Reactive & Functional Polymers 82 (2014) 103–110

Contents lists available at ScienceDirect

Reactive & Functional Polymers

journal homepage: www.elsevier.com/locate/react

Encapsulation of Congo Red in carboxymethyl guar gum–alginate gel microspheres

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ARTICLE INFO

Article history:
Received 28 January 2014
Received in revised form 17 June 2014
Accepted 18 June 2014
Available online 26 June 2014

Keywords:
Congo Red
Gel microspheres
Guar gum
Alginate
Coacervates
Infrared spectroscopy

ABSTRACT

Congo Red (CR) is a hydrophobic dye commonly used for diagnosis and potentially useful as therapeutic agent of beta amyloid plaques in neurodegenerative diseases. CR, as drug model, was encapsulated on Alginate–Carboxy Methyl Guar Gum (Alg–CMGG) blend microspheres. Guar gum 18% carboxymethylated (CMGG) derivative was synthesized in order to improve aqueous solubility, polymer blending and help reduce surface tension. The derivative was confirmed by FTIR spectroscopy, and elemental analysis. Surface tension of the new CMGG is reduced in about 50% compared with the native polymer. Lowering of Guar Gum (GG) aqueous solutions viscosity from 30,000 cps to 350–400 cps in case of CMGG is indicating pseudoplastic fluid behavior modifications. Vibrational spectroscopy analysis confirmed interactions among CR molecules in alginate–CMGG matrices ascribed largely to the aromatic motif of the dye and the biopolymer a polar regions. CR was encapsulated on 68/32% alginate/CMGG blend microspheres as the best formulation tested. The release of CR from the microspheres was not detected at pH = 1.2 in 25 min, but 62% of CR was found in the supernatant when the pH was raised to 7.4 at 37 °C after 8 h incubation.

1. Introduction

Congo Red (CR) is a linear anionic secondary diazo dye used as an acid-base indicator. Additionally, CR was used to detect fibril proteins enriched in β sheet conformation useful in histological studies of some neurodegenerative pathologies such as Alzheimer's, Creutzfeldt–Jacob's, Huntington's and Parkinson's diseases [1]. Moreover, CR delays appearance of clinical signs on experimental prion trials [2]. The compound could also be used as a palliative in neurodegenerative disease therapies. CR is soluble in many organic solvents, but yielding red colloidal fluorescent solutions in aqueous media because of its hydrophobicity made by the presence of biphenyl and naphthalene groups in the molecule [3]. The postulated mechanism for CR aggregation is by hydrophobic interaction involving the π–π bonds of the aromatic rings making planar structures [4]. Based on the CR physicochemical properties, the dye is an excellent candidate to test the potential encapsulation of common hydrophobic drugs, e.g. anthracyclines, taxanes, fluoroquinolones, into biopolymeric gel matrices.

In the last decades, biopolymers have received increasing attention both in the academia and in industry. Remarkable biopolymer properties like structural diversity, biological specific properties over a range of molecular dimensions and favorable non-covalent physiological interactions were unearthed. Additionally, tailorability, biodegradability, mild environmental synthesis, and convenient rheological modulations have made biopolymers very attractive tools for a myriad of applications [5]. Ultrapure Alginates (Alg) produced under GMP/ISO 9000 guidelines are one of most common biopolymer gels currently used in food and pharmaceutical industries. Algs are linear biopolymers composed of β-mannuronic acid (M) and α-guluronic acid (G) linked by 1–4 bonds, purified from the seaweeds and some bacteria. Alg hydrogels are formed by ionic crosslinking in presence of divalent cations (e.g. calcium and zinc), which cooperatively interact with different carboxylate ions forming ionic bridges between different polymer chains. Alg gel structure is commonly named as "egg box" because of the analogy with the egg containers. Alginate gels have been

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http://dx.doi.org/10.1016/j.reactfunctpolym.2014.06.006
1381-5148/

used extensively as a matrix for entrapment of many molecules and cells, and also in tissue engineering applications [6]. However, Alg gels are showing some drawbacks such as high hydrophilicity and erodability at alkaline pH, poor mechanical strength, and gel instability in freeze–thaw cycles which are preventing extensive applications. Some alternative strategies were developed earlier in order to improve Alg gel quality for many applications. Prior attempts in similar applications relied upon polymer chemical modification and covalent linking with a wide range of molecules like polyoles, chitosan, to sorbitan esters, amongst others [7]. Exhaustive chemical alterations have rendered Algs untenable in physiological or biological applications. Biopolymeric blends in non-covalent interactions are immerging alternatives and are more clearly understood in very recent years. Furthermore, inherent biomolecular blend interactions are low energy processes that are industrially more acceptable and easy to prepare. Novel properties of individual polymer blends can also add advantages in pin point applications [8].

A very attractive molecule to develop polymer blends is Guar Gum (GG). GG is a biopolymer synthesized in the endosperm of *Cyamopsis tetragonolobus*, a seed legume commonly found in the north of India and Pakistan. Chemically, GG is a galactomannan composed by a linear chain of β 1,4-D-mannopyranoses to which D-galactopyranoses residues are α 1,6-linked at every second mannosyl residue forming short side-branches (2:1 ratio) with a molecular weight of about 200 kDa. GG is also a robust engineering biopolymer that has found applications in concretes, cement settings and in petroleum oil drilling as a drilling mud. Relevant GG properties include thixotropy (the decrease in viscosity over time at a constant shear rate) above 1.0% concentration in water, the ability to retard ice crystal growth non-specifically by slowing mass transfer across the solid/liquid interface, good stability during several freezing–thawing cycles, and water swelling activities pH-dependently. Additionally, GG has approximately 8-times the water-thickening potency of corn starch, and can be used in multi-phase formulations as an emulsifier preventing oil droplets from coalescing, and/or as a stabilizer in order to prevent molecular aggregation and particles from settling, and it can form gels in presence of calcium and other polyvalent cations [9]. GG is commonly used in cosmetics and foods as a thickener in the USA and in the EU (E412 additive code). In foods, GG is an additive in the dairy industry (yogurts, kefir, and liquid cheese products), also to prevent ice crystal formation on sauces and dressing, meats, baked goods, ice creams, and in dry foods (e.g. soups and deserts). However, GG standalone presents some problems to be handled, like low hydrophilicity and concomitantly slightly solubility in aqueous solutions. A typical strategy to increase the solubility and polymer compatibility of GG is by introducing polar groups in the main structure of polymer.

The GG trimethyl amine derivative is currently used in the hair conditioner Jaguar® under patent protection. Alternatively carboxymethylation of GG, is possible where in, the reactive primary hydroxyl groups of galactomannan could be substituted with carboxylate functions. Consequently, the Carboxy Methyl Guar Gum (CMGG) derivative can became more soluble in water and making clear and low viscosity solutions. Despite of many advantages, CMGG impede in standalone gel formation in presence of multivalent ions due to functional groups distance geometry and biopolymer structural constrains. Similar galactomannan biopolymers however can help shape polymer blends and develop interpenetrating smart materials networks for biological and environmental application [10].

In previous study, hundred percent encapsulation efficiency of BSA (bovine serum albumin) in Alg–GG matrix crosslinked with glutaraldehyde was reported. Also, the BSA release from the gel matrix was pH-dependent, and unsusceptible to freeze and thawing procedures [7]. The same research group described the entrapment of crosslinked subtilisin crystal aggregates in Alg–GG gel matrix increasing the enzyme stability under harsh environmental conditions [11]. Furthermore, a successful purification of the Jacalin lectin using Alg–CMGG in a fluidized bed technique was described [12]. Later, the swelling and degradation of Alg–CMGG cross-linked with divalent barium ion at different pHs and pretreatments was also reported [8]. On the other side, both Alg and GG biopolymers has been reported individually to have beneficial effect of human health reducing serum cholesterol, and having positive effects on blood glucose [13,14].

The aim of the present work is to develop alginate–carboxy methyl guar gum hydrogel blend microspheres containing CR as molecular cargo for drug delivery. That model could also be used further as a therapeutic agent to reduce abnormal protein β folding associated to some neuronal disorders.

In order to develop the biopolymer blend, Carboxy Methyl derivative of GG (CMGG) was synthesized and characterized by centesimal composition, derivatization degree, viscosity, surface tension studies, and FTIR. Alg–CMGG gel microsphere blend composition was optimized by controlled release kinetic and swelling studies *in vitro* under different experimental conditions. The studies were complemented by optical and scanning electronic microscopy (OM and SEM), and the interactions between the hydrogel components and cargo were analyzed by viscosimetry and correlated with FTIR and Raman spectroscopies.

2. Materials and methods

2.1. Materials

Congo Red (CR), the sodium salt of benzidinediazo-bis-1-naphthylamine-4-sulfonic acid (purity > 99%) was purchased from Merck (AG. Darmstadt, Germany). Low viscosity sodium Alginate (Alg) (average Mn 1.0×10^5 Da) was obtained from Biochem S.A. (Buenos Aires, Argentina). All other reagents used were of analytical grade purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany). Guar Gum (GG, average Mn = $(2.20 \pm 0.20) \times 10^5$ Da) was kindly provided by Hindustan Gums & Chemicals (India).

CR stock solutions (1.0 g/L) and its dilutions were made in miliQ water and/or proper buffers. Calibration curve of CR was made at λ_{max} 497 nm in a Beckman DU 640 UV–Vis spectrophotometer (Beckman-Coulter, CA, USA).

2.2. Synthesis of Carboxy Methyl Guar-gum (CMGG)

50.0 g of guar gum was taken in a three neck round bottom flask fitted with a condenser, a nitrogen purging set up and a mechanical stirrer. Isopropanol (100 mL) was added in the reaction mixture and the biopolymer was allowed to soak under stir for 30 min. Afterward, 30.0 ml of 0.25% (w/v) aqueous NaOH solution was added through the condenser and the resulting mixture stirred for additional 10 min at room temperature. A dropping funnel was then attached through a fork followed by the addition of 150 mL of 170 mg/mL aqueous chloroacetic acid solution to the flask. The solution pH was adjusted to 7.0 ± 0.2 with 1.0% (w/v) NaOH under nitrogen purging. The addition time altogether was 1 h. The stirring was continued further for three hours and the reaction temperature was raised to 55 °C. The reaction mixture was cooled and a light yellow solid separated by filtration through a Buckner funnel. The precipitate was washed twice with 80% (v/v) isopropanol in water, and 50% ethanol–water solution, and dried in a vacuum desiccator. The mass obtained was 43.0 g.

CMGG was further purified for analysis and applications. Typically, 1.0 gm of CMGG was suspended in a solution containing 5.0 mL of isopropanol and 15 mL of concentrated hydrochloric acid for 10 min; followed by washing with water until free from chloride.

2.3. Determination of degree of substitution in CMGG

The degree of surface functionalization on Guar Gum (GG) structure was confirmed by standard alkali titration. A weighed half from 1.0 g of CMGG sample was dissolved in excess of 100 mM sodium hydroxide and stirred over a magnetic stirrer for 20 min. The unreacted excess of sodium hydroxide was back titrated against standard 100 mM hydrochloric acid with methyl orange as indicator. A titration blank was simultaneously carried out without a CMGG sample. The other half portion of CMGG was dried in an oven at 102 °C to constant weight. This weight was taken as equal to the sample weight in alkali titration. The degree of GG substitution was estimated as follows:

$$\mathrm{DS} = \frac{M_1 T(V_0 - V_1)}{W - [M_2 \cdot T(V_0 - V_1)]} = \frac{162T(V_0 - V_1)}{W - [58T(V_0 - V_1)]} \quad (1)$$

where W is the weight of CMGG taken for test (calculated on dry material basis); V_0, the volume of hydrochloric acid consumed in blank titration without CMGG sample; V_1, the volume of the hydrochloric acid in titration of the test sample; T, the titer of the 100 mM hydrochloric acid solution; M_1, the molecular weight of the unsubstituted monomer unit (162); M_2, the molecular weight of the substituting unit (58).

2.4. Percentage composition (C, H, N, S) analysis

Percentage C, H, N, S analysis was carried out by combustion technique in the CHNS analyzer (model CHNS-932, M/s Leco Co., Mi, USA). Samples weighted in semi-micro balance Paul Bunge (model 23, Hamburg, Germany) were taken in a tin cup and were completely combusted in a stream of oxygen from cylinder. Evolving element oxides were monitored in instrument IR devise and compared against standard supplied samples of acetophenon, and cystene from Leco Co. (Mi, USA).

2.5. Surface tension studies

Surface tension lowering in water due to guar-gum and its derivative were studied as this center in one of the principle application area for guar gum compounds. GG and CMGG solutions (0.1%, w/v) were used for determination of surface tension at defined pH in distilled water (carbon dioxide free) in Dynamic Contact Angle Meter and Tensiometer (DCAT, Data Physics, Germany).

2.6. Viscosity studies

Analysis of 1.0% and 2.0% (v/v) aqueous solutions of GG and CMG were prepared in HPLC grade water and were degassed under vacuum. Viscosities were measured using number LV-2 to LV-4 spindle in a Brookfield viscometer (model LVT, Brookfield, USA). The results were compared against the standard viscosity sample supplied by the manufacturer. The viscosity resultants were recorded at 30 °C (Table 2).

The apparent viscosities of Alg–CMGG blends were determined by an Ubbelobde viscosimeter (S-100) with or without CR at 37 °C. The results were expressed as relative viscosities of each blend were estimated by apparent viscosities of the solutions divided by the apparent viscosity of the solvent under the same experimental conditions.

2.7. Thermal analysis

The temperature effect on stability of the polymers was studied by differential thermal (DTA) and thermogravimetry analyses (TGA). The studies were performed in Pyris-Diamond TG/DTA instrument (Perkin–Elmer, USA).

Small amount of polymers were kept in a platinum crucible under continuous nitrogen flow. Samples were scanned in the temperature range 30–350 °C with a heating rate of 10 °C/min.

2.8. Molecular weight studies determined by Dynamic Light Scattering (DLS)

Extensive chemical reactions often affect the average molecular weight of polymers. In order to determine the average molecular weight of GG and CMGG, light scattering experiments were carried out in Zetasizer (nano-ZS, Malvern Instruments, UK).

2.9. Biopolymer formulations, microsphere formation

Different Alg:CMGG hydrogel blends (from 100:0 to 68.5:31.5) were tested to determine optimal conditions for CR entrapment and microsphere stability. Biopolymer blends containing percentages of CMGG higher than 31.5% were not able to form stable hydrogels in our experimental conditions, and disregarded for further experiments.

Hydrogel microspheres were made by extrusion of the biopolymer mixture through a 100 μm syringe attached to a pump (Watson-Marlow, UK). Alg (3.5%) and CMGG (0.5%) solution with or without CR (0.4%) were dropped into a solution containing 50 mM $CaCl_2$ (0 °C) under continuous stirring to avoid bead coalescence. Microspheres were aged in calcium chloride solution for 48 h, followed by filtration on paper (Whatman #1). Filtered microspheres were kept in solution containing 50 mM $CaCl_2$ and 10 μM NaN_3 at 5 °C. Alternatively, microspheres were lyophilized and/or air-dried at room temperature and then stored as mentioned before.

2.10. Vibrationals analysis: FTIR and Raman spectroscopies

The FTIR analysis of GG and CMGG were carried out in a FTIR Jasco (model 670 Plus, Jasco, Japan). Each compound was pressed into a pellet in FTIR grade Potassium Bromide (KBr, Pike technologies, USA) and scanned with background correction at 256 scans, against a high energy ceramic source and DLATGS detector. FTIR scans corresponding to GG and CMGG are showed in Fig. 2.

Infrared determination of biopolymer formulations in KBr pellets were recorded in the 4000–400 cm^{-1} range on a Bruker Equinox 55 FTIR spectrophotometer (Billerica MA, USA). The Raman spectra of the solids, run in the region between 3500 and 100 cm^{-1}, were obtained with a FRA 106 accessory mounted on a Bruker IFS 66 FTIR instrument (Billerica MA, USA) with an excitation line from an Nd–YAG laser of 1064 nm. Infrared and Raman spectra were scanned at 4 cm^{-1} resolution. The assignments were based on data found in previous literature [15–19].

2.11. Scanning electron microscopy (SEM)

The surface morphology of freeze- and air-dried microspheres was determined using scanning electron microscopes (Philips SEM 505, Netherlands; or FEI, Quanta 200 SEM equipped with a Falcon System running Genesis 1.1, USA). Microspheres were sputtered with gold and scanned at an accelerating voltage of 25 kV.

Table 1
Structural composition studies on guar gum and derivatives.

Compound	CHNS analysis (%)				Degree of substitution	Yield (%)
	Calculated[a]		Observed[a]			
	C	H	C	H		
Guar-gum	32.9	4.57	30.2	4.79	–	–
Carboxymethyl guar gum	43.8	5.48	45.0	5.79	0.18	96

[a] N and S were not detected in all samples.

Table 2
Physicochemical characterization of the Guar-gum derivatives.

Compound	TGA thermal decomposition (°C)	Viscosity (cps)	Surface tension (dynes/cm)
Guar-gum	275	30,000	62.15
Carboxymethyl guar gum	212	350–400	36.59

2.12. Optical microscopy (OM) of microspheres were performed in swelling characteristics of alginate–CMGG hydrogels

The optical microscopy of microspheres were carried out in a Leica microscope DM 2500 (Wetzlar, Germany).

Swelling characteristics of Alg–CMGG microspheres were determined by a method described previously [19]. Dried test samples were incubated in 5.0 mL of 100 mM sodium phosphate buffer solution (pH 7.4) for 12 h. Later, samples were filtered and blotted with a paper filter to absorb the excess water on the surface. Samples swelling ratios (*Q*s) were calculated from the equation:

$$Qs = \frac{Ws - Wd}{Wd} \quad (2)$$

where *W*s is the weight of the swollen sample and *W*d is the weight of the dried test sample.

2.13. CR Loading

CR entrapment efficiency was determined by dissolving 100 mg of microspheres with loaded CR in 5.0 mL containing 100 mM sodium phosphate buffer (pH = 7.4), and centrifuged at 10,000*g* for 10 min (5 °C). CR was spectrophotometrically assayed in the supernatant in a as previously mentioned. The CR loading efficiency was estimated following Eq. (3).

$$\text{Entrapment efficiency}(\%) = \frac{\text{CR concentration in the supernatant}}{\text{Theoretical CR concentration}} \times 100 \quad (3)$$

2.14. Time release kinetics

Alg–CMGG microspheres (200 mg) containing 0.4% CR were incubated in a 5.0 mL solution containing 154 mM NaCl and 20 mM buffer at 37 °C as follows: Clark and Lubs (pH 1.2), phosphate (pH 6.8, 7.4 and 8.0), TES (pH 6.8, 7.4 and 8.0) under soft magnetic agitation for specified times.

Samples of 750 μL were taken out from 5.0 mL test vial and the volume was replaced by the respective buffer. Samples were centrifuged (10,000*g* for 5 min at 5 °C). The supernatants were collected and measured spectrophotometrically (λ_{max} = 497 nm) as mentioned before. All experiments were carried out by duplicate.

2.15. Microspheres stability and CR release under different pHs

Alg–CMGG gel microspheres containing CR were incubated at pH 1.2 and 37 °C during 25 min, washed and transferred to 20 mM phosphate buffer (pH = 7.4). The amount of CR released from the microspheres was spectrophotometrically determined as mentioned before.

3. Results and discussions

The anionic derivative CMGG was synthesized from GG following the scheme presented in Fig. 1. Infrared spectra of GG and CMGG showed some minor differences, which can be interpreted in terms of only 18% GG derivatization combined with the low sensitivity of the technique. In the GG FTIR spectrum, the 3429 cm^{-1} band was assigned to the O—H stretching mode which is very wide probably because is highly hydrogen-bonds. The introduction of an additional carboxylic group in CMGG increased the hydrogen bonding interaction. Therefore, the band become wider and shifted to 3407 cm^{-1}. Moreover, C—H stretching at about 2930 cm^{-1} shifts to lower wavenumber with the introduction of the —CH_2— group (Fig. 2). Other spectral regions remain almost unchanged after methylation process. In fact, the infrared spectrum of the GG does not show significant changes except for the H bond interactions as suggested by the infrared spectra shown in Fig. 2. Elemental analysis and percentage of C, H, N, and S was carried out by combustion technique and the results were enlisted in Table 1. Additionally, the anionic derivative CMGG was readily soluble in water and more particularly in NaOH solution indicating the presence of polar groups ($RCOO^-$ groups) on the biopolymer surface.

Derivatization of GG to the anionic type of product CMGG has significantly reduced the surface tension of water in 42% (from 72 dynes/cm to 36.59 dynes/cm) using 0.36 g/L sample. Meanwhile, GG showed a drop of surface tension by 10 units (from 72.00 to 62.15 dynes/cm, about 13.7%) when concentration of guar gum was reduced to 0.28 g/L (about 22.6% less sample). Viscosity analysis of 2.0% (v/v) aqueous solution of GG was observed to be 30,000 cps as it forms strongly hydrogen bonded thixotropic gel in demineralized water. However, the surface modified variant CMGG evaluated in the same concentration and temperature (30 °C) showed a dramatically decrease of viscosity to 350–400 cps, indicating a pseudoplastic type fluidity.

Thermal analysis (TGA and DTA) of polymers can provide information about thermal stability, conformational alterations and structural constitutions. The GG native polymer appeared quite stable in DT and TG curves. Decomposition process started around 230 °C (in TG curve, Fig. 3S of Supplementary material). This feature may be associated with the endothermic peak observed at 310 °C in the DT curve. These processes could be possibly associated with carbon dioxide and structural water loss at around 310 °C (Figs. 1S and 3S of Supplementary material). The CMGG

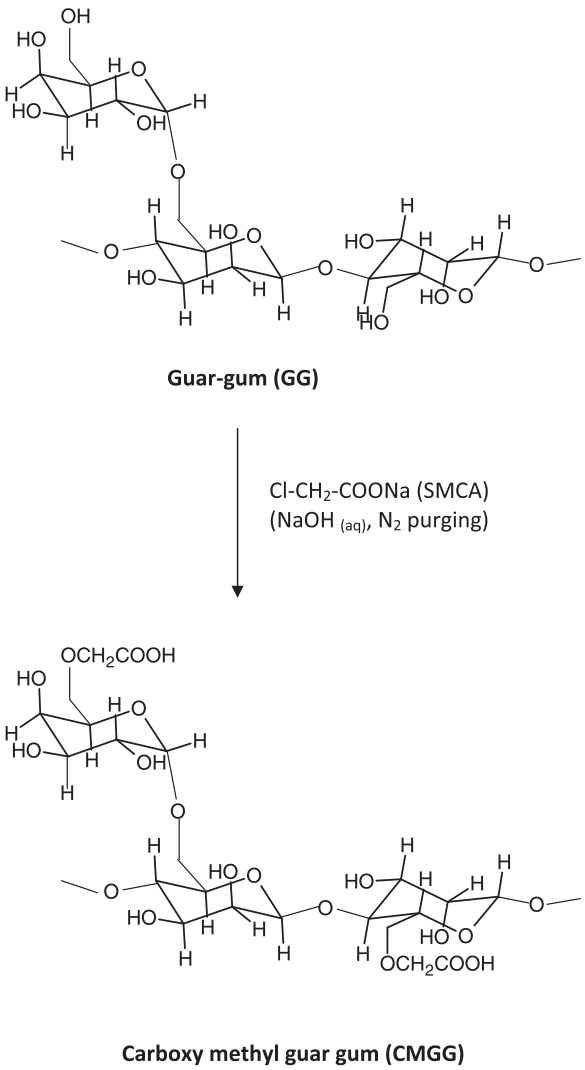

Fig. 1. Derivatization scheme of guar gum by carboxymethylation.

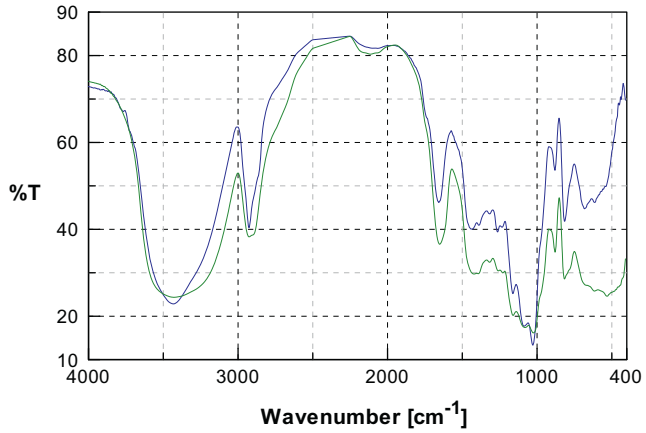

Fig. 2. FTIR spectroscopies of guar gum (blue line) and carboxy methyl guar gum (green line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

derived from the GG shows similar stability than GG because decomposition process started at around 210 °C. The polymer decomposition nature of GG indicates that the polymer is in a non-crystalline form while CMGG appeared partially crystalline, associating a nearly linear transition temperature in the temperature range 184.6–216 °C. This curve was leading to an exothermic range indicating the release of heat that corroborated the complete polymer degradation.

DTA curve of GG have an interesting endotherm peak at around 74 °C. This peak below 100 °C cannot be possibly associated to the water loss but it can be due to some polymer structural change. Both polymers were therefore appeared distinctly different in their DTA characteristics (Figs. 1S and 2S of Supplementary material).

Thermogravimetry involves the study of mass change with gradual increase in sample temperature. The thermogravimetric curve of guar gum showed the gradual loss of weight directly proportional to the increase of temperature in the range of 50–110 °C and associated to the gradual loss of water. The polymer becomes stable after this temperature range and up to 259.2 °C showing no mass change (Fig. 3S of Supplementary material).

The TGA curve of CMGG depicts a gradual and marked decomposition of polymer starting from the beginning up to 210 °C, later the curve reached a short plateau in the range of 210–240 °C indicating no-decomposition phase. After this range, rapid loss of CMGG weight is observed possibly due to the release of CO_2. The total decomposition of CMGG starts before 300 °C (Fig. 4S of Supplementary material). Hydrogen bonding between surface —OH functions perhaps is contributing to GG structural robustness that is significantly jeopardized anionic compound CMGG (Table 1S of Supplementary material). However, the carboxymethylation of GG do not produce a significant loss of polymer molecular weight, only approximately 4%, determined by DLS (Table 1S of Supplementary material).

Blends of Alg–CMGG in the range of 100:0–68:32 relative percentages were tested for optimal encapsulation of CR in presence of calcium chloride. On the other side, 100% Alg hydrogel microspheres are not able to keep gel morphology and constant cargo release kinetic under freeze and thawing procedures. Encapsulation of 0.4% CR was 89.7% in the 7:1 Alg:CMGG ratio (87.5:12.5%) in presence of 50 mM calcium chloride. Optical microscopy of microspheres showed spheroid morphology with a mean diameter of 25 μm and some heterogeneous CR dye distribution inside the gel (Fig. 6S of Supplementary material). CR aggregation was previously described from 0.3% and higher saline solution concentrations. CR supramolecular structures are engaged by stacking interactions along the main axis of the molecule tends to form rod-like or ribbon-like micellar structures [4]. However, micellar structures are thermodynamically unstable and time-course dependent. Molecular stability in such cases is strongly related to the chemical composition and physicochemical environmental factors. Considering this fact, gel structure was considered to play a key role in the controlled release of the model drug model (e.g. CR). *In vitro* swelling assays in water showed the incorporation of 135% (w/w) CR in dry Alg–CMGG microspheres without losing the gel structure. This results could potentially allow to target the large portion of the intestine (colon) with the Alg–CMGG blend for controlled release of CR-like drugs (e.g. doxorubicin), as because of the water exchange is the main mechanism of the organ physiological functions. CR-like drug molecular release may be controlled by the gel structure, drug dissolution and diffusion and the molecular interactions among the matrix components with the cargo.

SEM analysis of microspheres showed rougher structure of Alg gel microspheres rather than Alg–CMGG blend gel beads which are displaying smoother surfaces in both microscopy magnifications (Fig. 3). Also, the difference on image surface morphologies is confirming the presence of CMGG in the Alginate gel microspheres.

The relative viscosity in the selected Alg–CMGG blend solutions in presence or absence of CR were analyzed and showed linear

responses in both cases. However, incorporation of CR to the Alg–CMGG blend reduces the relative viscosity in about one third (Fig. 4). These results are suggesting strong interaction amongst CR and Alg–CMGG matrix solution. FTIR and Raman spectroscopies were further tested the hypothesis of CR and Alg–CMGG interaction.

FTIR and Raman spectroscopies were used to test the hypothesis of CR and Alg–CMGG interaction. The matrix formulation containing CR was analyzed simultaneously by infrared and Raman spectroscopies because both techniques are providing complementary information. CR bands are not observed in infrared spectra because it́s low concentration. Conversely, the strong dispersion observed for CR in Raman provided good quality spectra even in the low concentration level of CR. Consequently, infrared spectroscopy reveals interactions between matrix components while Raman evidenced interactions between CR and the matrix.

Wavenumbers and assignment of Raman spectra for the commercial CR and the CR in formulation are collected in Table 3. The Raman spectra are shown in Fig. 5S (see Supplementary materials). New bands were observed in the Raman spectra of the formulation containing CR at 1562, 1321, 1265 and 1176 cm^{-1}. The 1562 and 1176 cm^{-1} peaks corresponds to splitting bands of ν(phenyl ring) and ν(ϕ-N-azo), ν(—SO_2—O—)sym, respectively, while 1321 and 1265 cm^{-1} corresponds to GG bands contained in the matrix.

The naphthyl ring band at 1353 cm^{-1} undergoes appreciable shift to upper wavelength (+24 cm^{-1}) as consequence of CR-matrix interaction, while CR bands for ν(—N=N—)$_{azo}$ modes at 1453 and 1407 cm^{-1} does not shift significantly when the dye is inside the matrix, therefore this group does not interact with the biopolymer blend. The conclusion is also supported by structural considerations of the CR molecule considering the lone pair on nitrogen of azo groups is restricted by steric hindrance made by phenyl groups and naphthyl groups [19].

The main interaction between CR and the matrix is based on the aromatic motifs of CR associated with non-polar sections of the polymer blend determined by spectroscopic analysis [20]. Considering the "egg box" structure of alginate gels made by ionic gelation, and the low molecular weight and the planar structure of CR, it can be possible to hypothesize that the CR molecule could be located in the free non-ionic pockets in between two molecules linked by calcium ions [21]. Additionally, CMGG is not allowed to fit because of its molecular weight and hydrophilicity. This conclusion is relevant since the CR molecule entrapped inside the hydrophobic motif of alginate "egg-box" gel structure is not able to develop molecular stacking, and consequently the amount of CR released from the microspheres can be determined precisely increasing the CR biodisponibility concomitantly with a toxicity decrease.

Similarly, the adsorption of Direct blue 1 dye onto cellulose fibrils determined by Raman and UV–Vis spectroscopies was attributed to hydrogen bonding and by hydrophobic interaction between biphenyl groups of the diazo dye containing planar π-electrons and a polar regions of cellulose [22].

CR kinetic release from Alg–CMGG gel microspheres was studied *in vitro* under very acid (pH 1.2) and blood physiological (phosphate buffer, pH 7.4) conditions. No changes on microspheres morphology and CR release to the media were observed under acid conditions (pH 1.2) at 37 °C for 25 min. On the contrary, when the pH is increased above 6.0 a CR release from the microspheres to the media was detected. The CR kinetic release is showing hyperbolic behavior at pH 7.4 with 68.2% CR released in about 8 h (Fig. 5).

The changes in the CR release profile from the matrix can be explained based on the effect of environmental pH on the structure and chemical composition of alginate gels. Alginates are composed by beta-mannuronic acid (M units) and alpha-guluronic acid (G units) linked by 1–4 bonds with pKa of 3.38 and 3.65 respectively. Alginate hydrogels are formed by stacking of the G units in presence of multivalent ions, e.g. calcium, which cooperatively interact forming ionic bridges between different polymer chains making an structure known as "egg box" by analogy with egg containers. In between the crosslinking points, the mannuronic (M) units remain

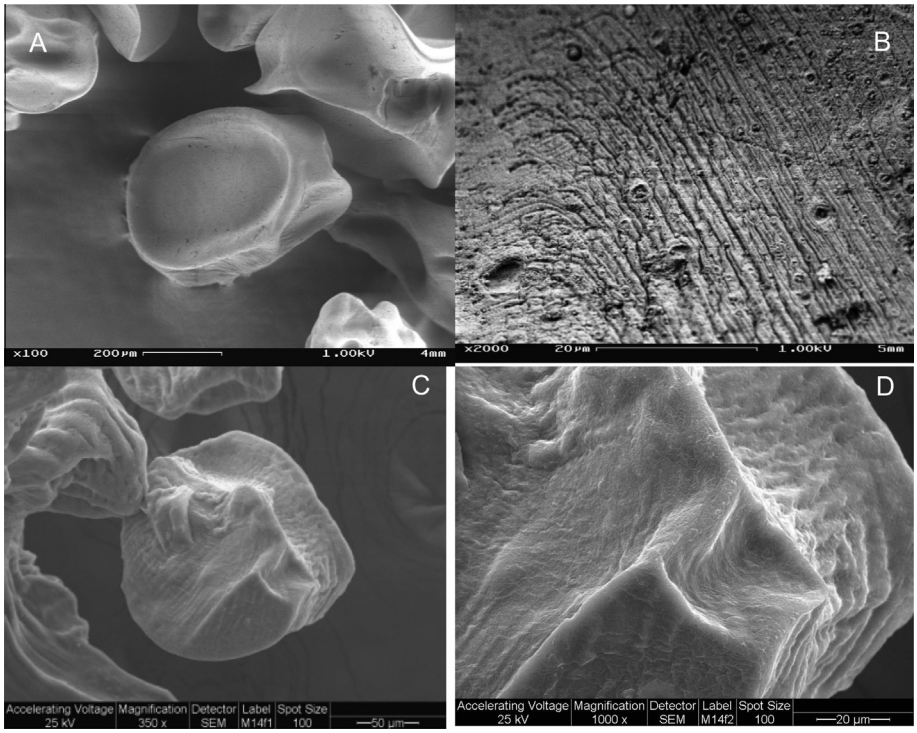

Fig. 3. SEM microscopy of Alg (A and B) and Alg–CMGG (7:1, C and D) microspheres.

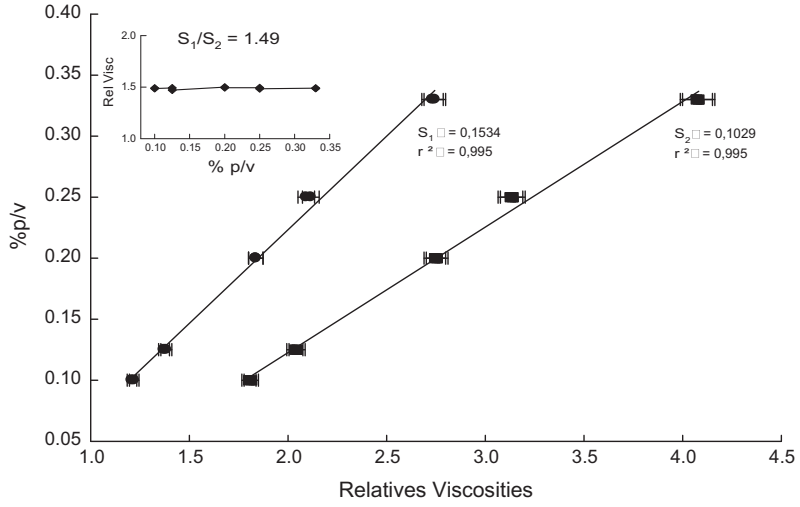

Fig. 4. Relative viscosity change of 1.0 and 2.0% Alg–CMGG blend solution in the absence (●) or presence (■) of CR, and the ratio between both curves (♦) at 37 °C.

Table 3
Raman assignments of CR molecule (solid state) pure and in the formulation (Alg–CMGG).

CR wavenumbers (cm^{-1})		Assignments
Pure	Formulation	
1590	1592	Phenyl ring
1453	1453	ν(—N=N—)azo
1407	1407	ν(—N=N—)azo
1353	**1377**	**Naphthyl ring**
1332	1332	Naphthyl ring
1279	1285	ν(ϕ-ϕ)
1155	**1176**	ν(ϕ-N-azo), ν(—SO_2—O—)sim

The idea was to highlight the major changes in the wavelengths betweeen the CR compared to the formulation.

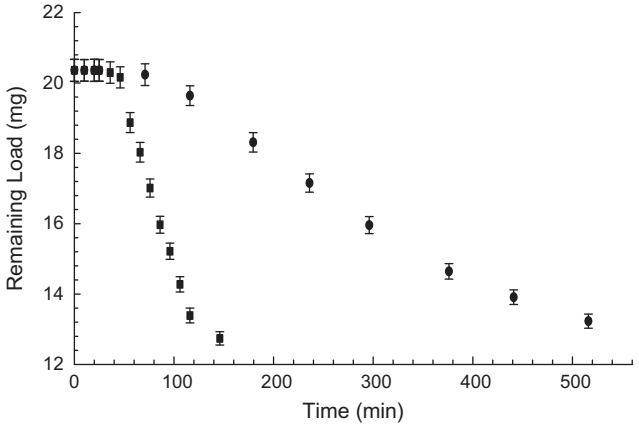

Fig. 5. Time release of CR from Alg (■) and Alg–CMGG (7:1, ●) in 154 mM NaCl and 20 mM phosphate buffer (pH 7.4) at 37 °C.

mostly free and the degree of ionization of carboxylate residues depends on external pH. At pH 1.2, the carboxylate of M unit is in the acid form (non-polar, pKa 3.38). Meanwhile at pH 6.8 to 8.0, the M carboxylate residue remains as anionic form. Based on the hydrophobic motifs of Congo Red and the interaction with the mannuronic acid residues, the dye is fast released from the matrix at alkaline pH compared to the acid one. Similar environmental sensitive gel matrices made of synthetic polymers from methacrylate derivatives containing different molecular cargoes were previously reported in the literature [23–25].

Additionally, the effect of CMGG coating on alginate microspheres is forming an additional diffusional barrier delaying the CR release from the gels.

4. Conclusions

CMGG derivative bring more soluble biopolymer compared to GG, easy to handle because of the low viscosity and making clear solutions, both are relevant properties for large scale use. CMGG stabilize Alg gels and let to perform freeze-dry procedures without losing gel structure allowing to slow release of the CR cargo.

Viscosimetric and spectroscopic analysis are revealing the interaction between the Alg–CMGG gel biopolymer blend and the aromatic rings of CR.

Encapsulation of CR in Alg–CMGG blend microspheres showed excellent stability at acid pH in where can be preserved with cargo release to the medium. The slow release of CR from the microspheres at pH 7.4 allows to control cargo delivery and demonstrating the advantage of encapsulation in Alg–CMGG to reduce the risk of molecular stacking favoring CR release as model for hydrophobic drugs. The present results could be also extrapolated to other molecules with pharmacological uses showing high toxicity and/or low biodisponibility.

Experiments in cell cultures and CR intracellular tracking studies are now under study in our laboratory.

Acknowledgments

Support from CONICET, Universidad Nacional de La Plata, and ANPCyT (PIP-0214, X/545, and PICT2011-2116 respectively) from Argentina to GRC; and from CONICET (PIP-0327), and Universidad Nacional de Luján to JAG is gratefully acknowledged. MEChV is member of Professional Staff of Comisión de Investigaciones Científicas, Provincia de Buenos Aires, Argentina (CICPBA). One of the author's (FA) would like to thank UGC, Govt. of India for a senior research fellowship grant.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reactfunctpolym.2014.06.006.

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