



Cross-linked carrageenan beads for controlled release delivery systems

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ABSTRACT

The objective of this study was to prepare and cross-link carrageenan beads that may be used as a controlled release delivery system. The influence of the bulk carrageenan and cross-linker concentrations on the bead size was studied in order to assess the mechanism of cross-linking between epichlorohydrin and the polysaccharide. The conditions were optimised on macroparticles (3.1 mm in diameter) for a better understanding of the cross-link density and its effect on the morphology and surface topography of the bead. It was shown that low epichlorohydrin concentrations led to unstable and weak beads with uneven and cracked surfaces. The optimum cross-linker concentration, which resulted in smooth and stable gel beads, was applied to microparticles (76 μm in diameter). The swelling/shrinking behaviour of these cross-linked microgels in saline solutions showed great potential for the application of these micro-sponges as delivery systems in food or pharmaceutical products.

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1. Introduction

Marine based polysaccharides are widely used in the food and pharmaceutical industry because of their biocompatibility and low toxicity. Some of these polysaccharides are polyelectrolytes and show great potential to interact with guest molecules and therefore are attractive materials as delivery vehicles for food products (Barreiro-Iglesias, Coronilla, Concheiro, & Alvarez-Lorenzo, 2005; George & Abraham, 2006; Lee et al., 2000; Moe, Skjak-Brak, Elgsaeter, & Smidsrod, 1993). κ -Carrageenan is a natural polysaccharide from marine seaweed whose gelling properties have been widely studied (Hjerde, Smidsrød, & Christensen, 1999; MacArtain, Jacquier, & Dawson, 2003; Sankalia, Mashru, Sankalia, & Sutariya, 2006) and consists of β -(1,3) sulphated D-galactose and α -(1,4)-3,6-anhydro-D-galactose. This gelling process depends on a coil-to-helix transition governed by the nature and amount of cations in solution as well as the temperature (Morris, Rees, & Robinson, 1980) which can potentially lead to interesting means of drug release by dissolution. Nevertheless, this thermal responsiveness seriously limits the application of carrageenan delivery systems in food products as the traditional pasteurisation or sterilisation processes involving high temperatures would melt and destroy them. Therefore, it seems that κ -carrageenan needs to be modified or chemically cross-linked so as to increase its stability in these harsh processing conditions. Biopolymers such as alginate or starch have been previously modified in order to confer higher resistance to extreme processing environments such as low and

high temperatures and low pH (George & Abraham, 2006; Hirsch & Kokini, 2002; Yeh & Yeh, 1993). Cross-linked hydrogels such as alginate and chitosan have demonstrated enormous swelling capability resulting in super-adsorbing matrices for delivery systems (Barreiro-Iglesias et al., 2005; Lee et al., 2000). Although carrageenan has been previously chemically modified in our laboratory (Jacquier, MacArtain, & Dawson, 2001), to our knowledge, cross-linking of carrageenan has never been attempted.

Epichlorohydrin is a well-known cross-linker for polysaccharides (De Miguel, Rieumajou, & Betbeder, 1999). The gel properties such as the swelling degree could be controlled by the cross-link agent concentration (Moe et al., 1993). It was also observed that the thermal stability of the cross-linked network increased by increasing the cross-linking degree (Denizli, Can, Rzaev, & Guner, 2004; Guner, Akman, & Rzaev, 2001).

The cross-linking degree in polysaccharides is usually very low as seen for example in food starches (Hirsch & Kokini, 2002). Although it proved difficult to measure chemically the extent of reaction and yield of cross-linked polymer, the physical properties of the cross-linked starches and their swelling behaviour as a function of cross-linking degree and salt concentrations indirectly provided conclusions about the cross-link density and success of reaction (Hirsch & Kokini, 2002).

In this study, the influence of carrageenan and epichlorohydrin concentrations on the swelling behaviour and surface topography of milliscale beads was investigated. The optimised cross-linking conditions were adapted to the microscale carrageenan spheres and the resulting microspheres' ability to respond to salt was studied as a potential delivery system for targeted release along the gut tract.

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2. Materials and methods

2.1. Preparation of beads

2.1.1. Carrageenan beads

Bulk κ -carrageenan (Gelcarin GP911, FMC Biopolymer, Philadelphia, PA, USA) gels were prepared by dissolving carrageenan powder in hot distilled water. The actual carrageenan content was analysed by drying samples ($n = 3$) to constant weight in a convection oven at 105 °C.

Homogeneous κ -carrageenan beads were made using a conventional dripping method (Moe et al., 1993; Zessen, Tramper, Rinze-
ma, & Beertink, 2005). Briefly, hot κ -carrageenan aqueous solutions were extruded from a syringe fitted with a 200 μ l pipette tip into a cold 100 mM KCl (Merck p.a. grade) hardening solution. To obtain spherical beads, cold rape seed oil (Millbrook Foods, Co., Meath, Ireland) was layered upon the hardening solution. After hardening, the beads were washed with general detergent (Teepol multi purpose detergent, Harvey Waddington, UK) diluted with KCl solution (100 mM) in a ratio of 1/100 (v/v) to ensure non-leaching of the gelling counterions and prevent bead dissolution. Images taken of the beads were analysed with image processing and analysis software (ImageJ, version 1.35s, <http://rsbweb.nih.gov/ij/index.html>). For a better contrast the surrounding solution was coloured with blue dextran polymer (~2,000,000 MW, Sigma). The sphericity of the beads was calculated as the ratio of the surface area of an equivalent sphere to the surface area of the bead, which in the case of oblate spheroids can be measured according to Eq. (1).

$$\psi = \frac{2\sqrt[3]{ab^2}}{a + \frac{b^2}{\sqrt{a^2 - b^2}} \ln \left(\frac{a + \sqrt{a^2 - b^2}}{b} \right)} \quad (1)$$

where a and b are the semi-major and semi-minor axes given by the ImageJ software respectively.

2.1.2. Microspheres

An emulsion of rape seed oil and 4% κ -carrageenan gel (5:1 v/v) was heated to 80 °C and stirred at 3000 rpm with a Silverson high speed rotor–stator mixer (model L4RT, Silverson Machines Ltd., Chesham, England) fitted with a fine emulsor screen (mesh: 800 μ m) for 20 min to ensure good homogeneity of the resulting emulsion. The emulsion was then cooled in an ice bath and stirred gently at 400 rpm with an overhead stirrer (Heidolph model RZR 2041, Schwabach, Germany) and kept at 10 °C or below for 30 min. As for the beads, the microspheres were then hardened with a 100 mM KCl solution and washed with general detergent solutions containing KCl to remove any remaining oil.

2.2. Cross-linking

The water in the beads or microgels was exchanged with 2-propanol (i-PrOH, Fluka, extra pure). The beads or microgels were washed with a mixture of water and 2-propanol where after each washing step the organic phase was increased until all water was removed (Jacquier et al., 2001). The beads or microgels were then mixed in an alcoholic alkaline solution (KOH 700 mM and i-PrOH 50% v/v). The cross-linking agent (Epichlorohydrin, from Aldrich, 99% GC) was added while stirring and left for 24 h to react. After cross-linking the beads were washed extensively with hot distilled water.

2.3. Particle size analysis

Particle size analysis was carried out using a Malvern Mastersizer S (Malvern Instruments Ltd., Morchester, UK) fitted with a 300

RF range lens (0.05–900 μ m) and a small volume sample dispersion unit. The Fraunhofer optical model was used as particle size was large enough for this model to give a good approximation of particle distribution without making any assumption about the optical properties of the microspheres (ISO13320 Particle Size Analysis, 1999).

The change of bead size is presented as swelling degree. The swelling degree or collapsing degree is defined as the ratio of the swollen or collapsed bead diameter to the native bead diameter D_0 .

2.4. SEM images

Images were produced by Cryogenic Scanning Electron Microscopy and carried out on Jeol 5410 (Akishima, Japan) scanning electron microscope with an Oxford Instruments (Oxford, UK) cryo-preparation chamber. The wet samples were loaded with a drop of microspheres and frozen in the liquid nitrogen slush, fractured under vacuum and sublimed at –80 °C for up to 30 min before sputter coating with gold and examined under an acceleration voltage of 15 kV. Dried samples were only sputter coated and examined (15 kV).

2.5. Statistical analysis

A multiple linear regression was used to analyse the results for each parameter studied based on correlation values and model significant levels. All means were subjected to Student's t -test to verify significant differences between the qualitative attributes investigated. Significance of differences was defined at $p < .05$. All statistical analyses were performed using SAS 9.1.3. (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. Gel beads

The conventional dripping method of bead manufacture provided significantly spherical beads with a homogenous surface within the carrageenan concentration range studied (1–6% w/w). Higher carrageenan concentrations did not produce spherical beads, probably due to the high viscosity of the dropping solution. By adjusting the dripping rate (10 drops/min), the carrageenan solution temperature (50 ± 4 °C) and an optimum distance of syringe tip to the hardening solution (2 cm), the bead size of the resulting native beads was found to be constant at a diameter (D_0) of 3.1 ± 0.1 mm with an excellent sphericity value ψ of 0.999 for the entire range of carrageenan concentrations. Although an increase in carrageenan concentration, which leads to a significant increase in dropping solution viscosity, should lead to larger beads according to Sankalia et al. (2006), the bead size was found to be not significantly different ($p > .005$) for all concentration used here, as reported by Sipahigil and Dortunc (2001). The implication for that phenomenon is probably the constant surface tension of the dripping solution. This leads to homogenous bead drops independently of the carrageenan concentration in the solution.

3.1.1. Effect of carrageenan concentration on the swelling degree of cross-linked beads

In order to assess the influence of bulk carrageenan concentration on the swelling behaviour of cross-linked beads, native beads of varying bulk gel concentrations (1–6%) were reacted with epichlorohydrin at low concentration (0.08 mol/L). Fig. 1 shows the evolution of the swelling degree of swollen cross-linked beads over bulk gel concentration compared to the untreated native beads represented as asymptote D_0 , which corresponds to the

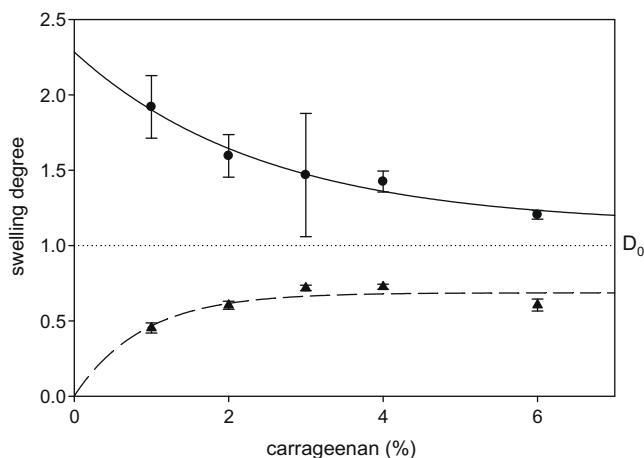


Fig. 1. Swelling degree of the cross-linked beads as a function of bulk carrageenan concentration. (●) Ratio of swollen and native beads, (▲) ratio of collapsed and native beads. The dotted line represents the diameter D_0 of native beads. The plain lines are visual fits only.

average diameter of 3.1 mm. The beads size during the cross-link reaction is shown as collapsing degree, ratio of collapsed bead diameter in reaction solvent and native bead diameter D_0 .

An apparent exponential decrease in swelling degree with bulk gel concentration is shown in Fig. 1, with the cross-linked beads made from the more concentrated carrageenan solutions showing less swelling, while the more dilute carrageenan beads demonstrated high swelling capability. Statistically it was found that the decrease in bead size with an increase of gel concentration was significant ($p < .05$), but that the sphericity of the cross-linked beads was not affected by the cross-linking reaction and remained very high (average ψ of 0.998).

Also shown on Fig. 1, the collapsing of the native bead in the alcoholic cross-linking solution was found to depend strongly on the polymer concentration and seemed to be at the highest degree at the lowest level of carrageenan concentration. Considering that the cross-linking reaction took place in the collapsed state one could assume that the collapsing degree influenced the final bead size, with the more collapsed native bead leading to denser carrageenan network and resulting in higher cross-linking efficiency. Nevertheless, it seems here that the extent of collapsing degree in the cross-linking medium does not supersede the native carrageenan concentration in determining the extent of cross-linked bead swelling.

3.1.2. Swelling degree as a function of epichlorohydrin concentration

In order to study the effect of cross-linker concentration on the swelling behaviour of the cross-linked beads, gel beads with fixed intermediate carrageenan concentration (3% w/w) were chosen. Fig. 2 shows the evolution of the ratio of diameters of the swollen cross-linked beads to the native beads (D_0) as a function of epichlorohydrin concentration.

At low epichlorohydrin concentration (<0.1 mol/L) the decreasing amount of epichlorohydrin resulted in an exponential increase in bead size ($>D_0$) with a defined degree of cross-linking. Very low epichlorohydrin concentration (<0.06 mol/L) resulted in unstable beads with a broken surface (Fig. 3B), while above this concentration beads were hard and possessed a smooth shape (Fig. 3A).

At intermediate epichlorohydrin concentrations, ($0.1 \text{ M} < [\text{ECH}] < 0.5 \text{ mol/L}$) no significant difference in bead size was noticed between cross-linked and native beads, while at very high epichlorohydrin concentration ($>0.5 \text{ mol/L}$), a slight increase in swollen bead size was seen.

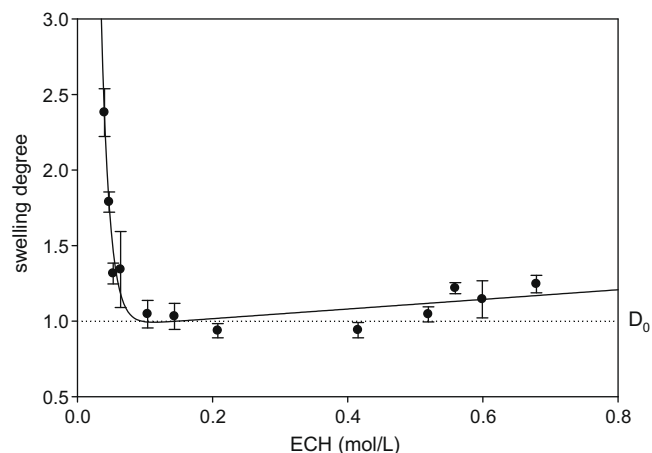


Fig. 2. Evolution of the swelling degree of the cross-linked beads as a function of epichlorohydrin concentration. The dotted line represents the native bead diameter (D_0). The plain line is visual fit only.

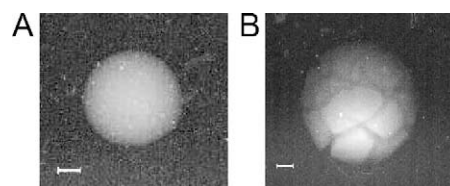


Fig. 3. Light microscopic images of beads with smooth shape (A) and with broken surface (B). White scale bar corresponds to 1 mm.

In previous studies about the cross-linking reaction of polysaccharides by epichlorohydrin it was assumed that one epichlorohydrin molecule built one cross-link between two polymer chains (De Miguel et al., 1999). The swelling degree, regulated by the cross-link density, was assumed to be controlled by the cross-linker molarity. The higher the cross-linking density the more concentrated and rigid the network becomes (Lee et al., 2000). In the case of cross-linked starch, Hirsch and Kokini (2002) showed how the viscosity and swelling degree could be influenced by changing the concentration of the covalently cross-linked bonds in the starch granule. The higher the cross-link degree was, the stiffer and smaller the starch granule became (Hirsch & Kokini, 2002). But in another study (De Miguel et al., 1999), it was found that at very high epichlorohydrin concentrations, the cross-linker was presumed to polymerise to form longer poly-oxyethylene-type cross-links between the polymer chains. The developed dimers or oligomeric structures could then be responsible for the higher swelling degree observed.

The results presented in this work tend to confirm this cross-linking mechanism, where very low epichlorohydrin concentrations ($<0.06 \text{ M}$) lead to highly swollen but unstable beads, probably due to an insufficient degree of cross-linking, while high epichlorohydrin concentrations ($>0.1 \text{ M}$) lead to poorly swelling beads until the cross-linker concentration is so high ($>0.5 \text{ M}$) that secondary polymerisation of the cross-linker occurs and results in a slight increase in the bead swelling degree.

As the object of this study was to examine the possibility of cross-linking carrageenan beads in order to obtain highly swellable “sponges” capable of encapsulating and target releasing bioactives upon swelling/shrinking, the range of optimum epichlorohydrin seems therefore to be between 0.06 and 0.1 M in order to yield beads with good swelling properties but yet stable structure.

3.2. Application of the optimised cross-linking conditions to microspheres

As delivery systems for food or pharmaceutical products are only applicable in micro scale, the next step in this study was the transfer of the optimised cross-linking conditions from millimetre scale beads to microscale spheres. The production of carrageenan microspheres of well defined size (100 μm) was based on an optimised emulsion technique previously developed in the laboratory (Ellis & Jacquier, 2009).

4% w/w carrageenan microspheres were then covalently cross-linked with epichlorohydrin (0.064 mol/L) in the same operating conditions as for the beads.

Fig. 4 shows the size distributions of native and cross-linked microspheres. Calculated from their respective De Brouckere Diameter ($D[4,3]$), the swelling degree or ratio of swollen microspheres ($D = 106 \mu\text{m}$) and untreated native microspheres ($D_0 = 76 \mu\text{m}$) was approximately 1.4, and showed very similar response in swelling behaviour than the milliscale beads (Fig. 1).

In Fig. 5, SEM micrographs show the structured network resulting from the cross-linking reaction. Fig. 5A represents a fractured bead where it can be clearly noticed that the cross-link reaction took place through the whole carrageenan network and not only on the surface which resulted in this sponge-like network formation. The image of the bead surface (Fig. 5B) reveals the characteristically rough topography of the bead when subjected to cryoscopic preparation.

The sponge-like cross-linked network formation led to the assumption that the matrix structure was stable and could endure harsh conditions such as drying process. The covalently cross-linked microspheres were collapsed and dried. The micrograph of the dried microspheres (Fig. 5C) shows that the microspheres were heat stable and kept their spherical shape even during the drying process. Re-swelling the dried spheres in aqueous solution resulted in no noticeable alteration in shape or size properties.

In conclusion, the cross-linking reaction which was developed on milliscale beads was successfully applied on microspheres with comparable resulting network properties.

3.2.1. Swelling degree as a function of salt concentration

The object of this study was to examine the possibility of cross-linking carrageenan microspheres in order to obtain highly swellable “sponges” capable of encapsulating and target releasing bioactives upon swelling/shrinking. As carrageenan is a highly charged

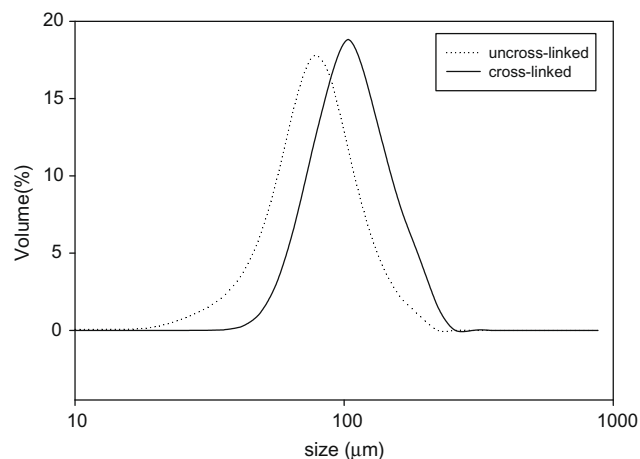


Fig. 4. Size distribution of native and cross-linked microspheres. Uncross-linked beads ($D[4,3] = 76 \mu\text{m}$) in 100 mM KCl, cross-linked beads ($D[4,3] = 106 \mu\text{m}$) in water.

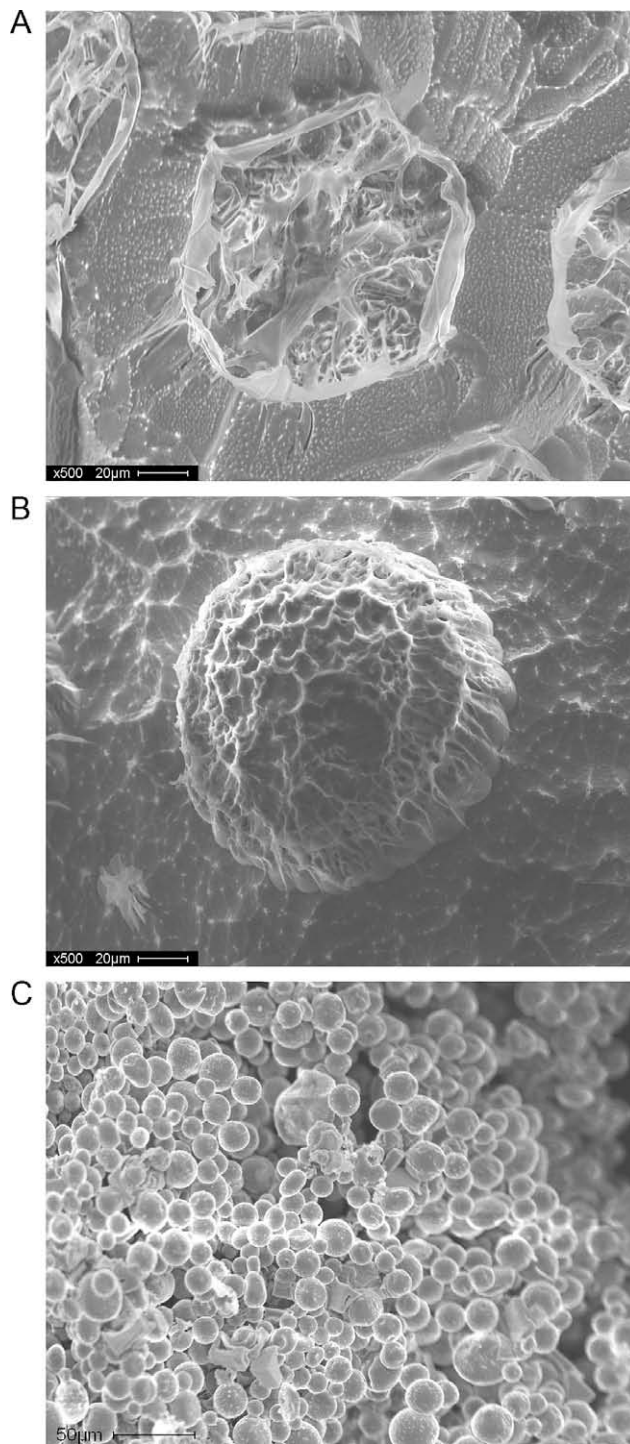


Fig. 5. Scanning electron microscopy images of cross-linked microspheres in water. Hydrated microspheres images taken under cryo SEM show fractured (A) and surface (B) of the bead and collapsed and dried at 40 °C (C).

polymer with affinity to specific metallic cations (MacArtain et al., 2003; Morris et al., 1980), the swelling behaviour of these microgels was studied as a function of salt concentration (Fig. 6).

At low salt concentration ($<10^{-3}$ M KCl) the beads swelled to their maximum De Brouckere Diameter $D[4,3]$ of 106 μm , while higher salt concentrations (10^{-3} M $<$ KCl $<$ 10^{-1} M) lead to a rapid decrease in microgel size to 66 μm . Further salt addition did not result in any further microgel shrinking, but it is noteworthy to mention here that the minimum shrunken diameter was significantly lower than the native microsphere diameter ($D_0 = 76 \mu\text{m}$).

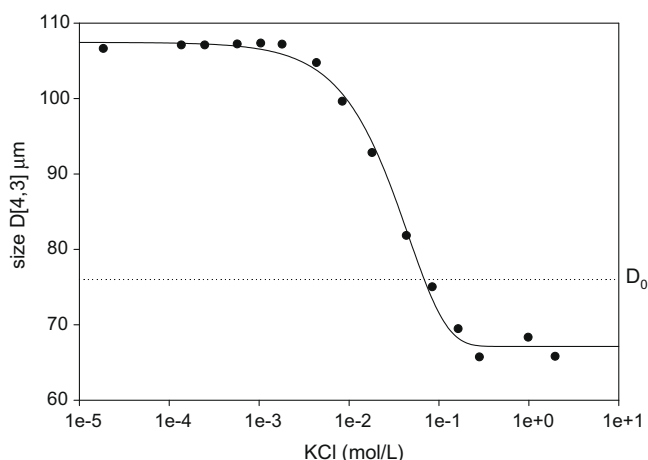


Fig. 6. Evolution of the diameter of cross-linked microspheres as a function of salt concentration. Dotted line represents the native bead diameter (D_0). The plain line is visual fit only.

This salt responsiveness was previously noted in the case of alginate (Moe et al., 1993) and gellan (Annaka, Ogata, & Nakahira, 2000), both highly negatively charged polymers like carrageenan. This common behaviour was attributed as being due to the ionic contribution to swelling and explained due to the effect of the Donnan equilibrium. Qualitatively, the cross-linked microspheres act as semi-permeable membranes with a relatively high concentration of fixed negative polymer charges which, in low salt solutions, will draw in a comparatively large concentration of free positive counterions from the solution and induce a concentration imbalance in counterions between the inside of the microsphere and the surrounding solution. In order to minimise this chemical imbalance, water then penetrates the microgel resulting in swelling up to the maximum network elastic potential. Higher free counterion concentrations in the surrounding solution will negate this effect and will result in microgel shrinking.

In conclusion, this phenomenon results in highly swelling microgels with a variation in volume of more than 400% between shrunken state in high salt and swollen state in dilute solutions which can be of tremendous interest for targeted release of the microgels contents in the gastrointestinal tract.

4. Conclusion

The objective of this study was to prepare and cross-link carrageenan beads that may be used as a controlled release encapsulation system. It was shown that both bulk polymer concentration and cross-linker concentration influence the size of the swollen cross-linked beads. With this knowledge it was possible to manufacture optimally cross-linked microspheres. These showed high

salt responsiveness which proves them to be very attractive smart delivery systems where a change in the surrounding saline conditions upon ingestion results in a substantial shrinking of these micro-sponges and a targeted release of their content in the gastrointestinal tract.

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