A novel ionic amphiphilic chitosan derivative as stabilizer of nanoemulsions. Improvement of antimicrobial activity of *Cymbopogon citratus* essential oil

Maria Cristina Bonferoni[1,4*], Giuseppina Sandri[1,4], Silvia Rossi[1,4], Donatella Usai[3], Ioannis Liakos[2], Alice Garzoni[1], Maura Fiamma[3] , Stefania Zanetti[3], Athanassia Athanassiou[2], Carla Caramella[1,4], Franca Ferrari[1,4]

[1] Department of Drug Sciences, University of Pavia, Vle Taramelli 12, 27100 Pavia, Italy

[2] Smart Materials Group, Nanophysics Department, Italian Institute of Technology (IIT), Via Morego 30, 16163 Genoa, Italy

[3] Department of Biomedical Sciences, University of Sassari, V.le San Pietro 43/C, 07100, Sassari, Italy

[4.]Center for Health Technology (CHT), University of Pavia, Via A. Ferrata 1, 27100 Pavia, Italy

* Corresponding author:
Maria Cristina Bonferoni
Department of Drug Sciences, University of Pavia
Vle Taramelli 12, 27100 Pavia
E-mail: cbonferoni@unipv.it
Tel +39 0382 987375, FAX +39 0382 422975

Highlights:

Ionic chitosan oleate stabilizes low energy nanoemulsions

Chitosan oleate nanoemulsions show polymer bioactive properties

Nanoemulsion improves essential oil antimicrobial activity

Abstract

Amphiphilic chitosans have been recently proposed to improve delivery of poorly soluble drugs. In the present paper a derivative obtained by ionic interaction between chitosan and oleic acid was for the first time studied to stabilize o/w nanoemulsions of an antimicrobial essential oil, *Cymbopogon citratus* (Lemongrass), in a low energy and mild conditions emulsification process.

The novel combination of spontaneous emulsification process and of chitosan oleate amphiphilic properties resulted in stable dispersions of a few hundred nanometer dimensions. Positive zeta potential confirmed the occurrence of a chitosan shell around the oil droplets, responsible for nanoemulsion stabilization and

for their acquisition of chitosan bioactive properties, such as mucoadhesion behaviour. Cytotoxicity test was performed on four different cell lines (HEp-2, Caco-2, WKD and McCoy cells) showing biocompatibility of the systems. The maintenance and in some cases even a clear improvement of essential oil antimicrobial activity was verified for Lemongrass nanoemulsion on nine bacterial and ten fungal strains, all of clinical relevance.

Keywords: Amphiphilic chitosan; Antimicrobial essential oil; Lemongrass oil; Mucoadhesion; Nanoemulsions; Oleic acid

1. Introduction

In the formulation of o/w emulsions and nanoemulsions, a number of advantages come from the employment of amphiphilic polymers as stabilizers. The hydrophilic portions of the polymers are hydrated in aqueous media associating steric stabilization to interfacial activity. In grafted polymers hydrophobic portions are represented by multiple pendant chains so that the high number of contact points with the oil droplets results in especially strong anchoring at the interfaces [1]. An example of grafted hydrophobically modified (HM) polymer with such successful features is represented by modified inulin, now commercially available as a surfactant agent [2].

HM chitosan derivatives have been studied as emulsion stabilizers in only a few cases [3], although in literature they are widely proposed to prepare polymeric micelles [4 -7]. A further advantage of polymeric surfactants is linked to the occurrence in an aqueous environment of an outer shell represented by macromolecules with potential peculiar biological activities. In this perspective, particular advantages can be associated to the use of derivatives of chitosan, since this polysaccharide is largely described in literature for mucodhesive behavior, absorption enhancement properties, wound healing and peptidase inhibition effects [8-13]. In the case of glycol palmitoyl chitosans, it has been demonstrated that in spite of derivatization, amphiphilic derivatives maintained mucoadhesion [14] and penetration enhancement properties [15].

Most of N-acyl modified chitosans described in literature are covalent conjugates obtained by means of amide linkages between the hydrophobic moiety and amino groups of the polysaccharide backbone [16].

An alternative approach involves the preparation of HM chitosans by electrostatic interaction between the polysaccharide chain, in this case cationic chitosan, and the hydrophobic moiety represented by fatty acids. This kind of reaction has been demonstrated to obtain polymeric micelles suitable to improve the solubility of hydrophobic and poorly soluble drugs. The employment of ionic derivatives (polymer salts) instead of covalent ones presents the advantage of easy preparation and lower regulatory burden with respect to new chemical entities. On the other hand, the ionic derivatives are characterized by relatively low stability

in presence of environmental ions with respect to covalent ones. This limits their employment for systemic delivery following parenteral administration, although they can be suitable for topical administration, for example in ocular pathologies, mucosal applications and skin wounds [17-18].

The goal of the present study was to verify the ability of an amphiphilic chitosan derivative obtained by ionic interaction between chitosan and oleic acid (CsO) to stabilize essential oil O/W emulsion, according to a recently patented technology [19], in a spontaneous emulsification process. This is a low energy method based on addition to the water phase of a solution of oil and surfactant in water miscible solvent under moderate stirring [20]. To our knowledge there is limited experience in literature about the use of polymeric surfactants in the preparation of nanoemulsions with this mechanism. *Cymbopogon citratus* more commonly known as lemongrass (LG) was selected as oil phase. Like other essential oils, lemongrass is characterized by antimicrobial and anti-inflammatory activity [21-25], and it is therefore possible to envisage a topical application to skin or mucosal lesions.

The antibacterical activity of lemongrass can be supported by the same CsO used as a stabilizer of the dispersion, as both chitosan and oleic acid are described as capable of antibacterial activity [26-27]. A covalent amidic derivative of chitosan and oleic acid was moreover studied for antibacterial activity that was maintained despite the derivatization reaction [28].

In the present work two different LG/chitosan oleate (LG:CsO) ratios, and different LG and chitosan concentrations were studied. The antimicrobial and antifungal activity of chitosan oleate salt, and of LG nanoemulsions was assessed on on nine bacterial strains and ten fungal strains, all of clinical relevance. Some of these microorganism are involved in ophthalmic or vaginal infections, where chitosan mucoadhesive properties can improve residence time and therefore efficacy of the formulation.

2. Materials and Methods

2.1 Materials

The following materials were used: Chitosan (CS) was obtained as HCl salt from low molecular weight (LMW) chitosan base, deacetylation degree 80% (Sigma Aldrich, Milan, I), by addition of HCl 0.5 N to chitosan until complete dissolution, dialysis in bidistilled water for 24 h and freeze-drying (HetoDrywinner, Analitica de Mori, Milan, I). Oleic acid was from Fluka (Milan, I), Lemongrass essential oil (LG) from Maitreya Natura (Carpegna, I), D-(+)-trehalose dihydrate was from Sigma Aldrich (Milan, Italy). Acetone, Acetic acid, Sodium acetate, Sodium chloride, were all from Carlo Erba (Milan, I)

2.2 Methods

2.2.1 Preparation of nanoemulsions

Chitosan oleate (CsO) was obtained, as previously described [17, 18] in situ by self assembling during the preparation of the samples. The ratio between chitosan and oleic acid was maintained the same in all cases, corresponding to 1:1 molar ratio taking into account the theoretical free aminogroups of chitosan. Taking into account the deacetylation degree (about 80%), 1.4 mg oleic acid were used per each mg of chitosan. Different systems were prepared by varying the ratio between LG and chitosan oleate (LG:CsO) that was set to 0.5:1 or to 1:1, corresponding to the theoretical loading of 33 and 50% of the oil in the systems. Chitosan (CS) concentration varied between 0.05% and 0.5% as reported in Table 1.

Table 1. Composition of the nanoemulsions based on different concentrations of chitosan (CS) and two different LG:CsO ratios (0.5:1 and 1:1).

	CS 0.05% 0.5:1	**CS 0.05% 1:1**	**CS 0.1% 0.5:1**	**CS 0.1% 1:1**	**CS 0.2% 0.5:1**	**CS 0.2% 1:1**	**CS 0.5% 0.5:1**	**CS 0.5% 1:1**
	Final concentration (mg/ml)							
Lemongrass	0.6	1.2	1.2	2.4	2.4	4.8	6.0	12.0
Chitosan	0.5		1.0		2.0		5.0	
Oleic acid	0.7		1.4		2.8		7.0	

Solutions in acetone of LG and oleic acid were prepared at 10 mg/ml concentration in the case of CS 0.05% (w/v) and 0.1% (w/v), at 20 mg/ml concentration in the case of CS 0.2% (w/v) and 50 mg/ml in the case of CS 0.5% (w/v). Different volumes of these solutions were mixed and therefore added drop by drop, according to spontaneous emulsification [20], to aqueous solutions of chitosan under stirring to obtain the final concentrations as seen in Table 1. Acetone was removed under nitrogen at room temperature. The samples were sonicated 15 minutes (Elmasonic S 80 H, Elma Hans Schmidbauer GmbH & Co, Singen, Germany).

2.2.2 Dimensional characterization of dispersed phase

The particle size and the Polydispersity Index (PI) of the dispersed phase were measured by Photon Correlation Spectroscopy (PCS) (N5 Submicron Particle Size Analyser Beckman Coulter, IL, Milan, Italy). Samples were diluted in filtered bidistilled water and analyzed at 90° detection angle. PI indicates the width of the size distribution ranging between 0 (monodispersity) and 1. Dimensional characterization was performed on samples maintained at 25 °C up to three months.

2.2.3 LG loading characterization

LG in nanoemulsions was evaluated by spectrophotometric analysis at 240 nm wavelength (Perkin Elmer Instrument Lambda 25 UV/VIS Spectrometer, Monza, I) by comparison with a calibration curve of LG. Both

the standards and the samples were diluted (usually 50 µl to 2 ml) in CH_3CN:acetate buffer pH 4.0 in 80:20 ratio. This medium was previously found to be capable of dissolving all the nanoemulsion components. The final LG loading % was calculated using the following ratio:

$$LG\ loading\% = \frac{amount\ of\ LG\ quantified\ in\ nanoemulsion}{amount\ of\ CsO\ in\ the\ system} \times 100$$

2.2.4. Freeze drying

A freeze drying step (Heto Dry-Winner, Analitica De Mori, Milano, Italy) was performed on all the samples after the addition of trehalose (2% final concentration) as a crioprotectant. Dimensional analysis and LG loading were verified after resuspension of the samples in distilled water to the initial volumes by means of 5 minutes in vortex agitation (Maxi-Mixer, Apparecchi scientifici Riccardo Passoni, Pavia, I) and 10 minutes sonication in ice (Elmasonic S 80 H, Elma Hans Schmidbauer GmbH & Co, Singen, Germany)

2.2.5 Morphological analysis of dispersed phase

Morphological analysis of the dispersed phase was performed by means of Atomic Force Microscopy (AFM) in True Non Contact Mode (Park System AFM instrument XE-100). AFM images were obtained in air using an anti-vibration table (TableStable TS-150) and within an acoustic enclosure. For the data acquisition Ultra Super Sharp Silicon cantilevers were used with nominal diameter less than 20 nm and 42 N/m elastic force constant for high sensitivity. The resonance frequency was defined around 272.7 kHz and the scan rate was maintained at 0.2 Hz.

2.2.6 Zeta potential measurements

Zeta potential measurements were performed by means of a Zetasizer Nanoseries (Malavern Instrument) with a zeta DTS1060C cell. Five measurements were performed from 10 to 100 runs.

2.2.7. Ex vivo mucoadhesion test

Mucoadhesion behavior was evaluated on ex-vivo porcine buccal mucosa obtained from a local slaughterhouse. After washing it twice with saline solution, the donor ring of Franz cells (9 mm internal diameter) was clamped over the mucosa to define a constant area. The mucosa samples were put in Petri dishes (35 mm diameter), maintained wet with 1 ml physiological solution and kept at 37 °C in thermostatic chamber 5 minutes before and during the test.

The samples (150 µl) were layered on the mucosa for 1 minute. Washing was then performed once with 350 µl and twice with 500 µl to a final volume of 1 ml.

The amount of sample that adhered to the mucosa was calculated as the difference between the amount of LG initially loaded on the mucosa and the amount found in the washing volume. In all cases the LG concentration was quantified spectrophotometrically by diluting the samples in CH_3CN:acetate buffer pH 4.0 in 80:20 ratio, as previously described in section 2.2.3.

2.2.8. Evaluation of citotoxicity

The cytoxic effect of Lemongrass essential oil, nanoemulsion (sample CS 0.2-LG:CsO 1:1) and unloaded CsO was evaluated on 4 cell lines: HEp-2 (human laryngeal carcinoma), Caco-2 (human colon carcinoma), WKD (human conjunctiva) and McCoy (mouse connective tissue) cells were all from ATCC. Cells were grown in the appropriate media (supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin) and incubated at 37°C and 5% CO_2. 1,5x 10^5/mL cells were seeded in 96-well plates and incubated with serial dilutions (from 16% to 0.0005%) of the samples prepared in culture medium with the addition of Tween 80 (0.5%). Wells were washed twice with PBS and 100 µl of culture medium without serum plus 1/10 MTT solution (3-[4,5- dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide)/PBS were added. After 4 hours incubation, M-8910 MTT solubilization solution - 10% Triton X-100 plus 0,1N HCl in anhydrous isopropanol was added. The quantity of formazan (presumably directly proportional to the number of viable cells) was measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. The percentage of viability was calculated according to the following formula:

$$\% \text{ cell viability} = \frac{absorbance\ \ of\ sample}{absorbance\ of\ control} \times 100$$

Percentage values higher than 50% (60% for WKD cell line), correspond to the absence of cytotoxicity.

2.2.9. Antimicrobial activity

The Minimum Bactericidal Concentration (MBC) of the same samples was evaluated on both Gram-positive and Gram-negative bacteria from ATCC. Escherichia coli and Gardnerella vaginalis strains were from vaginal swabs. The three Pseudomonas aeruginosa were multi-drug resistant strains isolated from eyes with very severe post-operative endophthalmitis in India. The other filamentous fungal strains were isolated from desert sands [29-31]. Samples were diluted in Luria Broth (LB) with 0.5% Tween 80 added at concentrations ranging from 16% to 0.0005% (v/v). The bacterial inoculum was performed at the concentration of 10^6 CFU/mL. An inoculum of 100 µL of microbial culture was added to 100 µL of each concentration of the different samples in 96-well plates and incubated at 37°C for 24 h. Cultures that showed no visible turbidity were sub-cultured on the surface of a Plate Count Agar for colony counting. MBC was considered as the lowest concentration that could inhibit 99% of bacterial growth. Each experiment was performed in duplicate and repeated three times.

2.2.10. In vitro evaluation on Chlamydia trachomatis

McCoy cell monolayers (2.0 × 105 cells/well) grown on glass coverslips in 24-well plates were infected with Chlamydia trachomatis by centrifugation at 1500 rpm at 37°C for 1 h. Chlamydia inoculum was removed and infected cells were incubated with a concentration of 0.004% Lemongrass, prepared in culture medium. After 48 h of incubation at 37°C and 5% CO_2 in cycloheximide-free medium, the coverslips were fixed with methanol and stained with fluorescein isothiocyanate conjugated monoclonal (FITC) antibody against C. trachomatis MOMP (Pathfinder™ Chlamydia Culture Confirmation System, Bio-Rad Laboratories, Inc.). The number of IFUs was enumerated by counting all fields using a fluorescence microscope [32].

2.2.11 Statistical analysis

Statistical evaluations were performed by means of Stat Graphics 5.0, Statistical Graphics Corporation, MD, USA. Differences were determined according to One-way ANOVA as for the results of dimensional stability are concerned. To put in evidence the effects of CS concentration or LG:CsO ratio, statistical differences were evaluated by means of a multifactor ANOVA. In both cases differences were considered significant at $p < 0.05$.

3 Results and discussion

3.1 Dimensional characterization of dispersed phase

Figure 1a illustrates the results of the dimensional characterization as mean volume diameter and Polydispersion Index (PI) for the nanoemulsions based on different chitosan concentrations and for the two LG:CsO ratios. It must be remembered that the increase of chitosan concentration corresponded to a proportional increase in oleic acid and LG concentrations, while LG:CsO ratios were maintained constant. The relevance of LG:CsO ratios can be seen by comparing 0.5:1 and 1:1 formulations. The concentration of chitosan and of the dispersed phase affects the dimensions that increase slowly until CS 0.2% (w/v) remaining below 400 nm. When polymer concentration increases to 0.5% (w/v), the dimensions become close to 1 µm, and the PI increases above 0.5, indicating that the samples are more heterogeneous. No significant differences in dimensions can be attributed to the different LG:CsO ratios, while the following differences were found statistically significant concerning chitosan concentration: 0.05 vs 0.2; 0.05 vs 0.5; 0.1 vs 0.2; 0.1 vs 0.5; 0.2 vs 0.5 (Multifactor ANOVA, Post hoc Fisher's test, $P<0.05$).

As previously observed [17, 18], during removal of acetone chitosan and oleic acid can interact to give HM chitosan nanoparticles with hydrophobic domains typical of a micelle like structure. In the present case, HM chitosan, while forming, thanks to its amphiphilic properties conceivably reorganizes at the surface of

the finely dispersed oil phase, resulting in homogeneous, stable samples. A relevant aspect of spontaneous emulsification method is that the preparation of nanoemulsions do not involve the application of high energy steps, as the addition of oil phase is performed under magnetic stirring. This represents a clear advantage with respect to preparation of nanoemulsions by high energy methods, in which a relevant portion of energy is dissipated as heat [1]. This quite easy occurrence of finely dispersed oil droplets is conceivably due to the diffusion of acetone into aqueous phase, that contributes to lowering interfacial phenomena and to helping the fine subdivision of oil. It is however recognized that the surfactant nature can be critical for this process [20]. In the present case, the spontaneous emulsification method was efficiently associated to the self-assembling properties of chitosan oleate, as oleic acid can be easily added, dissolved in the organic phase, to aqueous phase containing chitosan.

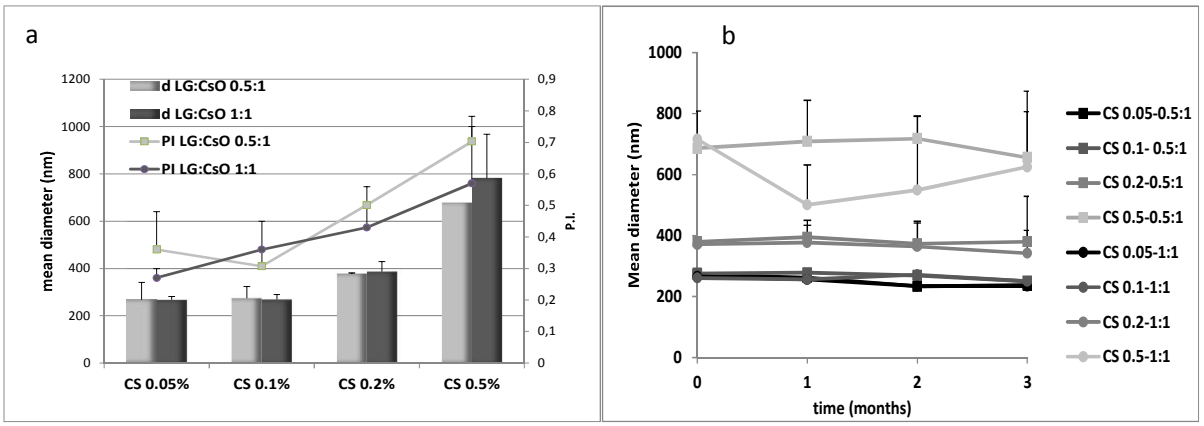

Figure 1. Dimensional characterization of the dispersed phase of nanoemulsions at the two LG:CsO ratios (0.5:1 and 1:1) for the different chitosan (CS) concentrations (mean±sd; n=3). a) Mean diameter (d) and Polydispersion Index (PI). b) Mean diameters of the nanoemulsions at different storage times (mean±sd; n=3)

Figure 1b shows the results of the dimensional characterization after different times of storage at room temperature for all the CS concentrations and the two LG:CsO ratios, in a preliminary evaluation of physical stability. The dimensions of all the less concentrated samples, based on CS 0.05% and 0.1 %, remained at about 300 nm during the entire period of evaluation. The samples based on CS 0.2% and 0.5% show larger dimensions that, however, do not increase with time, although some variability can be observed. This can be explained with the higher dishomogeneity of these samples, as previously evidenced by the increase of PI values with increase of concentrations (Figure 1a). In all cases, the statistical evaluation did not show significant differences for the dimensions of each sample in time (One-way ANOVA, $P<0.05$).

3.2 LG loading and sample freeze drying

Figure 2 illustrates the final concentration of LG in the obtained nanoemulsions. By comparing the concentrations found for the different systems with the theoretical ones in Table 1, it is possible to see that in all cases some decrease with respect to the theoretical values can be observed, more importantly for the systems prepared with higher chitosan concentrations. These are the systems characterized by larger dimensions of dispersed phase and possibly by lower droplet stability. A greater loss of non-encapsulated oil during the preparation steps (especially during removal of acetone) can be envisaged for more concentrated samples. This phenomenon is, of course, reflected by decrease in LG loading. Taking into account the increasing amount of HM chitosan, the system loading can be calculated, and in this case too a decrease could be observed passing from the lowest to the highest concentrations. The loading levels ranged between 50% and 10%. The values obtained are illustrated in Figure 3a, and correspond to the system loading values before freeze drying. Even for the highest CsO concentrations, the LG loading at the preparation (before FD) is in line with literature data [33]. Especially good values obtained with the systems based on the two lowest CS concentrations (0.05 and 0.1%) suggest the possibility to prepare nanoemulsions with quite high LG levels.

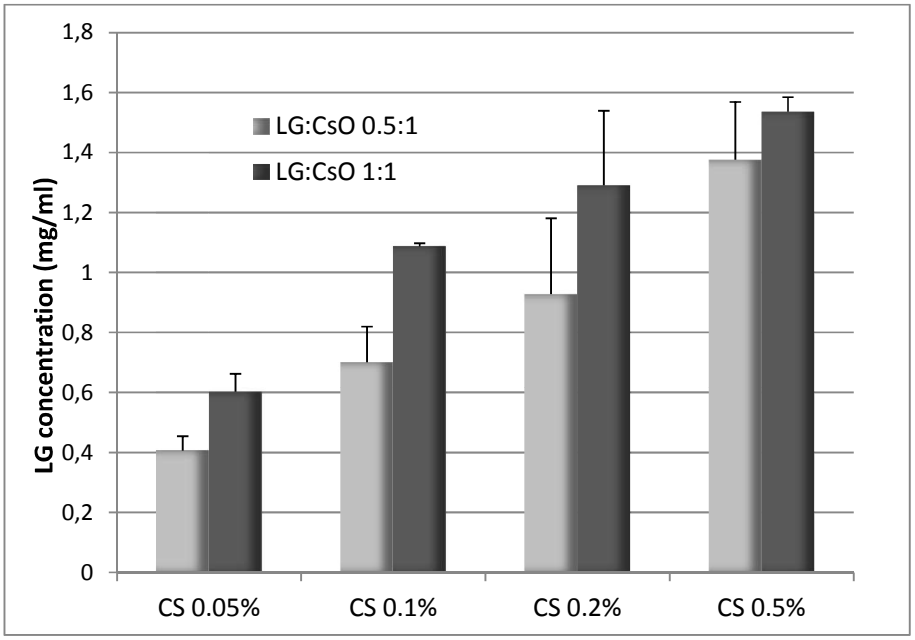

Figure 2. LG concentration in nanoemulsions at the two LG:CsO ratios, for the different chitosan (CS) concentrations (mean±sd; n=3)

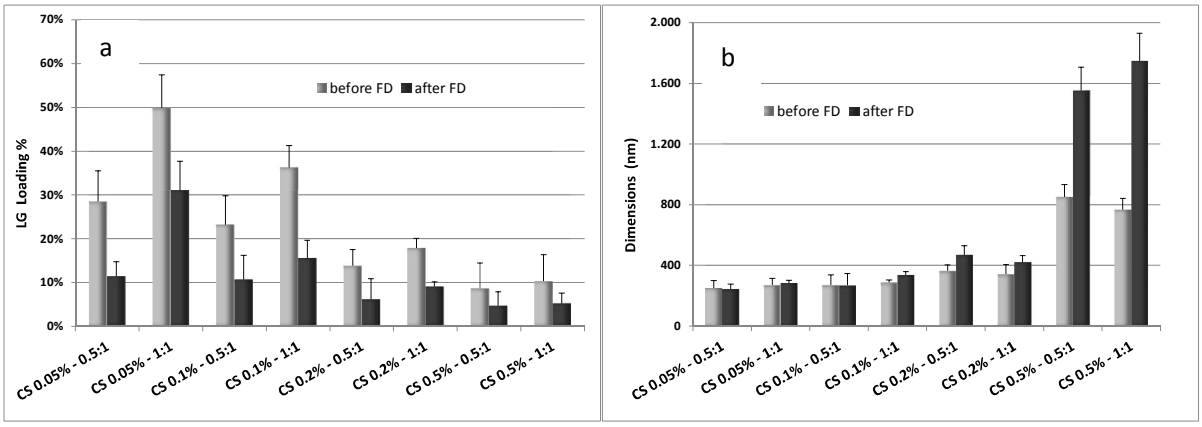

Figure 3. Nanoemulsions before and after freeze drying (FD) at two LG:CsO ratios and different chitosan (CS) concentrations (mean±sd; n=3). a) LG loading %, b) mean diameter of the dispersed phase.

In Figure 3 (a and b) the LG loading and the dimensions before and after freeze drying are compared, respectively. The samples after freeze drying were reconstituted with distilled water to the initial volume. The effect of freeze drying on the dispersed systems was studied to assess the possibility to improve their stabilization and to introduce them in formulations such as powders for skin delivery or eventually for inhalation. As was conceivable for the oil volatility, all the systems were subject to a decrease in the content of oil, which was reduced to about 50% of the initial one. The final loading of CS 0.05% and CS 0.1% systems was maintained above 10%, however, even after freeze drying, which is still in line with comparable systems described in the literature [33]. The comparison of the dimensions before and after freeze drying is given in Figure 4b. An increase in dimensions can be observed only for nanoemulsions based on CS 0.5%, while for all the other systems, and especially the most diluted ones, the resuspension was complete.

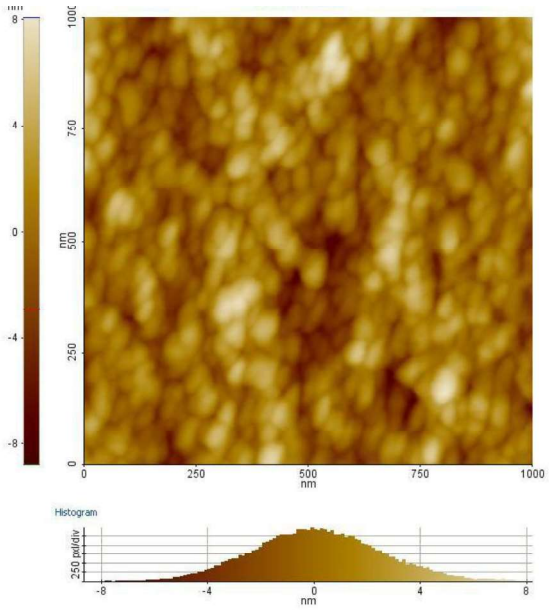

Figure 4. AFM image of the sample CS 0.5-0.5:1

3.3 Morphological analysis of disperse phase of nanoemulsions

Figure 4 shows, as an example, the image of sample CS 0.5 -0.5:1 performed by means of Atomic Force Microscopy. Nanoparticles are characterized by spherical morphology and homogeneous dimensions, indicating the presence of a unique population. Dimensions of single particles appear very small in comparison with the results obtained with PCS, that are probably affected by the presence of some aggregates which could not be completely avoided in spite of the dilution, as already observed with different samples [34].

3.4 Zeta potential

The zeta potential of non-encapsulated lemongrass was negative, with a value of -8.2 (±1.3) mV. Unloaded chitosan oleate gave a dispersion with positive zeta potential values, of 42.6 (±3.0) mV, conceivably due to the self assembling behavior of the amphiphilic ionic chitosan derivative. This, in fact, in water environment gives micelle like structures with the hydrophobic chains to give an inner core and hydrophilic chitosan chains to form an outward shell. As shown in Figure 5a, all the encapsulated LG samples showed positive values increasing from about 40 mV to almost 70 mV with the increase of concentration. It can be supposed that the samples CS 0.05% with lower dimensions and higher loading also have higher surface/volume ratios and in turn relatively lower concentrations of chitosan on their surface.

3.5. Mucoadhesion properties

Figure 5b illustrates the percentages of samples that remained adhered onto the buccal porcine mucosa in ex-vivo mucoadhesion test. In all cases the percentage adhered is lower for the 1:1 ratio. It must be considered, however, that the absolute amount of oil layered on the mucosa increases with the increase of LG:CsO ratio. A decrease in adhered percentage can be observed with the increase of the sample concentration. Also in this case the total absolute amount of layered oil increases with the sample concentration. A statistically significant effect ($P<0.05$) of both concentration and LG:CsO ratio was verified by a multivariate ANOVA. The lower adhered percentage can be explained by taking into account the increase of dimensions observed passing from Cs 0.05% to CS 0.5%. The highest dimensions probably correspond to lower surface/volume ratio and lower surface of chitosan which is responsible for mucoadhesion. The positive interaction with the mucosa obtained in all the samples is in line with the presence in all of a shell of chitosan, as demonstrated by the strongly positive zeta potential values.

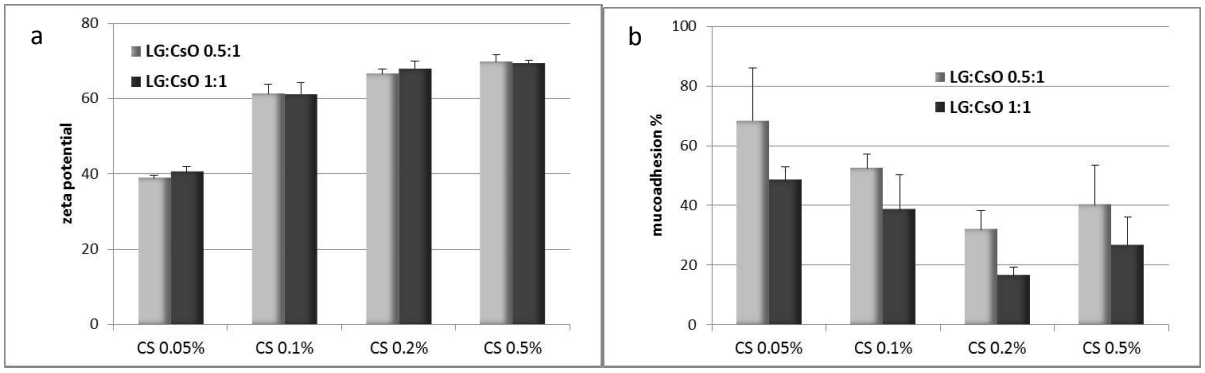

Figure 5. Zeta potential values (a), and mucoadhesion on porcine buccal mucosa (b) for samples at two LG:CsO ratios and different chitosan (CS) concentrations (mean±sd; n=6)

3.6. Cytotoxicity

Table 2 gives the results of the cytotoxicity test for the essential oil (LG) and for the nanoemulsion CS 0.2-LG:CsO 1:1 obtained starting from chitosan 0.2%. The final concentration of LG in this sample was quantified before the test and corresponded to 1.5 mg/ml. As was conceivable, the levels of LG that showed no cytotoxicity on the cell cultures were quite low, in line with the low dosages therapeutically used for essential oils. Chitosan oleate corresponding to CS 0.2%, with a final concentration of 4.8 mg/ml according to Table 1, resulted as non toxic in Caco-2 and in WKD cells, while in HEp cells only relatively diluted samples can be used. McCoy cell line resulted especially sensitive to the CsO blank. The nanoemulsion sample CS 0.2% LG:CsO 1:1 contains CsO with the same concentration of the CsO blank and a concentration of LG much lower than that tested in the case of the essential oil as such: this explains why the toxicity results are intermediate between LG and CsO for Caco-2, and the low toxicity of loaded samples

for HEp-2 and WKD. The lowest toxicity observed in McCoy cells for the loaded samples, with respect to the blanks and the pure LG, deserves further studies to be explained. It can be supposed that in the case of unloaded CsO, the oleic acid moieties can be especially critical for the cells. In the case of loaded samples, since the oleic acid anchored at the LG interface is being released more slowly during time, it is probably less available for cell toxicity. The "in vitro" effect of Lemongrass essential oil on the Chlamydia trachomatis reference strain ATCC® -VR88 was also investigated by adding a concentration of Lemongrass 0.004% to the medium. No further studies were performed because no differences in the infectivity were observed when compared to uninfected control cells [32].

Table 2: Cytotoxicity assay results on different cell lines (serial dilutions from 16% to 0.0005%).

	HEp-2 50% cell viability	Caco-2 50% cell viability	WKD 60% cell viability	McCoy 50% cell viability
CsO (blank)	1	16	16	0.008
CS 0.2% LG:CsO 1:1	1	4	16	0.5
Lemongrass (LG)	0.008	0.004	0.06	0.008

3.7. Antimicrobial activity

Table 3 gives the results of Mean Bactericidal Concentrations (MBC) for Lemongrass (LG), unloaded CsO and CS 0.2% - 1:1 loaded system. Pure Lemongrass (LG) was active against most Gram + and Gram- and fungi. For the unloaded CsO, in the case of fungi no activity at the tested concentrations could be observed, while CsO showed some activity, according to literature data [26, 27] towards *E. coli* and *S. aureus*. This result is in line with data reported in literature for amide derivatives of chitosan and oleic acid [28].

The nanoemulsion showed a good effect on almost all the tested strains, and especially on fungi, that being largely diffused in the environment can give severe ophthalmic, cutaneous and pulmonary affections [30, 31]. Negative results were obtained only with one strain of *Aspergillus* and the three strains of *P. aeruginosa.* It must be noted that towards the three *P. aeruginosa*, that were multi-drug resistant strains, even LG was relatively less active than towards other microorganisms. In all the other cases, MBC values for nanoemulsion were comparable or only a few times higher than those of the pure LG oil, while it must be remembered that in nanoemulsion LG was present at almost one thousand lower amount (1.5 mg/ml). Therefore, taking into account its LG concentration, nanoemulsion appears much more active than the pure essential oil. This strong activity could be explained with the dispersion in the nanometric scale, that results in better contact of the samples on microorganisms. In the case of bacterial strains, moreover, some synergic effect between CsO and LG can be envisaged, as supported by literature [26-28]. The advantage of the administration of LG as nanoemulsion appears evident by comparing the MBC values (Table 3) and the cytotoxicity results (Table 2). Pure LG cannot be used on the tested cell lines at concentrations as high as those required by its antimicrobial activity (MBC) without a cytotoxic effect. The nanoemulsion

formulations results instead biocompatible on all the cell lines at concentrations comparable with most of MBC values, with clear improvement of efficacy to safety balance.

Table 3. MBC values (serial dilutions from 16% to 0.0005%)of lemongrass (LG), nanoemulsion CS 0.2%-1:1 containing LG at 1.5 mg/ml concentration, and blank CsO corresponding to CS 0.2%.

	CsO	CS 0.2%-1:1 (LG 1.5 mg/ml)	LG
E. coli ATCC 25922	0.5	0.5	‹0.12
E. coli ATCC 35218	1	1	0.5
E. coli (vaginal swab)	2	4	2
Gardnerella vaginalis	›16	4	1
S. aureus ATCC 29213	16	1	0.5
S. aureus ATCC 43300	1	0.5	0.25
P. aeruginosa	›16	›16	2
P.aeruginosa	›16	16	4
P.aeruginosa	›16	›16	1
Aspergillus spp.	›16	›16	‹0.12
Penicillium glabrum	›16	‹0.12	‹0.12
Botrytis cinerea	›16	1	0.5
Aspergillus niger	›16	2	0.25
Ceriporia spp.	›16	‹0.12	‹0.12
Fusarium	›16	2	0.25
Aspergillus spp.	›16	0.25	‹0.12
Aspergillus spp.	›16	0.25	‹0.12
Aspergillus spp.	›16	0.25	1
Aspergillus tubigiensis	›16	‹0.12	‹0.12

4. Conclusions

A nanometric dispersion of lemongrass essential oil in water was easily obtained, without high energy homogenization, thanks to the self-assembling properties of ionic chitosan and oleic acid derivative during spontaneous emulsification process. This result confirms moreover the suitability of the amphiphilic chitosan derivative as a stabilizer of the nanoemulsion. This derivative is obtained, at the same moment of

the oil in water dispersion occurrence, by ionic interaction between the cationic polysaccharide and oleic acid, and it is therefore likely to present lower regulatory concerns than covalent acyl modified chitosans, more largely described in literature. The dimensions of the dispersed phase depended on the concentration of both polymer and hydrophobic phase (oleic acid and essential oil). They were maintained without significant variations during the three months of the preliminary stability study. Chitosan stabilization is likely to be due to the amphiphilic character of the derivative, but also to steric protection towards coalescence. The presence of a shell of chitosan chains outside the droplets was confirmed by zeta potential measurements, and gives the droplets the same properties that characterize the polymer, such as mucoadhesion, as confirmed in the present study.

The nanoemulsions resulted biocompatible with different epithelial human cell lines at concentrations compatible with the antimicrobial activity.

The antimicrobial properties of Lemongrass essential oil were not only maintained but even enhanced in the encapsulated samples, clearly improving the risk to benefit ratio. This result is probably due both to the fine dispersion and stability of the nanoemulsion, and to the synergic antimicrobial effect of chitosan and of oleic acid.

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