *E. coli* and *S. aureus* revealed that the inhibition of bacterial growth is observed for the same CPC concentration regardless of TTO content. This lack of synergisms between the CPC and TTO indicate that the dispersed phases do not fuse to the negatively charged bacterial cell envelope, even when they exhibit high positive zeta potentials. Instead, the CPC molecules seem to move from colloidal structures towards the bacterial cell envelope, while the TTO molecules remain preferentially solubilized within their hydrophobic cores. Based on these findings, we can state that TTO (up to 0.5% w/w) cannot be used as antibacterial agent in pharmaceutical formulations (microemulsions and emulsions) stabilized with polysorbate 80 and CPC. This suggests that further investigations with TTO containing mouthwashes are needed in order to demonstrate their efficiency against odor-causing bacteria.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. Effect of CPC on the scattering intensity distributions of colloidal systems formed with (A) 0.125% and (B) 0.375% TTO.

Figure S2. Scattering intensity distribution of pure CPC micelles. The larger structures correspond to the presence of impurities or artifacts within the samples.

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