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Development of pH-responsive biopolymer-silica composites loaded with *Larrea divaricata* Cav. extract with antioxidant activity

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ABSTRACT

A detailed study of biomaterials is mandatory to comprehend their feasible biomedical applications in terms of drug delivery and tissue regeneration. Particularly, mucoadhesive biopolymers such as chitosan (chi) and carboxymethylcellulose (CMC) have become interesting biomaterials regards to their biocompatibility and non-toxicity for oral mucosal drug delivery. In this work, pH-responsive biopolymer-silica composites (Chi-SiO_2, Chi-CMC-SiO_2) were developed. These two types of composites presented a different swelling behavior due to the environmental pH. Moreover, the nanocomposites were loaded with aqueous *Larrea divaricata* Cav. extract (Ld), a South American plant which presents antioxidant properties suitable for the treatment of gingivoperiodontal diseases. Chi-CMC-SiO_2 composites showed the highest incorporation and reached the 100% of extract release in almost 4 days while they preserved their antioxidant properties. In this study, thermal and swelling behavior were pointed out to show the distinct water-composite interaction and therefore to evaluate their mucoadhesivity. Furthermore, a cytotoxicity test with 3T3 fibroblasts was assessed, showing that in both composites the addition of *Larrea divaricata* Cav. extract increased fibroblast proliferation. Lastly, preliminary *in vitro* studies were performed with simulated body fluids. Indeed, SEM-EDS analysis indicated that only chi-SiO_2 composite may provide an environment for possible biomineralization while the addition of CMC to the composites discouraged calcium accumulation. In conclusion, the development of bioactive composites could promote the regeneration of periodontal tissue damaged throughout periodontal disease and the presence of silica nanoparticles could provide an environment for biomineralization.

1. Introduction

Stimuli-responsive materials have an emerging importance in nanotechnology and medicine [1,2]. Temperature, pH and ionic strength are some of the variables in which materials can respond differently and therefore they are able to modulate their behavior. Indeed, external stimuli may modify material properties, structure, interactions and dimensions. The pH-responsive materials are a subgroup of stimuli-responsive materials in which structure and material properties change according to the environmental pH. The presence of acidic and basic groups in this type of biomaterials can influence the degree of swelling and drug delivery rates [3].

In the drug delivery field, stimuli-responsive materials can be used for the controlled release of bioactives [4]. The pH-responsiveness has a special interest due to the fact that the pH in the human body varies between many target tissues and in some pathological conditions such as periodontal disease [5], cancer [6,7] and infections [8].

Today, it is important to develop new drug delivery systems that not only play their role as carriers, but can also have other functions, such as regenerating tissues [9–11]. In this sense, silica has gained great interest in the past few years [12,13]. Silica is a bioactive ceramic material, which can promote bone formation [14]. SiO_2 is a documented differentiation promoter and its incorporation to delivery systems improved osteoblast adhe-

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sion, differentiation, proliferation and mineralization [15]. Silanol groups from silica could provide a suitable environment for biomineralization. Indeed, the supersaturation of Ca^{++} near a negative surface could trigger the nucleation of hydroxyapatite [16].

Periodontal disease is caused by periodontopathogenic bacteria [17] which triggers an inflammatory response that eventually leads to periodontal tissue destruction. Initially, gingivitis takes place, the early stage of periodontal disease, which consists in the inflammation of the gingiva. Later, the chronic stage appears, where not only inflammation is present, but also alveolar bone and periodontal ligament destruction are observed [18]. In this context, silica can play a key role in bone periodontal regeneration.

Additionally, mucoadhesive biopolymers [19] such as chitosan (Chi) [20] and carboxymethylcellulose (CMC) are considered good candidates for oral local delivery [21]. Chitosan is a marine polysaccharide composed by glucosamine and N-acetyl-glucosamine and carboxymethylcellulose is a chemically modified biopolymer, derived from cellulose. Although both biopolymers have different structures and properties they present a suitable mucoadhesive profile for oral local drug delivery. CMC is an anionic polymer with outstanding mucoadhesive properties due to the formation of strong hydrogen bonds with the mucin layer. While Chi is a cationic biopolymer which can bind to the mucosal epithelium via ionic bonds regards to its amino groups and the sialic acid residues of the mucosa [22].

Periodontal disease can be locally treated with antioxidants, antimicrobials and anti-inflammatory drugs. In this sense, natural products like the extracts obtained from a South American plant called *Larrea divaricata* Cav. emerged as good candidates mainly due to their antioxidant and anti-inflammatory properties [23–26]. Its aqueous extract has promising applications for the therapy of gingivoperiodontal diseases that present marked inflammation and periodontal tissue damage. An interesting feature of plant extracts is that their therapeutic effect can be caused by: i) a single active ingredient, ii) a particular phytochemical group or iii) the synergistic action among various compounds. Particularly, one of the main compounds present in *Larrea divaricata* Cav. leaves is nor-dihydroguayaretic acid (NDGA). Evidently, the use of antioxidants such as *Larrea divaricata* Cav. could treat the harmful effects of chronic inflammation, improving the signs and symptoms of patients with gingivoperiodontal diseases.

Herein, pH-responsive composites obtained from Chi-CMC-silica nanoparticles loaded with aqueous *Larrea divaricata* Cav. extract are presented. The composites could have a differential mucoadhesive profile throughout periodontal disease which may be convenient as it is known that saliva from patients with gingivitis present a higher pH than saliva from patients with periodontitis. Additionally, plant extract incorporation in two types of composites was analysed by HPLC as well as the release profile are presented highlighting the potentialities of the composite which showed the highest incorporation. Finally, cytotoxicity studies with fibroblasts and *in vitro* biomineralization analysis further confirm the potential application of these composites in alveolar bone regeneration.

2. Materials and methods

2.1. Materials

Low-viscosity chitosan obtained from crab shells, structural viscosity 66 mPas, deacetylation >75.0%, lysozyme from chicken egg white (100,000 U/mg), thiazolyl blue tetrazolium bromide reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl) and epinephrine were purchased from Sigma–Aldrich (St Louis, USA). Carboxymethylcellulose sodium of medium viscosity with a degree of substitution of 0.65–0.90 was obtained from Noviant, Netherlands. Colloidal silica (aerosil 200) of SiO_2 content >99.8% and specific surface 200 $m^2\ g^{-1}$ was provided by Evonik Resource Efficiency GmbH, Germany. Dulbeccoís modified Eagleís medium, foetal bovine serum; penicillin and streptomycin were purchased from Gibco. All other reagents were of analytical grade.

2.2. Plant material and extract

Leaves of *Larrea divaricata* Cav. were collected in the province of Cordoba, Argentina and identified using morphological, anatomical and histochemical analysis. A voucher specimen was deposited in the Museum of Pharmacobotany, School of Pharmacy and Biochemistry, University of Buenos Aires. The aqueous extract of the leaves was prepared as follows: the air-dried leaves were extracted for 10 min with boiling distilled water, then the extract was filtered and lyophilized. The aqueous extract was aliquoted and stored at −20 °C until used.

2.3. Composites synthesis

A solution of 20 mg mL^{-1} of chitosan in acetic acid 1% was prepared. Afterwards, 1 mL of chitosan solution was placed in each well of a 24 wells plate and neutralized with 1 N NaOH solution. The hydrogels obtained were washed with ${}_dH_2O$ until neutral pH was reached. Later, hydrogels were incubated for 24 h with 1 mL of two ethanol-water dispersions (10:90): the first one was composed of glycerol 350 mg mL^{-1} and 14 mg mL^{-1} SiO_2 while the second one contained 350 mg mL^{-1} glycerol, 14 mg mL^{-1} SiO_2 and 15 mg mL^{-1} CMC. For composites loaded with *Larrea divaricata* Cav. extract, the lyophilized plant extract was added in a concentration of 6.6 mg mL^{-1} to both ethanol-water dispersions previously described. Afterwards, the dispersions were removed and the composites were washed with deionized water.

2.4. SEM ultrastructural characterization

Chitosan hydrogels were analysed by scanning electron microscopy (SEM). Samples were fixed with a solution of glutaraldehyde (10% v/v) for 1 h at 4 °C. Following fixation, samples were washed three times with deionized water and frozen at −80 °C. Finally, the samples were freeze-dried and gold sputter-coated for analysis using a Zeiss SUPRA 40 microscope.

2.5. FTIR characterization

Fourier transform infrared (FTIR) spectra from freeze-dried composites were performed with an FTIR-Raman Thermo Scientific Nicolet model 50 computer IS, that is coupled with an attenuated total reflection device (ATR). Scanning range was 4000–500 cm^{-1}. Spectra were processed by Thermo Nicolet OMNIC software.

2.6. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements were carried out on a Shimadzu DSC-50 instrument. Temperature was calibrated with In (157 °C, 3.3 J mol^{-1}). Approximately 20 mg of the freeze-dried composites and chitosan hydrogels were first equilibrated at 25 °C and then heated from 25 to 240 °C at a constant rate of 10 °C min^{-1}, under a nitrogen flow of 50 mL min^{-1}.

2.7. Rheological measurements

Chitosan hydrogels and biopolymer-silica composites rheology was analysed. Amplitude sweeps were carried out first to determine the linear viscoelastic range (LVR). The elastic or storage

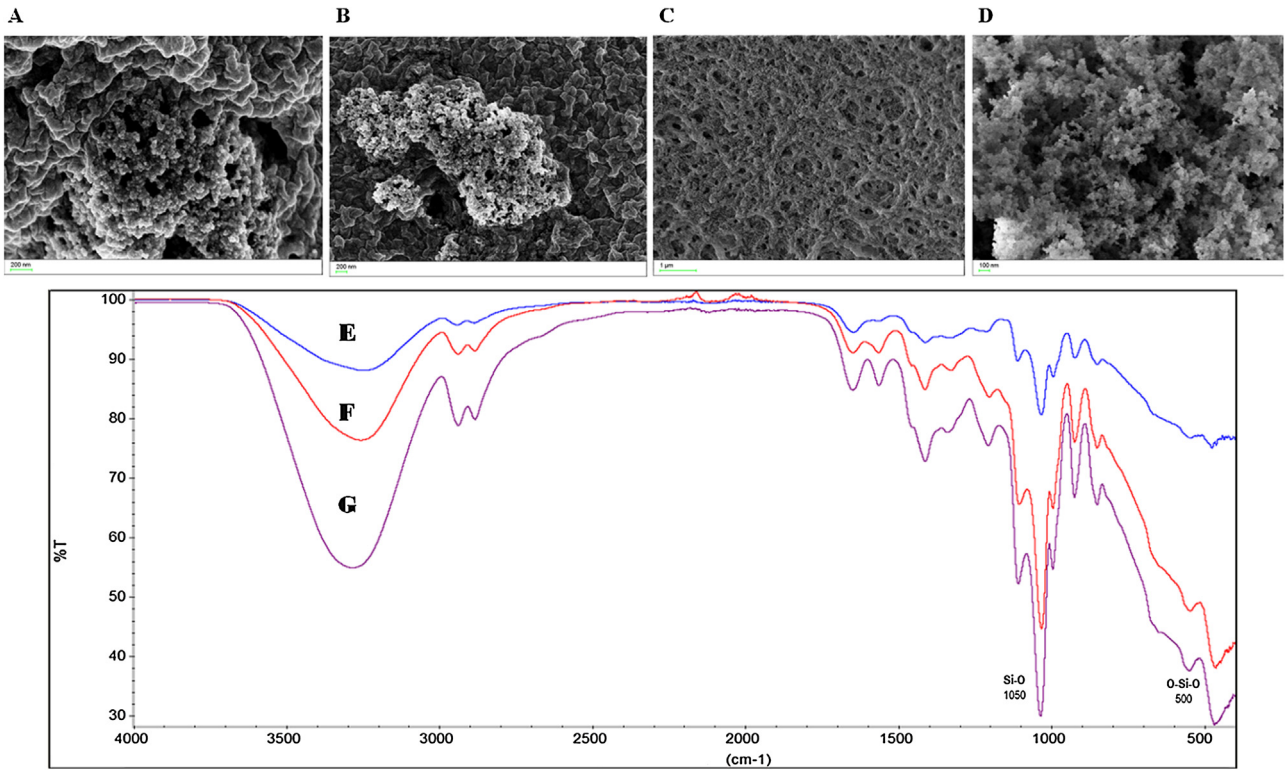

Fig. 1. Representative scanning electron microscopy images of (A) chi-SiO_2 composite (B) chi-CMC-SiO_2 composite (C) chi hydrogel (D) SiO_2 nanoparticles. Representative Fourier transform infrared spectra of (E) hybrid chi-CMC (F) chi-CMC-SiO_2 composite (G) chi-SiO_2 composite.

modulus, G′, the viscous or loss modulus, G″ and complex viscosity (η^*) of the studied materials were obtained in small-amplitude oscillatory shear flow experiments using a rotational rheometer from Anton Paar (MCR-301) provided with a CTD 600 thermo chamber. The tests were performed using parallel plates of 25 mm diameter, a strain of $\gamma = 1.0\%$ and a frequency range of 0.1–500 s^{-1}. Measurements were performed at room temperature (20 °C). All tests were carried out using small strains to ensure the linearity of the dynamic responses. All runs were repeated using different samples. The gap width used was 1.8–2 mm.

2.8. Swelling studies

Composites and chitosan hydrogels were freeze-dried, weighted (W_o) and stored in buffer pH 6.5 or 7.5 to allow water uptake. Hydrogels and composites were removed from the buffer pH 6.5 or 7.5 and weighted (W) at different time points (t) after removing the excess of water. The degree of swelling was calculated by the next equation:

$$W\% = [(W - W_o)/W] \times 100$$

Afterwards, the water content at equilibrium (W_∞) and the kinetic rate constants were calculated according to the following equation:

$$t/W = 1/(KW^2{}_\infty) + t/W_\infty$$

2.9. Composites biodegradability

Chitosan hydrogels and composites were freeze-dried and weighted (W_o). Afterwards, they were immersed in PBS (pH 7.4) with 1 mg mL^{-1} of lysozyme (100,000 U mg^{-1}) at 37 °C. After 7 days, the hydrogels and composites were washed, lyophilized and weighted (W_{7days}). The percentage of weight was assessed to evaluate chitosan hydrogels and composites biodegradation according to following equation:

$$W\% = (W_{7days}/Wo) \times 100$$

2.10. Larrea divaricata *Cav. incorporation and release*

After characterization of the composites, the incorporation of *Larrea divaricata* Cav. aqueous extract into the silica nanoparticle-mucoadhesive polymer composites was analysed by a high performance liquid chromatography (HPLC) technique. The HPLC analysis was performed in a Varian Pro Star instrument equipped with a Rheodyne injection valve of 20 μL and photodiode array detector set at 280 nm. A reversed-phase column C18 Shodex 150 × 4.6 mm (5 μ dp) was used. As the mobile phase, a two-gradient solvent system was employed. The system was composed by solvent A, water and acetic acid (98: 2) and solvent B, methanol and acetic acid (98: 2) according to the following gradient: 30% B at 100% B in 30 min. The chromatographic procedure was performed at room temperature and the flow rate was set at 1 mL min^{-1}.

The release study of the composites loaded with *Larrea divaricata* Cav. extract was performed adding 1 mL of buffer phosphate pH 6.5 in each composite to perform the release assay. The release medium was removed at different time points from the composites and replenish with fresh media. The method employed to evaluate the concentration of the plant extract in the solutions was the technique of HPLC described above.

2.11. Antioxidant activity

2.11.1. DPPH assay

The scavenging activity on the stable free radical DPPH was assayed by the modified Blois' method in which the bleaching rate of DPPH is monitored at a characteristic wavelength in presence

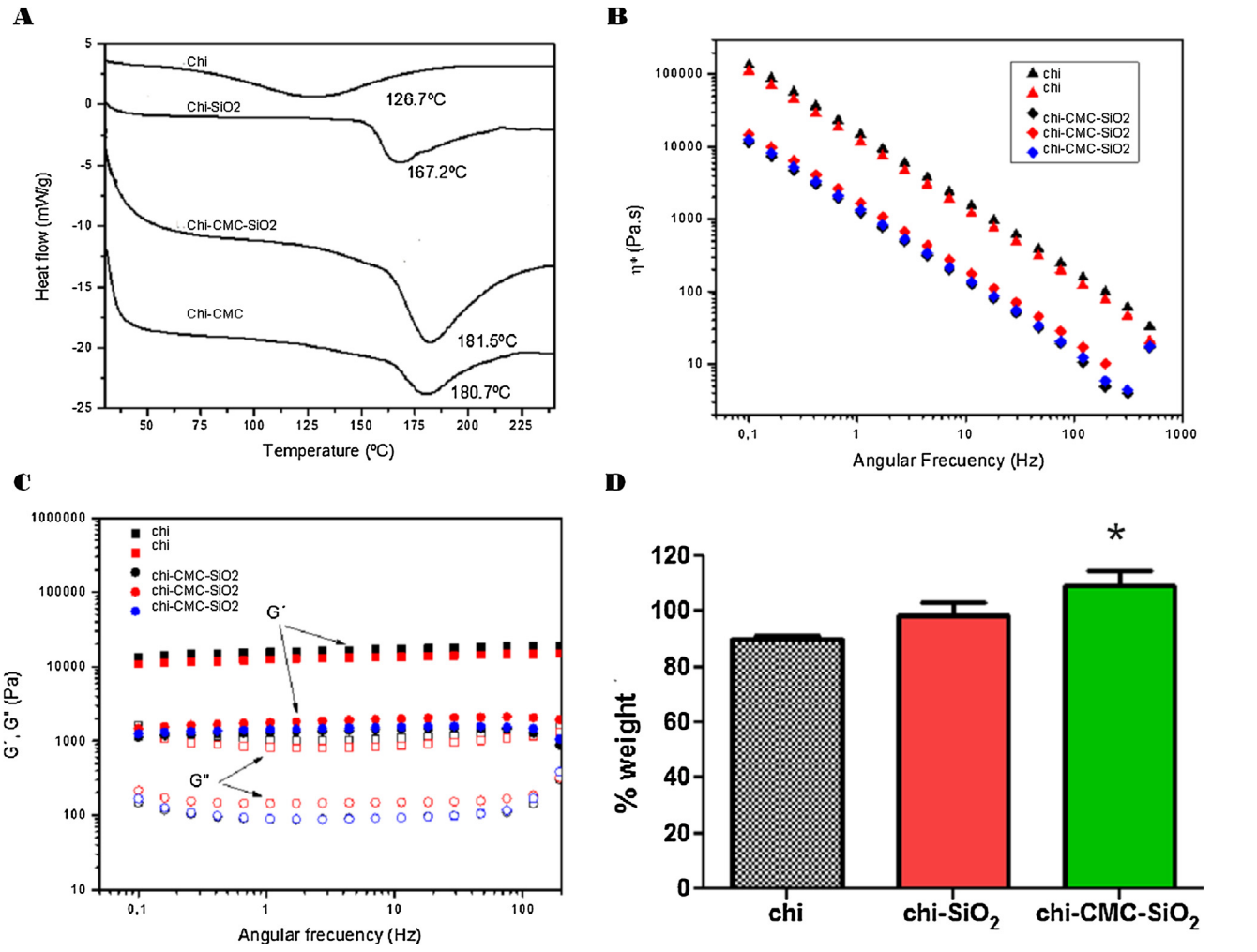

Fig. 2. (A) Differential scanning calorimetry thermograms at a heating rate of 10 °C min^{-1} under a nitrogen flow of 50 mL min^{-1} chi hydrogel, chi-SiO_2 composite, chi-CMC-SiO_2 composite and chi-CMC hybrid (B) Complex viscosity of chitosan hydrogel and chi-CMC-SiO_2 composite against frequency (C) Viscoelastic response of chitosan hydrogel and chi-CMC-SiO_2 composite against frequency (G′ and G″) (D) Biodegradation of chitosan hydrogels and composites chi-CMC-SiO_2 and chi-SiO_2 in a period of 7 days with 1 mg mL^{-1} of lysozyme in phosphate-buffered saline. The differences were analysed using one-way ANOVA, followed by Bonferroni multiple comparisons test, $p < 0.05$ was considered significant.

of the sample [27]. The assay was performed as follows: 100 μL of *Larrea divaricata* Cav. extract removed from the biopolymer-silica composites were mixed with 400 μL of Tris buffer pH = 7.4 and 500 μL DPPH 100 μM. Absorbance was measured at 517 nm. DPPH inhibition was calculated by the equation:

$$\%\text{inhibition} = [1 - (A_{\text{sample}}/A_{\text{DPPHsolution}})] \times 100$$

where A is the absorbance measured.

2.11.2. Simil superoxide dismutase activity

The simil superoxide dismutase activity was determined by the capacity to inhibit epinephrine auto-oxidation, to adrenochrome, in presence of atmosphere oxygen [28]. For this purpose, 50 μL of *Larrea divaricata* Cav. extract removed from the composites, 910 μL phosphate buffer pH = 10.7 and 40 μL 2 mM epinephrine were mixed. The resulting absorbance was measured at 480 nm every 10 s for 5 min Δabsorbance/min was calculated. The antioxidant activity of samples was evaluated as the% of epinephrine auto-oxidation inhibition by the following equation:

$$\%\text{inhibition} = [(\Delta\text{Abs}/\text{mi}_{\text{epinephrine}} - \Delta\text{Abs}/\text{min}_{\text{sample}})/\Delta\text{Abs}/\text{min}_{\text{epinephrine}}] \times 100$$

2.12. In vitro *bioactivity*

Preliminary *in vitro* biomineralization studies were performed in the biopolymer silica composites. In this sense, we evaluated hydroxyapatite formation on the surface of the composites in simulated body fluid (SBF) which has nearly the same concentration of ions as plasma (Na^+ 142 mM; K^+ 5.0 mM; Mg^{2+} 1.5 mM; Ca^{2+} 2.5 mM; Cl^- 147.8 mM; HCO_3^- 4.2 mM; HPO_4^{2-} 1.0 mM; SO_4^{2-} 0.5 mM, pH 7.40). The preparation of SBF was carried out as detailed in *Kokubo* et al. [29]. After soaking the composites in SBF prepared for 3, 7, 14 days they were washed, fixed in a glutaraldehyde solution (10% v/v) for 1 h at 4 °C and analysed by SEM-EDS (Scanning Electron Microscopy with Energy Dispersive Spectroscopy)

2.13. Cell culture and cytotoxicity test

[3T3] mouse fibroblast cells were grown in adherent culture flasks with low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin–streptomycin. Cells were kept at 37 °C in a humidified 5% carbon dioxide chamber until confluence was reached. Harvesting was done with a trypsin–EDTA solution. Cells were stained with trypan blue and counted with a Neubauer cam-

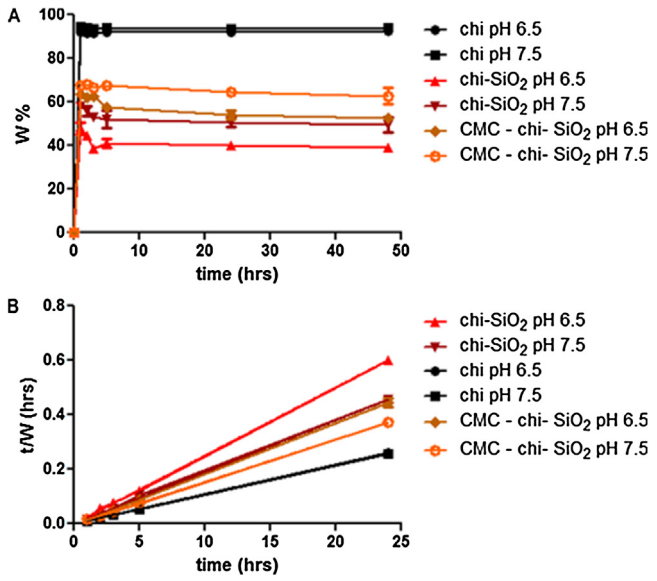

Fig. 3. (A) Swelling profile of chitosan (chi); composite chitosan-SiO_2 (chi-SiO_2); composite carboxymethylcellulose-chitosan-SiO_2 (chi-CMC-SiO_2) at pH 6.5 and 7.5 (B) Biopolymer-silica composites and chitosan hydrogels experimental data of water content and time plotted according to a second order kinetic.

era. Fibroblast cells (4.0×10^4) were seeded in each well followed by the addition of 1 mL complete low glucose DMEM medium. After 48 h of incubation, both types of composites were added to 24-well plate on top of the fibroblast layer and cell metabolic activity was measured 24 h later using the MTT assay. The medium was first removed and replaced by 0.5 mL of a 0.5 mg mL^{-1} MTT solution in fresh media. Samples were incubated in a humidified 5% carbon dioxide chamber for 4 h. Subsequently, MTT solutions were removed, 1 mL of absolute ethanol was added and the mixtures were incubated for 30 min at room temperature. The absorbance was recorded at 570 nm in a UV–visible spectrophotometer (Cecil CE 3021, Cambridge, England).

2.14. Statistical analysis

Data are represented as means ± SD of at least triplicate experiments. The differences were analysed by ANOVA (analysis of variance), followed by a Bonferroni multiple comparison test or a Student's *t*-test. Statistical evaluation $p < 0.05$ or $p < 0.01$ was considered significant.

3. Results and discussion

3.1. Composites synthesis and characterization

The structure of the hydrogels was analysed by SEM. Indeed, SEM images indicate that the hydrogels consist of a highly porous structure formed by homogeneous chitosan networks. Moreover, SEM micrographs showed that the composites of a) chi-SiO_2 composite, b) chi-CMC-SiO_2 composite and c) chi hydrogel have a porous and uniform structure indicating that the addition of silica nanoparticles to the acid chitosan solution did not modify the hydrogel formation after neutralization. (Fig. 1**a**–**c**). In addition, silica nanoparticles of a size of 10 nm were clearly visible. (Fig. 1**d**)

The FTIR analysis reveals the energies of possible stretching and vibrational deformations within the composites. The assignment of these specific vibrations to specific chemical groups allows the identification of chemical components of the sample giving a better understanding of these new materials. Therefore, infrared absorption analysis revealed the characteristic vibrational bands of the chitosan (Fig. 1**e**). The spectrum shows a band at 1660–1620 cm^{-1} corresponding to the amide I band. This band is composed of two broad peaks at 1655 cm^{-1} and 1625 cm^{-1} (C=O and C—N stretching, respectively). Amide II band (1540 cm^{-1}, N—H stretching) is detectable together with amide III band (1390 cm^{-1}). In addition, as it was expected, silicon oxide band were detected in nanocomposites. Indeed, the bands at 500 cm^{-1} (O—Si—O) and another one at 1050 cm^{-1} (Si—O) in both Chi-CMC-SiO_2 and Chi-SiO_2 composites indicating the presence of colloidal silica were observed (Fig. 1**f** and **g**).

Additionally, a thermal analysis of the composites was carried out (Fig. 2**a**). When analysing the thermograms obtained by the DSC technique, there were some differences in the area and position of the endothermic peak caused by the loss of water from the composites. These findings indicate that composites differ in the strength of the water-composite interaction and, therefore, in their water retention capacity. In the case chi-SiO_2, a shift of 40 °C was observed by the addition of glycerol and silica nanoparticles. On the other hand, in the case chi-CMC-SiO_2, where the polysaccharide sodium carboxymethylcellulose was added to the composite, the shift was even greater (60 °C). Clearly, the addition of hydrophilic silica nanoparticles, glycerol and sodium carboxymethylcellulose increases the water retention capacity of chitosan.

In general, the magnitude of the G' and G" values versus frequency (within a frequency range) and the ratio of G' to G" can be used to estimate the resistance of hydrogels. In principle, rheologically strong hydrogels are associated with higher values of G ', and in turn these values of G' should be one or two orders greater than G ". Considering this, it can be said that the chi-CMC-SiO_2 composites are predominantly elastic, because the storage modulus G′ values (*c.a.* 1000 Pa) were always greater than the loss modulus (*c.a.* 100 Pa) G″ values with a probable good swelling profile, but not as resistant as chitosan hydrogels. (Fig. 2**b**,**c**)

A biodegradability test was also performed to the composites. Saliva is a natural fluid which contains electrolytes and enzymes like lysozyme [30], which is capable to degrade chitosan because it can hydrolyze β-1,4 bonds between the monomers *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine. Chitosan hydrogels and composites were exposed to lysozyme to evaluate biodegradation. Composites chi-CMC-SiO_2 showed no degradation but composites chi-SiO_2 and chitosan hydrogels presented *c.a.* 10% of biodegradation ($p < 0.05$) (Fig. 2**d**). Evidently, the biodegradability was modified by the addition of CMC which probably influences lysozyme hydrolytic degradation performance.

In parallel, we performed the swelling profile of the composites and chitosan hydrogels at two different pHs (Fig. 3**a**,**b**). The swelling test evaluates the affinity for water of the material, and therefore estimates their mucoadhesivity properties. Although in every case the swelling profile followed a second order kinetics and swelling ratio reached the equilibrium in almost one hour, the two types of composites presented a different swelling behavior due to the environmental pH. Chitosan polymer is probably ionized at pH 6.5, encouraging the formation of ion pars with carboxylic acid groups and silanol groups which can modify the composites affinity to water. In the case of chitosan hydrogels, despite the affinity for water was greater, no influence for the pH was observed. In the case of both composites a higher amount of water was absorbed at a pH of 7.5. The water content at equilibrium (W_∞) calculated for the composites and chitosan hydrogels are detailed in Table 1 (Table 1). Chitosan hydrogels presented *c.a.* 93% of water content while the water content at equilibrium of the composites Chi-CMC-SiO_2 and Chi-SiO_2, at a pH 7.5 were *c.a.* 64% and *c.a.* 52% respectively. These results were almost 10% higher than those observed at pH 6.5 for the same composites.

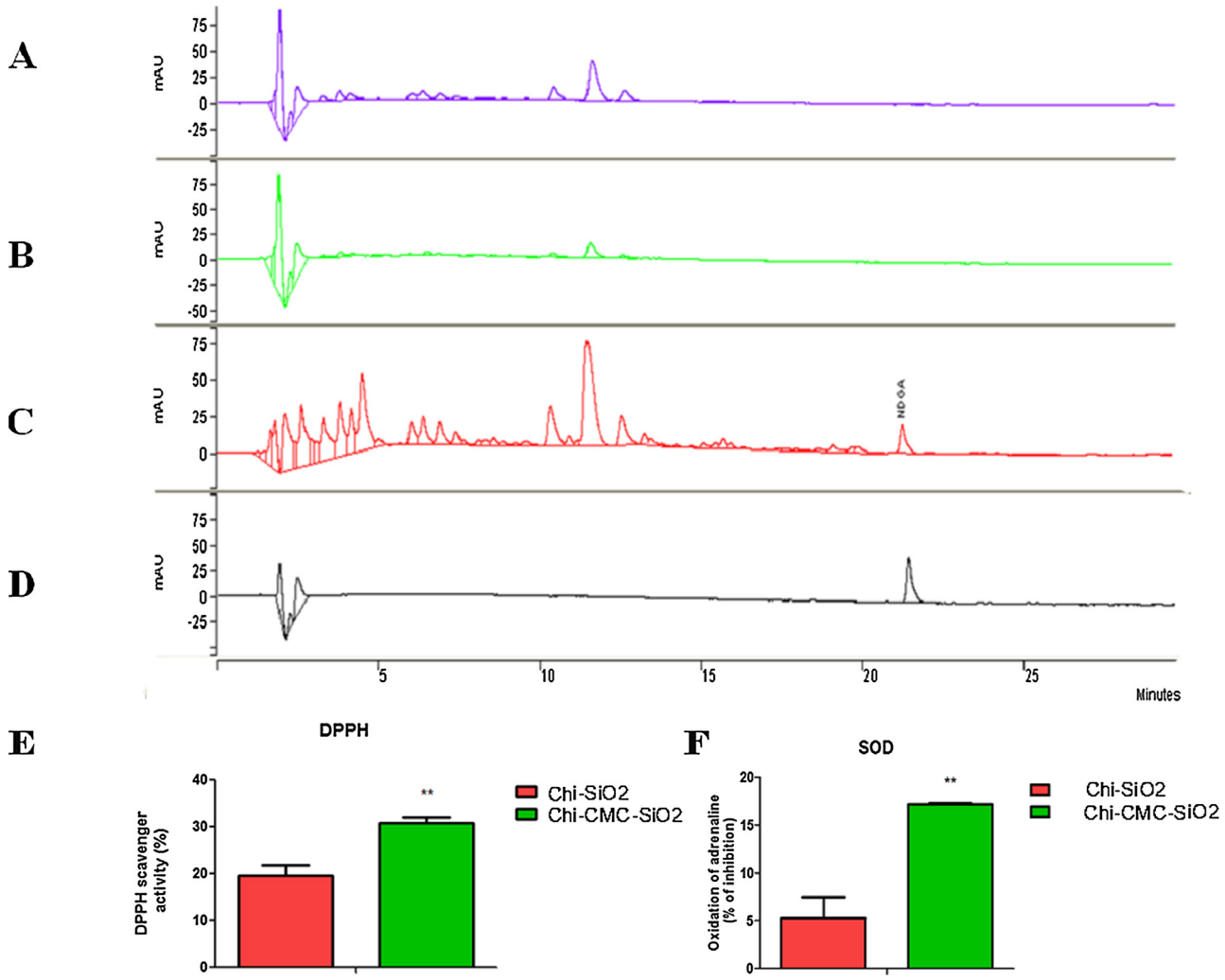

Fig. 4. HPLC chromatograms (A) *L. divaricata* Cav. extract incorporated to chi-CMC-SiO_2 composite (B) *L. divaricata* extract incorporated to chi-SiO_2 composite (C) *L. divaricata* Cav. control extract (D) biomarker nor-dihydroguayaretic acid (NDGA) the figures are representative of three determinations. Antioxidant activity of biopolymer-silica composites (E) DPPH scavenging activity (F) simil radical SOD activity of *L. divaricata* Cav. extract incorporated into chi-SiO_2 composite and chi-CMC-SiO_2 composite. Results are expressed as mean ± SEM of three determinations performed in triplicate. ** ($p < 0.01$) significant differences between chi-SiO_2 composite and chi-CMC-SiO_2 composite according to statistics Student *t*-test.

Table 1
Biopolymer-silica composites and chitosan hydrogels water content at equilibrium (W_∞) and coefficient of determination (r^2) of water content and time plotted according to a second order kinetic.

	W_∞	r^2
chi-SiO_2 pH 6.5	39,91	0,9985
chi-SiO_2 pH 7.5	52,63	0,9963
chi pH 6.5	92,04	1,0000
chi pH 7.5	93,52	0,9999
CMC – chi- SiO_2 pH 6.5	53,39	0,9975
CMC – chi- SiO_2 pH 7.5	64,26	0,9996

3.2. Larrea divaricata *Cav. extract incorporation and release studies from biopolymer-silica composites*

We evaluated the incorporation of *Larrea divaricata* Cav. aqueous extract in the composites by a HPLC (High Performance Liquid Chromatography) method as well as the HPLC extract profiles. Previous HPLC studies in *L. divaricata* Cav. infusions showed fingerprints compounds such as cinnamic acid and nordihydroguaiaretic acid [31]. It was demonstrated that both composites could incorporate many actives present in the aqueous extract and most of them are polar compounds. This is consistent to the fact that the composites showed to be highly hydrophilic and therefore, polar compounds would be much easier incorporated. For this reason, the biomarker nor-dihydroguayaretic acid (NDGA), one of the main compounds of *Larrea divaricata* Cav. extract, was not present in the chromatograms obtained (Fig. 4**a**–**d**). According to the HPLC results, compounds which are more polar that NDGA contribute to the antioxidant activity observed in the composites. These findings support that *Larrea divaricata* Cav. antioxidant properties are not only attributed to the biomarker NDGA. Therefore, not only NDGA is responsible of the antioxidant activity of the extract, but evidently there are other compounds involved in its antioxidant properties.

In terms of the quantitative analysis, the Chi-CMC-SiO_2 composites showed a higher incorporation of the extract compared to the Chi-SiO_2 composites. The degree of incorporation of the extract for Chi-CMC-SiO_2 composites was 44.36% CV: 3% and for Chi-SiO_2 composites 18.32% CV: 11%. These percentages express the amount of extract incorporated with respect to the amount of extract incubated with the composites after 24 h. The incorporation of aqueous *Larrea divaricata* Cav. extract for the Chi-CMC-SiO_2 composites is almost two times higher compared to the Chi-SiO_2 composites indicating that the addition of CMC increased the incorporation of the extract to the composites. Due to the differences observed

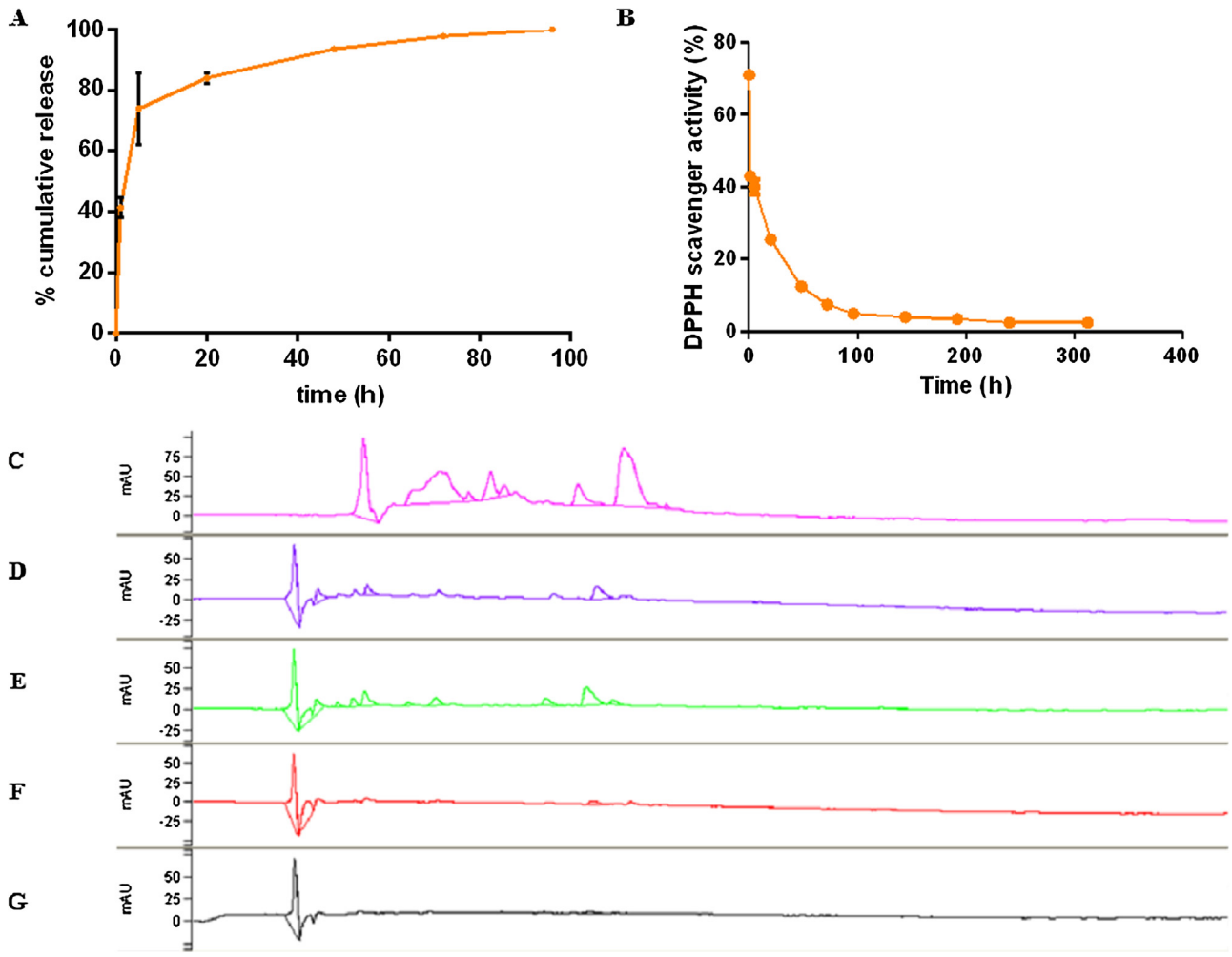

Fig. 5. (A) Cumulative release in buffer pH 6.5 of *L. divaricata* Cav. extract by HPLC of composite carboxymethylcellulose-chitosan-SiO_2 (CMC-chi-SiO_2) (B) DPPH scavenger activity of *L. divaricata* Cav. extract released in buffer pH 6.5 of composite chi-CMC-SiO_2 Representative HPLC chromatograms of *L. divaricata* Cav. extract released from composite chi-CMC-SiO_2 in buffer pH 6.5 (C) total extract incorporated (D) 1 h (E) 5 h (F) 48 h (G) 72 h.

between both composites in terms of the extract incorporation, it was decided to perform the release study of the extract only from the Chi-CMC-SiO_2 composites. Considering that normal saliva is a natural fluid with a pH range between 6.5 and 7, we decided to carry out the release profile in phosphate buffer pH 6.5 which is the lowest pH found in literature for patients suffering periodontitis. The *Larrea divaricata* Cav. extract present in the composite reached the 100% release in almost 4 days (Fig. 5**a**). Within the first five hours, almost 80% of the extract was released. Beyond that point, the pattern of release changed, and the amount of the extract left in the composite was released gradually until the fourth day. The HPLC profile of the *Larrea divaricata* Cav. extract released from the composite Chi-CMC-SiO_2 was preserved throughout the release studies as shown in Fig. 5**c–g**.

3.3. Composites antioxidant studies

Periodontal disease is initiated by the presence of subgingival biofilm and it is modulated by inflammatory and immune response. Many studies sustain that the dynamic equilibrium between reactive oxygen species and antioxidants could play a role in periodontal disease patogenia [32]. Indeed, gingival biopsy *in vitro* studies revealed a diminished activity of superoxide dismutase (SOD) and catalase (CAT), encouraging the hypothesis that these enzymes prevent excessive ROS production and therefore periodontal healing [33].

Antioxidants represent the first line of defense against free radical damage. They can be classified according to its mode of action: preventive antioxidants, like SOD, CAT and DNA repair enzymes which modulate redox processes and scavenging compounds such as carotenoids and polyphenols which are chain breaking antioxidants.

Furthermore, antioxidants may have positive effects in the regulation of fibroblasts migration and proliferation along the processes of gingival healing and periodontal repair [34]. Indeed, they can reduce the production of cytokines and pro-inflammatory proteins, neutralize reactive oxygen species and promote the process of wound healing [35].

Due to the relationship between ROS and periodontal disease patogenia, antioxidant adjuctive supplementation could help treating signs and symptoms of periodontal disease [36]. In this sense, several plant extracts have proven to be helpful for oral cavity inflammation [37–39]. Therefore, a detailed study of a candidate plant extract in a suitable carrier for the treatment of periodontal disease may have a significant relevance.

For the evaluation of the composites antioxidant capacity loaded with *L. divaricata* Cav., two different methods were carried out (Fig. 4**e** and **f**). Firstly, their capacity to eliminate free radicals by the DPPH free radical scavenging method was assessed and sec-

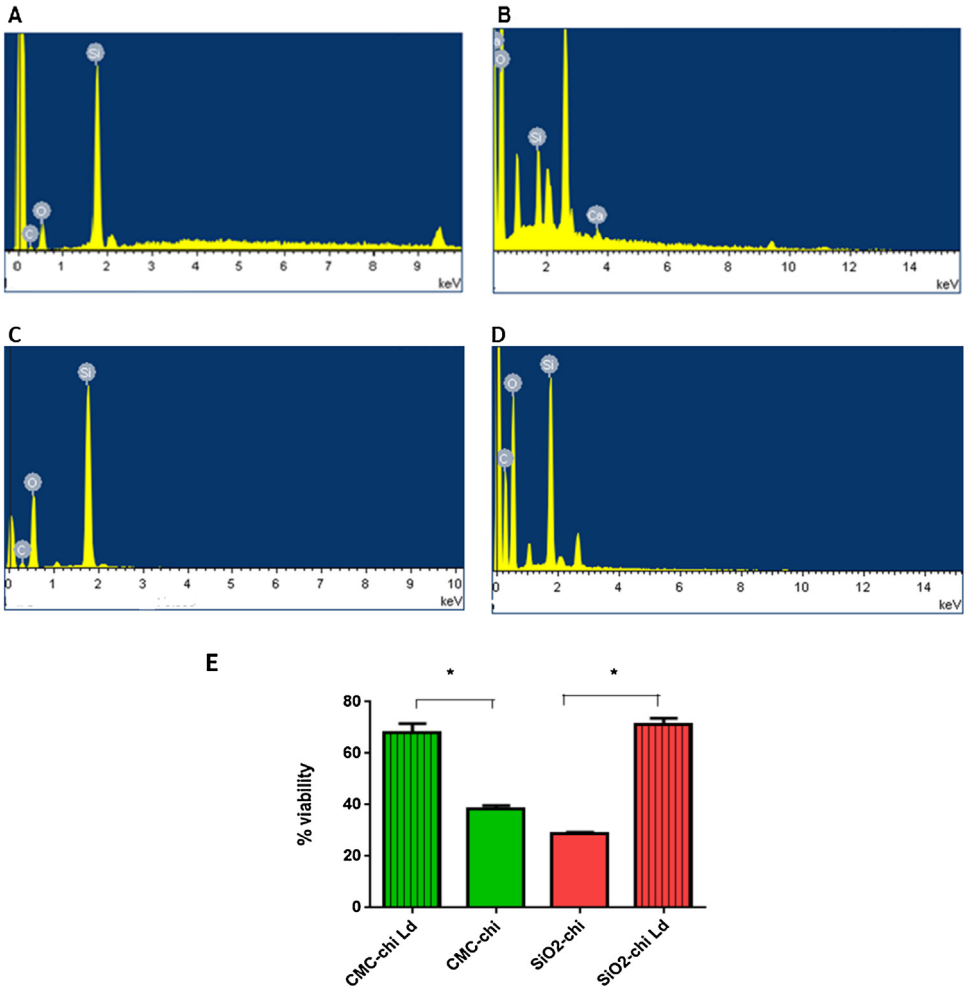

Fig. 6. (A) EDS-SEM of composite Chi-SiO_2 day 0 in simulated body fluid (B) EDS-SEM of composite Chi-SiO_2 day 14 in simulated body fluid (C) EDS-SEM of composite Chi-CMC-SiO_2 day 0 in simulated body fluid (D) EDS-SEM of composite Chi-CMC-SiO_2 day 14 in simulated body fluid (E) [3T3] Mouse fibroblast cytotoxicity for 24 h in composites Chi-SiO_2 and chi-CMC-SiO_2 by a colorimetric thiazolyl blue tetrazolium bromide assay for assessing cell proliferation. Differences were analysed using one-way ANOVA, followed by Bonferroni multiple comparisons test, $p < 0.05$ was considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ondly, their simil SOD activity was evaluated as described in Section 2.11.2.

It was observed that the Chi-CMC-SiO_2 composites presented a 3.5-fold higher activity of simil SOD and 1.5-fold higher capacity to eliminate free radicals by the DPPH assay when compared to the Chi-SiO_2 composites. These results are in close agreement with the differences observed in the incorporation of the extract of *Larrea divaricata* in the two types of composites.

Furthermore, during the whole release study, we evaluated simultaneously, by the DPPH method, the antioxidant properties of the extract released from the composite Chi-CMC-SiO_2, to verify that its activity was preserved. Fig. 5**b** shows that the antioxidant activity associated to the plant extract was also observed for a 4 day-time period in concordance with the release studies.

3.4. Composites bioactivity and cytotoxicity studies

Preliminary bioactivity studies demonstrate the ability to promote mineralization in the surface of the composites. Polymer-ceramic composites have gained a lot of attention in bone regeneration because of their similarity to bone [40,41]. Considering that in periodontal disease, particularly its advanced stage, periodontitis, tissue damage reaches alveolar bone, we intended to assess the role of silica nanoparticles in the composites. Bone is constituted by organic extracellular proteins and inorganic material, so polymer-ceramic composites microstructure may help to stimulate remodelling [42–45]. The nucleation of hydroxyapatite is influenced by electrostatic accumulation of cations and must follow structural and stereochemical requirements. Indeed, negative surfaces encourage the formation of a Ca^{2+} rich scaffold surface which can attract HPO_4^{2-}, facilitating the formation of nucleation sites [46]. Soaking the composites in simulated body fluid for 14 days revealed that only the composite Chi-SiO_2 presented a calcium accumulation in its surface (Fig. 6**a**,**b**) while in Chi-CMC-SiO_2 the addition of CMC discouraged the accumulation of calcium (Fig. 6**c**,**d**).

A cytotoxicity study was performed to the composites with fibroblast cells for 24 h. For that matter, we decided to perform the study on a fibroblast cell layer. Fibroblast cells were employed because of their importance in oral soft tissues, either as a cell component of the oral mucosa or because of their role in wound healing. In periodontal disease, the oral mucosa is seriously damaged, to the point that tissue remodelling becomes a crucial factor in terms of periodontal conservation. Cytotoxicity of materials can be rated based on cell viability relative to controls, where an activity level relative to controls of <30% is severe cytotoxicity, between 30 and 60% is moderate cytotoxicity, between 60 and 90% is slight cytotoxicity, and >90% is no cytotoxicity [47,48]. Accordingly, Chi-SiO_2 and

Chi-CMC-SiO_2 composites presented a moderate cytotoxic effect and materials with a moderate toxic effect on MTT assay can be considered as potential materials for biomedical applications [49]. However, it is worth to mention that when they were loaded with *Larrea divaricata* Cav. their cytotoxicity was decreased because the extract promoted fibroblast proliferation (Fig. 6**e**) ($p < 0.05$).

4. Conclusions

A variety of polymers are interesting biomaterials because of their biocompatibility, non-toxicity and easy processing for a medical application [50–52]. The understanding of the particularities of each type of biopolymer may help to design suitable biomaterials to treat oral diseases such as periodontal disease.

The oral cavity is an environment with a high percentage of humidity with natural secretions which tend to dilute the drugs, so that multiple applications are required to achieve a continuous action. Thus, a study of a bioactive carrier is mandatory in terms of mucoadhesivity and bioactive release. Herein, two biopolymer-silica composites loaded with aqueous *L. divaricata* Cav. extract are presented along with a detailed characterization. Interestingly, their swelling profile could be modulated by the pH of the saliva of patients who suffer from periodontal disease. The ionization of chitosan amino groups, carboxylic acid groups from CMC and silanol groups from silica nanoparticles are responsible for the distinct swelling behavior between pH 6.5 and 7.5. Thermal behavior also indicated differences in their water retention capacity and DSC thermograms revealed that composite Chi-CMC-SiO_2 presented the highest water affinity.

Furthermore, both composites could incorporate *L. divaricata* Cav. aqueous extract. Nevertheless, Chi-CMC-SiO_2 composites showed a greater incorporation and of course a higher antioxidant activity. HPLC *L. divaricata* Cav. fingerprints demonstrated that several actives were incorporated to the composites. This is quite interesting, because there are some discrepancies in literature towards NDGA beneficial effects. Renal and hepatoxicity have been reported for its chronic use [53]. Accordingly, the development of composites without NDGA may avoid these side effects.

The extract release from the composites at pH 6.5 was achieved in four days and both composites can be considered as potential materials for biomedical applications.

Lastly, due to silica bioactive properties [54], the presence of silica in the composites provides an environment for biomineralization. In periodontal disease, it is not only important the local delivery of bioactives but also to achieve the recovery of lost tissue. Bioactive materials may promote the regeneration of periodontal tissue damaged throughout this disease. Results showed that only composite chi-SiO_2 could provide an environment for possible biomineralization.

In conclusion, *in vitro* results indicate that composite Chi-CMC-SiO_2 could be an efficient carrier of *Larrea divaricata* Cav. extract as an adjunctive antioxidant supplementation for periodontal disease. In the case of chi-SiO_2 composite, further studies are needed to evaluate the role of this type of composite in bone periodontal regeneration while *in vivo* studies are in course.

Conflicts of interest

The authors report no conflict of interest.

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