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Controlled release and antioxidant activity of chitosan or its glucosamine water-soluble derivative microcapsules loaded with quercetin

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ABSTRACT

The controlled release and antioxidant properties of the flavonoid quercetin (Qr) incorporated into crosslinked microcapsules using chitosan (Ch) or its derivative modified with glucosamine by Maillard reaction (GACh) as wall materials were evaluated. The microcapsules containing Qr (Qr-MC) were obtained by the spray-drying technique with high microencapsulation efficiency of Qr, and with spherical shape of average size of 2.0 ± 1.5 μm. Under gastrointestinal simulated conditions, the Qr-MC showed controlled release within few hours, being the release rate faster under gastric than intestinal conditions. The rate of release of Qr by GACh-MC was almost double than those made with Ch under gastric conditions, but the same release rate was observed for both Qr-MC under intestinal conditions. Efficient antioxidant activity of the Qr-MC against reactive oxygen species (ROS) including hydroxyl radical $HO^{\bullet}$, anion superoxide $O_2^{\bullet-}$ and singlet oxygen $^1O_2$ was observed, indicating that Ch biopolymers are also suitable functional coating materials for flavonoid microencapsulation, regarding the gastro resistance, antioxidant activity and controlled release properties that could increase the bioavailability of the flavonoid.

1. Introduction

Chitosan (Ch), a natural amino-polysaccharide biopolymer, has received much attention due to its unique biocompatible properties, e.g. nontoxic, immunostimulant, anticancer, antimutagenic, mucoadhesive and antimicrobial, yielding a large variety of applications in food industry, including the production of nutraceutical and functional foods [1–3]. Chemically, Ch is composed of glucosamine and N-acetylglucosamine and produced by the alkaline N-deacetylation of chitin, the major component of the shells of crustaceans such as crab, shrimp, and crawfish [4,5]. The physical properties of Ch depend on a number of parameters including molecular weight (MW), deacetilation degree (DD) and sequence of the amino and the acetamido groups [6]. However, the intrinsic insolubility of Ch at neutral or high pH limits often its application in aqueous media under those pH conditions [1,7,8]. Recently, it has been shown that aqueous solubility of Ch is improved by the covalent attaching through Maillard reaction (MR) of mono- or disaccharide residues such as glucosamine (GA), preserving the global properties of the polysaccharide [8–11], even enhancing its antioxidant properties under neutral pH conditions [12].

On the other hand, the application of polyphenols in the preparation of functional foods and pharmaceutical formulations, especially including flavonoids such as quercetin (Qr) is of superior commercial interest, on account of their health benefits to humans [13]. Qr (3,3′,4′,5,7-pentahydroxyflavone) is widely distributed in the vegetal kingdom, and due to the large hydroxyl substitution onto the aromatic ring system presents remarkable free radical scavenging and metal cation chelating capacities, inhibition of the activity of oxidases, and also singlet molecular oxygen quenching ability [14–18]. Hence, Qr is a biocompatible molecule with potent antioxidant properties. However, the effectiveness of polyphenols depends on preserving the stability, bioactivity and bioavailability of the active ingredients [19]. At the

Abbreviations: Ch, Chitosan; ChMC, Chitosan Microcapsules; DD, Deacetylation Degree; GRAS, Generally Recognized as Safe; GACh, Glucosamine chitosan derivative; $HO^{\bullet}$, Hydroxyl Radical; MR, Maillard reaction; MC, Microcapsules; ME, Microencapsulation efficiency; MY, Microencapsulation yield; MW, Molecular Weight; % RS, Percent of Radical Scavenged; Qr, Quercetin; Qr-MC, Quercetin loaded Microcapsules; ROS, Reactive Oxygen Species; $^1O_2$, Singlet molecular oxygen; $O_2^{\bullet-}$, Superoxide Anion Radical.

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same time, as opposed to quercetin glycosides, Qr is poorly absorbed in the intestine, being preferentially absorbed in the stomach, like occurs with others aglycone flavonoids [20].

Therefore, there is considerable interest on the development of formulations of Qr capable of improving its chemical and biological stability and its bioavailability. In this regard, microencapsulation using biocompatible polymers is an excellent tool to vehiculate bioactive compounds, and depending on the properties of the polymeric coating, it can be controlled the release of the core material in a specific site [19]. Among microencapsulation techniques, spray-drying is an economical, flexible, and continuous operation procedure widely used for the production of high quality microparticles in food and pharmaceutical industries and lab scale [21].

In the present study, the effectiveness of both Ch and GACh as coating material for the spray-drying microencapsulation of Qr was evaluated, with the purpose to design a gastro-resistant microcapsules, in order to enhance the bioavailability and antioxidant properties of the flavonoid in the whole gastrointestinal apparatus, since it is well-known that Ch is resistant to gastric digestion [3,22–24]. The morphology, release kinetic, and antioxidant activity against reactive oxygen species (ROS) including hydroxyl radical ($HO^{\bullet}$), anion superoxide ($O_2^{\bullet-}$) and singlet molecular oxygen ($^1O_2$) of both Qr loaded MC were also studied.

2. Materials and methods

2.1. Materials

Ch medium MW (583 kDa, 78% DD), Qr (aglycone, MW 302.24 Da), glucosamine hydrochloride (GAHC), nitrotetrazolium blue chloride (NBT), 2-Deoxy-D-ribose (DoR), hydroxylamine hydrochloride (HAHC), trichloroacetic acid (TCA), tris(bipyridine)ruthenium(II) dichloride ($Ru(bpy)_3Cl_2$), sodium tripolyphosphate (TPP) were from Sigma-Aldrich (MO). Ferric chloride ($FeCl_3$), monobasic potassium phosphate (KH_2PO_4), sodium chloride (NaCl), sodium hydroxide (NaOH), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$) and ascorbic acid, all analytical grade, were obtained from Cicarelli (Argentina). The thiobarbituric acid (TBA) and ethylenediaminetetraacetic (EDTA) were provided by Merck (Germany) and hydrogen peroxide (H_2O_2), hydrochloride acid (HCl), isopropanol and glacial acetic acid (CH_3COOH) by Anedra (Argentina). Aqueous solutions were prepared with ultrapure water.

2.2. Preparation of glucosamine chitosan derivative

GACh derivative was obtained by Maillard reaction (MR) between native Ch with GAHC as described by Vanden Braber et al. (2017) [12]. Briefly, 1% (w/v) Ch in 0.25 M CH_3COOH was mixed with 1% (w/v) GAHC and kept in an orbital shaker at 65 °C for 48 h at pH 3. Then the sample was centrifuged with a Sorval LST 16R centrifuge (Thermo Scientific, USA) at 5.000 rpm for 20 min. The supernatant was dialyzed against distilled water through a membrane with MW cutoff of 12–14 kDa (Sigma-Aldrich) for 96 h at 4 °C. Detailed procedures for their preparation and characterization were described in our previous paper [12].

2.3. Spray-drying microencapsulation of Qr

0.5% (w/v) Ch and GACh solutions in 0.5% (v/v) CH_3COOH were used as encapsulant material. Qr was added into encapsulant solutions in a proportion of 5% (w/w) of the biopolymers, under continuous stirring. The mixture was homogenized at 18.000 rpm for 5 min protected from light using an Ultraturrax homogenizer (IKA T18, Germany). Then, under constant stirring, 1% (w/v) TPP was added as ionic crosslinker agent in a ratio of 5% of encapsulant material solution volume. MC loaded with Qr (Qr-MC) were obtained by spray-drying process in a BÜCHI Mini Spray Dryer B-290 (BÜCHI Labortechnik AG, Switzerland), equipped with a two-fluid nozzle with a cap pinhole diameter of 0.5 mm. The working conditions such as inlet temperature, liquid flow, aspiration rate and compressed spray air flow (represented as the volume of the drying air input) were set at 130 °C, 4 mL/min, 100% and 1.05 m^3/h, respectively. Empty MC were prepared in the same conditions without adding Qr in the mixture to be spray-dried. The powders obtained after spray-drying were stored at room temperature in desiccator containing silica pearls to avoid moisture.

2.4. Qr microencapsulation efficiency and yield

To quantify the total content of microencapsulated Qr ($[Qr]_T$), an weighted amount of Qr-MC was dissolved in 0.1 N HCl in isopropanol: water (1:1). This solution was sonicated during 2 h to ensure complete release of the flavonoid. Instead, for the quantification of the adsorbed content of Qr onto the MC surface ($[Qr]_S$), an isopropanol:water mixture (1:1) was used to disperse a known amount of Qr-MC and the supernatant was immediately separated by centrifugation (500 rpm). In both cases, the flavonoid content was quantified by registering the absorbance at 366 nm (absorption maximum for Qr) using a Specord S600 UV–Vis diode array spectrophotometer (Analytik Jena, Germany), after subtracting the background absorbance and/or scattering of a same concentration MC solution without Qr. A calibration curve made with Qr solutions in water at different working pH was used. The microencapsulation efficiency (ME), defined as the percentage of Qr molecules inside the microcapsule in relation to its total (inside + surface) concentration, was calculated with Eq. (1) [25].

$$ME(\%) = 100 \times ([Qr]_T - [Qr]_S)/[Qr]_T \quad (1)$$

In turns, the microencapsulation process yield (MY) was calculated according to Eq. (2),

$$MT(\%) = 100 \times (m_{MC}/m_T) \quad (2)$$

where m_T and m_{MC} are the mass of solids obtained before and after spray-dried encapsulation.

2.5. Morphology and size of Qr-MC

Morphology and size distribution of the spray-dried powders were evaluated by scanning electronic microscopy (SEM) with a ZEISS SIGMA VP Field Emission Scanning Electron Microscope (FE-SEM) (ZEISS, Germany), using an acceleration voltage of 5 kV. The MC were fixed in stubs containing a double-faced adhesive metallic tape and coated with gold in a CED 010 vacuum evaporator (Balzers Union, Liechtenstein). Size distribution of spray-dried powders was evaluated with the ImageJ 2014 software (Rasban, National Institute of Health, USA).

2.6. Qr release in simulated gastrointestinal digestion conditions

The in vitro Qr release kinetic patterns were studied during 7 h using an orbital shaker at a rotational speed of 150 rpm and at 37 °C [23]. Two different digestion stages were performed: firstly, 0.05 g of Qr-MC were mixed with ultrapure water and placed into dialysis membrane with MW cutoff of 12–14 kDa (Sigma-Aldrich) and disposed in 60 mL of 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 0.1 N HCl at pH 1.2 used to simulate the gastric fluid conditions during 2.5 h. In the second stage, the same dialysis membrane was disposed in 60 mL of 50 mM KH_2PO_4, 22.4 mM NaOH at pH 6.8 during 4.5 h in order to simulate intestinal fluid conditions. At the indicated time points, samples were collected and the volume replaced with fresh medium to maintain the same conditions. Collected samples were analyzed spectrophotometrically at 366 nm to determine Qr content using calibration curves as described in sub-section 2.4. In addition, the stability of Qr in

gastrointestinal conditions was evaluated, for which ≈20 μM Qr solutions were stored at 37 °C in simulated gastric and intestinal fluids, the concentration changes being determined by the absorbance at 366 nm.

The Qr release profiles were fitted according to the mathematical models more used to describe this phenomena [26], zero order ($Qr_t = Q_0 + k_0\ t$), first order ($Qr_t = Qr_0 e^{kKt}$), Higuchi ($Qr_t = k_H t^{1/2}$), and Krosmeyer-Peppas ($Qr_t/Qr_\infty = k_K t^n$). In this equations, Qr_0 is the initial concentration of Qr in dissolution, Qr_t is concentration of Qr released at time t, Qr_t/Qr_∞ is the fraction of Qr released at time t, k_0 is the zero-order rate constant, k_1 is the first order rate constant, k_H is the Higuchi constant of dissolution, k_K is the Korsmeyer-Peppas constant (incorporate structural and geometric characteristics), and n is a diffusional exponent that depends on the release mechanism and the geometry of the tested system.

2.7. In vitro antioxidant assessment

2.7.1. Hydroxyl radical scavenging activity

$HO^\bullet$ was generated via Fenton reaction at pH 7.4. In presence of 2-deoxy-D-ribose (DoR), the $HO^\bullet$ reacts producing malonaldehyde (MDA) among other products, which forms a pinkish adduct in presence of TBA allowing its quantification by UV–Vis spectroscopy. The $HO^\bullet$ scavenging effect of Qr-MC with Qr, empty MC and pure Qr was determined as previously described [27]: the reaction was performed in 50 mM phosphate buffer (pH 7.4) containing 10 mM DoR, 100 mM H_2O_2, 1 mM $FeCl_3$, 5 mM EDTA, in presence and absence of the samples. The reaction started with the addition of ascorbic acid in a final concentration of 5 mM. The reaction mixture was incubated for 1 h at 37 °C in a water bath; then, 1% (w/v) TBA and 5.6% (w/v) cold TCA were added and heated up to boiling temperature (95–100 °C) for 20 min to cause the colored adduct formation, which was measured at 532 nm. The radical scavenging percentage RS(%) was calculated with Eq. (3):

$$RS(\%) = 100 \times (1 - A_X/A_0) \quad (3)$$

where A_0 is the absorbance of the control and A_X is the absorbance in presence of MC samples.

2.7.2. Superoxide radical scavenging activity

The scavenging of $O_2^{\bullet -}$ was performed, as previously described by Boiero et al. (2014) [27], using NBT as colorimetric reagent. The assay is based on the $O_2^{\bullet -}$ generation by HAHC autoxidation, which reduces NBT to nitrite ($NBT^{+\bullet}$). In aqueous media, the organic radical-cation $NBT^{+\bullet}$ yields the stable cation monoformazan (MF^+), which shows maximum absorption at 560 nm. The competitive scavenging of $O_2^{\bullet -}$ was studied as follows: in a test tube the desired amounts of MC with Qr, empty MC as well as pure Qr were weighted and dissolved in 50 mM phosphate buffer (pH 8) containing 1 mM NBT, 1 mM EDTA, and 5 mM HAHC. The samples were incubated for 1 h at 37 °C and afterwards the absorbance of MF^+ was measured at 560 nm. The RS(%) of $O_2^{\bullet -}$ was calculated with Eq. (3). For both radicals, all samples were evaluated in the same MC content (mg/mL) from a 5 mg/mL stock solution in 0.5% (v/v) CH_3COOH. The antioxidant activity was expressed as the effective concentration of pure or released Qr (μM) to reach 50% of radical scavenging (EC_{50} value). The Qr content in the MC solution was determined spectrophotometrically at 366 nm subtracting the background absorbance and/or scattering of the MC solution without Qr at the same biopolymer concentration. EC_{50} value was calculated from the slopes of the graph of RS(%) vs. Qr concentration.

2.7.3. Reactivity against singlet molecular oxygen

The photosensitized O_2-uptake in Qr-MC, MC and Qr solutions was monitored by measuring the consumption of dissolved oxygen with a stainless-steel 1/16" FOXY-R-AF fiber-optic luminescent sensor of $^3O_2$ coupled to a CCD USB2000 fluorimeter (OceanOptics, Dunedin, FL) as described by Vanden Barber et al. (2017) [12]. A high-power blue LED (466 ± 30 nm, 1 W) was as steady-state excitation source of the photosensitizer $Ru(bpy)_3Cl_2$ (57 μM), which produces singlet molecular oxygen ($^1O_2$) with approximately 33% of quantum efficiency. At the excitation LED wavelength region, the Qr and Qr-MC solution are transparent avoiding flavonoid or chitosan excitation. Simultaneously, absorbance spectral changes of the solution were also registered with an extra CCD-USB2000 spectrometer (OceanOptics, USA), with the analyzing UV–Vis beam at right angle to the photolysis beam from the blue LED. The experiments were performed in air-saturated aqueous solutions mimicking gastric conditions (pH 1.2) containing the photosensitizer and 5 mg/mL of empty MC, Qr-MC, or pure Qr in equivalent concentration to that provided by the outside of the MC (approximately 20 μM). The test was repeated after having exposed the samples 2 h at 37 °C under gastric conditions.

2.8. Statistical analysis

The experimental data were processed and analyzed with OriginPro 8.5 (OrginLab Software Corporation, USA). All experiments were done by triplicate and data were presented as mean value ± standard deviation (SD). In all cases, linear slope calculations were subjected to statistical analysis of variance (one way ANOVA) followed by Tukey test as *post-hoc* test using the InfoStat 2014/e statistical package (Di Rienzo, Casanoves, Balzarini, Tablada & Robledo. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). ANOVA was considered statistically significant when P-value < .05.

3. Results and discussion

3.1. MC characterization

Table 1 collects the %w/w of total, inner and adsorbed Qr relative to the total mass of material to be spray-drying, and the respective microencapsulation efficiency (ME) and yield (MY) calculated according to Eqs. (1) and (2). Several factors may affect the ME of a bioactive compound, e.g. its chemical affinity to the coating material, bioactive/polymer concentration ratio, mechanical conditions, etc. Previous works have shown that ME increases with Ch concentration, due to the larger viscosity of the polysaccharide solution preventing the loss of bioactive molecules from the sprayed droplet [28]. Indeed, at the same biopolymer mass concentration, the small differences of amount of Qr incorporated into the GACh-MC and Ch-MC, resulted in almost the same ME as expected for coating materials of similar chemical nature [28,29]. However, the MY was larger for MC made of GACh than Ch (P < .05, Table 1). As the molecular weight of GACh (98 kDa) is much lower than for Ch (583 kDa) [12], a plausible explanation of the larger MY for GACh-MC is the result of lower adherence and final viscosity of the suspension to be dried [23].

On the other hand, the total content of flavonoid in both Qr-MC was evaluated during a period of 12 months under storage as dry powder in desiccator at room temperature, and Qr content was almost constant (data not shown), suggesting the microencapsulation of Qr in Ch matrices is an efficient stabilization method for flavonoids.

Fig. 1 shows the SEM images obtained for both Qr-MC, and it can be observed individual spherical-like MC with smooth surface and

Table 1
Microencapsulation process efficiency and yield of Qr in Ch matrixes.

Sample	Qr % (w/w) in total MC mass			ME (%)	MY (%)
	Total	Outer	Inner		
ChMC	3.88 ± 0.25[a]	0.84 ± 0.01[b]	3.04 ± 0.20[a]	78 ± 5[a]	60 ± 5[a]
WSChMC	3.23 ± 0.25[a]	0.74 ± 0.01[a]	2.49 ± 0.25[a]	77 ± 5[a]	75 ± 5[b]

Each value is expressed as mean ± SD (n = 3). Means with different letters, for the same column, are significantly different (P < .05).

indentions. The last ones appear on the surface of microspheres due to the rapid evaporation of the solvent during the spray-drying process. Despite of the size disparity due to the nature of the preparation method, similar average diameters of 1.94 ± 0.70 μm and 1.86 ± 1.90 μm were calculated for Qr-MC of GACh and Ch, respectively.

3.2. Qr stability and release kinetics in simulated gastrointestinal conditions

Previous to the release study, the stability of Qr in aqueous solutions simulating gastric and intestinal conditions at 37 °C was evaluated. After 4 h of incubation, <5% of the initial Qr was degraded under gastric conditions while about 25% was consumed in intestinal conditions, as illustrated in Fig. 2A by the UV–Vis absorption spectral fade of Qr in aqueous model solutions of intestinal fluids and the respective Qr-degradation kinetics in both conditions (inset of Fig. 2A). The intrinsic flavonoid degradation was taken into account for the calculation of the % Qr release from MC under both different gastrointestinal conditions.

Considering that in their cationic forms both Ch and GACh are hydrophilic polymers and swells well in aqueous media, polymer crosslinking is desirable to obtain a more controlled release of a bioactive compound from Ch microcapsules [30]. Control releases experiments of Qr in both Ch-MC and GACh-MC without treatment with the nontoxic ionic cross-linker TPP indicated a very rapid release (few minutes) of Qr due to the prompt dissolution of the MC (data not shown). Hence, in order to improve the controlled release of Qr, TPP was used to stabilize both Ch-MC and GACh-MC, and Fig. 2B shows the respective cumulative release kinetic of Qr in a time window of 7 h. They were performed during the first 2.5 h under gastric conditions (pH 1.2, ionic strength = 0.28), and later continued at intestinal conditions (pH = 6.8, ionic strength 0.07).

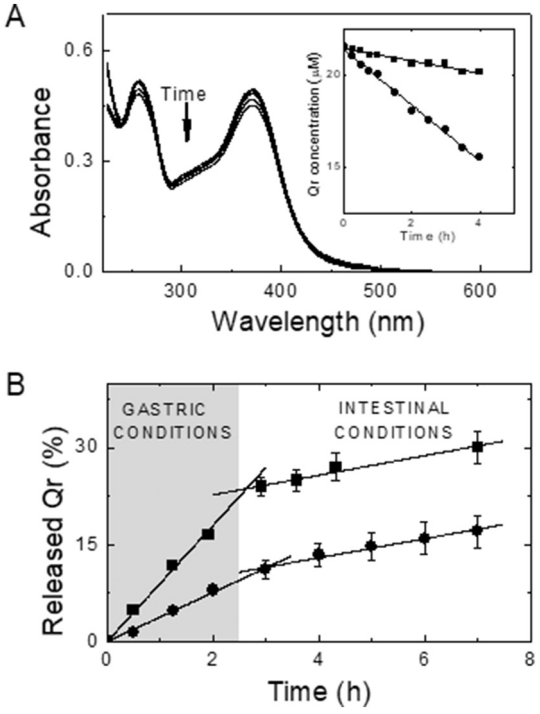

Fig. 2. (A) UV–Vis absorbance spectra of 20 μM quercetin (Qr) stored at 37 °C in simulated intestinal fluid solution. Inset: Stability plot for Qr stored at 37 °C under (■) gastric conditions, (●) intestinal conditions. (B) Release kinetic curves of Qr in simulated gastrointestinal fluid solutions at 37 °C from microcapsules made of (●) Ch, (■) GACh. Solid lines represent the zero-order fit of Qr releases under both gastrointestinal conditions, and the respective zero-order rate constants (k_0) are collected in Table 2.

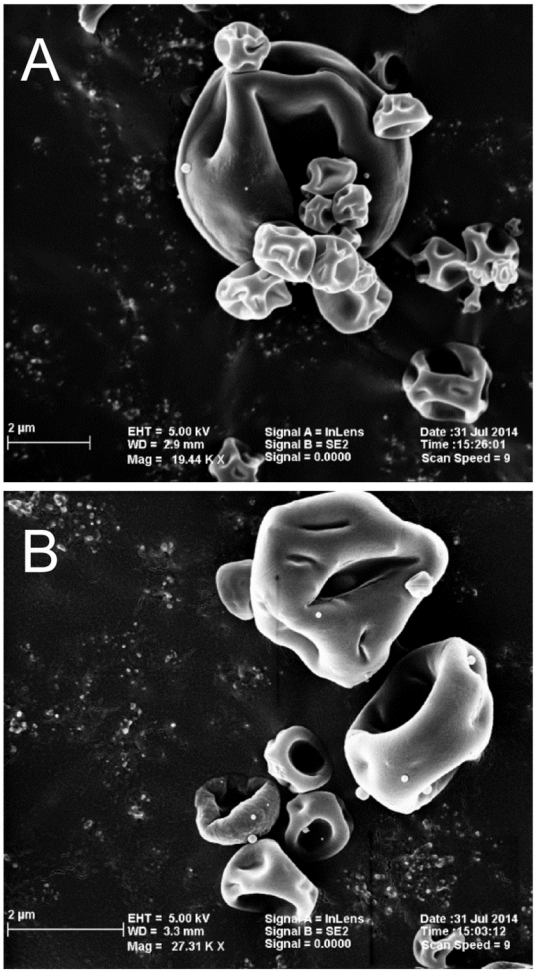

Fig. 1. SEM images of spray-dried crosslinked microcapsules containing quercetin using as wall material: (A) Chitosan (Ch) and (B) glucosamine chitosan derivative (GACh).

The data obtained from Qr release in gastric and intestinal conditions were fitted into the mathematical models previously mentioned in experimental section. The data obtained did not fitting with Higuchi's square root of time equation, while for zero order, first order and Korsmeyer-Peppas equations, good fitting were obtained with regression coefficient (R^2) > 0.88. The values of the release exponent (n), the kinetic rate constant (k) and R^2 are presented in Table 2. The R2 value, obtained for the microcapsules of both polymer's and for each gastrointestinal condition, indicating that Qr release followed zero-order kinetics, corresponding to systems where the rate of drug release is independent of its concentration and with non-Fickian diffusion mechanism. Under gastric conditions the release k_0 of Qr was almost twice greater in GACh-MC than Ch-MC, but it was much lower and independent of the biopolymer under intestinal conditions. Thus, the releases of Qr under gastric simulated conditions reached about 17% and 8% for GACh-MC and Ch-MC, respectively, but it was reduced to only 6% for intestinal conditions for both types of MC. This particular pH-dependent behavior can be associated with the fully protonation of the glucosamine moiety in both polymers, the highest solubility and swelling at very acidic conditions, what hence facilitating the delivery of the Qr molecule. On the contrary, at neutral pH conditions, both GACh and Ch show similar deprotonation degree and therefore it can be expected lower solubility and swelling in both types of MC reducing in similar fashion the release of Qr. Srinatha et al. (2008) [30] also found a pH-sensitivity in the release mechanisms of ciprofloxacin from ionic crosslinked Ch beads. Additionally Gârlea et al. (2010) [31] obtained zero-order release kinetics of tannic acid as polyphenol model from nanocapsules of chitosan–surfactant matrices and a drug release being controlled by pH. Recently Alam et al. (2016) [32] designed furazolidone

Table 2
Comparison of different Qr release kinetics models.

Sample	Medium Conditions	Release model						
		Zero-order		First-order		Korsmeyer-Peppas		
		k_0	R^2	k_1	R^2	k_K	n	R^2
Ch-MC	Gastric	3.8 ± 0.2	0.993	1.68 ± 0.09	0.932	0.17 ± 0.01	1.16 ± 0.06	0.995
	Intestinal	1.5 ± 0.2	0.973	0.05 ± 0.01	0.941	0.50 ± 0.05	0.33 ± 0.05	0.883
GACh-MC	Gastric	9.1 ± 0.3	0.997	1.08 ± 0.06	0.986	0.29 ± 0.01	0.84 ± 0.09	0.973
	Intestinal	1.4 ± 0.2	0.972	0.11 ± 0.01	0.941	0.33 ± 0.01	0.47 ± 0.02	0.952

(antimicrobial agent) -chitosan based spray dried microparticles with zero-order release kinetics and controlled release pH-dependent.

3.3. Radical scavenging capacity

Fig. 3 shows the scavenging activity of hydroxyl radical ($HO^{\bullet}$) superoxide anion radical ($O_2^{\bullet-}$) comparing empty and Qr-loaded MC, freshly prepared and dissolved in solutions mimicking the intestinal fluid condition. In all cases GA-ChMC showed better radical scavenging activity than Ch-MC. In presence of Qr loaded into the MC, the average radical scavenging activity increased relative to empty MC, which themselves contribute to radical scavenging in about 30%–57% and 70–80% against $HO^{\bullet}$ and $O_2^{\bullet-}$, respectively.

Free radical scavenging capacity of Qr is primarily attributed to the high reactivity of -OH groups on the B-ring, which donate one hydrogen and one electron to free radicals stabilizing them and giving rise to a relatively stable flavonoid radical [15,35]. The 3′4′-catechol structure in the B-ring strongly enhances lipid peroxidation inhibition through H-bonding and electron delocalization [33]. Another function of the catechol moiety in the B-ring is the possible chelation of transition metal ions that may cause radical oxygen species formation via Fenton-type reaction [36]. Interestingly, the studied Qr-MC and empty MC showed approximately three-times lowest scavenger capacity against $HO^{\bullet}$ than for $O_2^{\bullet-}$. In the case of Qr-MC, this behavior can be explained by the pro-oxidant mechanism of flavonoids that interfere in the Fenton reaction for the $HO^{\bullet}$ generation. In fact, Puppo (1992) [36,37] have demonstrated that flavonoids with the highest number of -OH in ring B can act in the "redox" cycle of Fe, restoring Fe^{2+} in the system and cooperating in the $HO^{\bullet}$ formation. In any case, the antioxidant effect of Ch biopolymers is associated with its ability to scavenge free radicals or chelate metal ions through a donation mechanism of hydrogen or a lone pair of electrons. In fact,-OH and -NH_2 groups in Ch are key functional groups for its antioxidant activity [8].

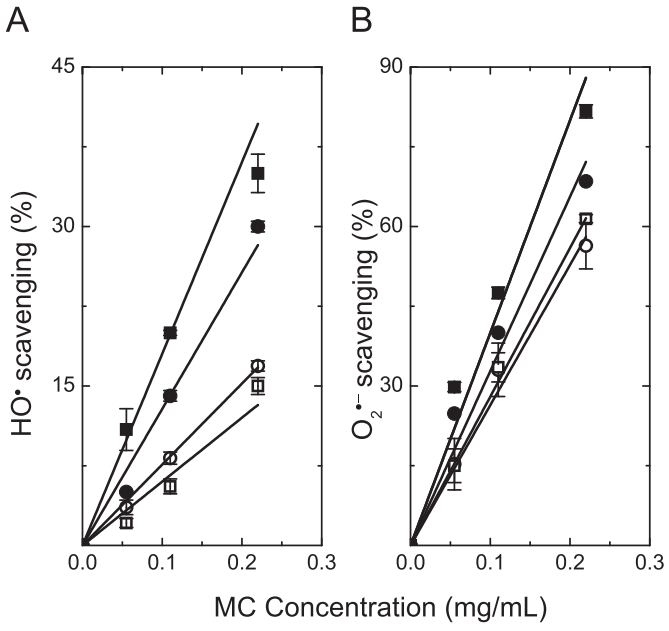

Fig. 3. Percent scavenging activity of radicals $HO^{\bullet}$ (A) and $O_2^{\bullet-}$ (B) by (○) empty Ch-MC, (●) QrCh-MC, (□) empty GACh-MC, and (■) QrGACh-MC.

3.4. Singlet oxygen quenching by Qr-MC

In aqueous solutions, the triplet excited-state of the metal-to-ligand charge transfer (^{3}MLCT*) of the coordination complex $[Ru(bpy)_3]^{2+}$ by molecular oxygen $^3O_2$ mainly involves energy-transfer quenching process, generating molecular singlet oxygen ($^1O_2$) with quantum yields between 0.3 and 0.5, depending of the concentration of dissolved $^3O_2$ [37,38]. On the other hand, it is well-known that Qr is an efficient sacrificial quencher $^1O_2$, since the channel is almost through charge-transfer mediated chemical reaction producing the flavonoid oxidation [18,34], Eq. (5).

$$Qr + {}^1O_2 \xrightarrow{k_r} QrO_2 \tag{5}$$

Therefore, if $^1O_2$ is deactivated, both the Qr and $^3O_2$ concentrations must be deplected by the above reaction. Fig. 4A shows the first-order plot for Qr consumption as monitored by absorbance fade at 366 nm in air-saturated solutions containing the photosensitizer $[Ru(bpy)_3]^{2+}$ and simulating the gastric fluid conditions at 37 °C (acid pH). In absence of blue-light, no photosensitization process occurred, and the observed slight degradation of pure Qr is due to the thermal instability of the flavonoid (inset of Fig. 2). On the contrary, under photosensitization

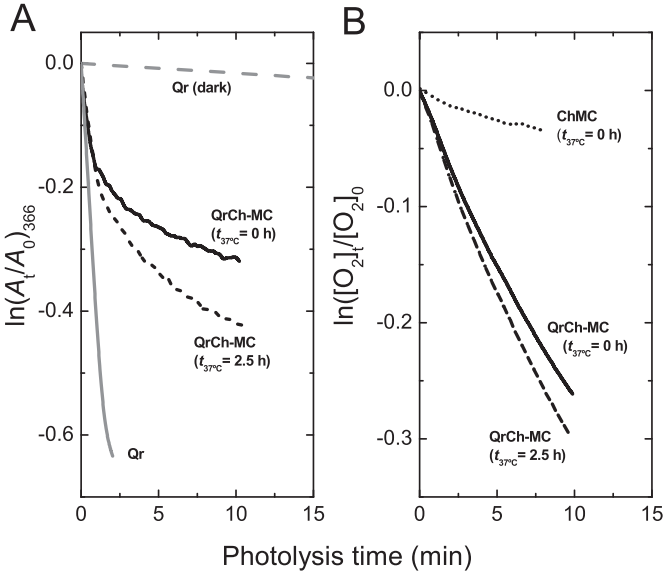

Fig. 4. Qr-uptake (A) and O_2-uptake profiles (B) produced by steady-state photolysis at 466 ± 30 nm of $Ru(bpy)_3Cl_2$ (57 μM) in simulated gastric conditions. Pure Qr in darkness (gray dash line), pure Qr (gray solid lines), (black solid line) QrCh-MC t = 0 h, (black short dash line) t = 2.5 h at T° = 37 °C, (black dot line) empty ChMC, (black line) QrCh-MC t = 0 h, and (black short dash dot) QrCh-MC t = 2.5 h at 37 °C.

conditions, the flavonoid is largely consumed due to the reaction 5. In solutions containing Qr-MC, there is a prompt photosensitized degradation of Qr, similar to that observed for pure Qr, that can be related with the reactivity of the outer or more solvent exposed flavonoid adsorbed onto the MC. The prompt Qr-degradation in the MC solutions is followed of a slower degradation of the flavonoid, which is dependent on the incubation time at 37 °C of the solution prior to photosensitization. For the solution incubated 2.5 h, a faster and larger photosensitized degradation of Qr than in the case of freshly prepared solutions was observed, indicating a better accessibility of $^1O_2$ into the MC and/or more efficient flavonoid release after swelling of the MC.

Fig. 4B compares the uptake kinetics of dissolved O_2 for empty and Qr loaded Ch-MC. It can be observed that empty Ch-MC was able to uptake of O_2, in agreement with previous results that demonstrated that both Ch and GACh biopolymers in acidic solutions were able to react with $^1O_2$ with reactive rate constants of $\approx 10^8$ $M^{-1}s^{-1}$, oxidizing the biopolymer [12].

For QrCh-MC the larger O_2-uptake is due to the parallel reaction of $^1O_2$ with the flavonoid (Eq. (5)). In the same fashion that for the flavonoid consumption, the O_2-uptake was more efficient and larger for pre-incubated during 2.5 h at 37 °C QrCh-MC than freshly solutions, confirming that the swelling of the MC favors either diffusion of $^1O_2$ or interaction with reactive quenchers into the MC. Similar results were found for GACh-MC (data not shown).

4. Conclusions

In the present work, we showed that both Chitosan (Ch) and its glucosamine derivative (GACh) are suitable biopolymers for quercetin (Qr) microencapsulation by spray-drying, yielding microcapsules with diameters around 2 μm with efficiency of encapsulation of about 70%. The flavonoid loaded microcapsules also showed interesting antioxidant functions, being able to scavenge efficiently several reactive oxygen species (ROS), such $HO^{\bullet}$, $O_2^{\bullet-}$ and $^1O_2$. The antioxidant effect was even more efficient when the MC were previously swelling by incubation in solution at 37 °C, suggesting that microcapsule dissolution favors the accessibility of the carried flavonoid to the ROS. Altogether, besides the feasibility to carry antioxidant molecules as flavonoids, the present results exert the utilization of chitosan biopolymers as functional coating material for microencapsulation not only by their biocompatibility and mechanical properties but also by their intrinsic antioxidant properties, in particular the GACh derivative, which MC showed better release and swelling than those made with native Ch due to the larger solubility of the derivative [12].

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