

Kinetics of ascorbic acid transport from alginate beads during in vitro digestion

NATALYA GORBUNOVA – ALEKSANDR EVTEEV – IVAN EVDOKIMOV – ANNA BANNIKOVA

Summary

This work aimed at the behaviour of multilayer alginate beads loaded with antioxidants depending on the type of preparation and at characterization of their transport rates under simulated gastrointestinal conditions. Changes in mechanical properties reflected changes in the gel microstructure with the beads becoming stronger in gastric phase (values of Young's modulus of approx. 4×10^4 Pa) and weaker in intestinal conditions (Young's modulus of approx. 0.5×10^4 Pa). Investigation of the swelling behaviour showed that all beads shrank in gastric conditions and swelled in intestinal conditions, with multilayer capsules displaying more significant shrinkage and swelling (by 40%). Results on the release of the antioxidant from the capsules suggested that the preparations were able to withstand harsh gastric conditions retaining more than a half of ascorbic acid at the end of gastric phase, and facilitate its transport to the simulated small intestine. A direct relationship between mechanical characteristics of the alginate beads and transport of the bioactive compound was established within the experimental time. It was shown that 90% of the bioactive transport was governed by the nature of the capsules, where their mechanical properties are considered to be a basis of optimal utilization of the controlled delivery.

Keywords

alginate; multilayer capsules; release; in vitro hydrolysis; mechanical properties

Development of novel functional foods provides an opportunity to improve the quality of foods available to consumers and to provide benefit to their health [1]. Functional food may be prepared by application of capsules to deliver bioactive compound(s) in order to protect the active compound(s) from environmental conditions and enhance stability, thus maintaining properties as well as masking undesirable odours or tastes [2–4]. Alginate is a non-toxic, versatile and economical hydrogel. Its most attractive property is that the gel formation is induced by the addition of various divalent cations [5–7]. For alginate gelation, the most widely used encapsulation technique is the extrusion method, which consists of dropping an aqueous solution of polymer and active principle into a gelling bath of calcium chloride [8]. Thus,

when ionic-induced gelation occurs, alginate forms a polymeric matrix trapping the active component inside [9–11].

Physico-chemical properties of alginate gel beads utilized as a carrier for encapsulation of bioactive compounds in simulated gastro-intestinal conditions were recently widely studied. RAYMENT et al. [12] reported that the beads shrink in gastric conditions and swell in intestinal conditions due to changes in electrostatic forces in the gel matrix at different pH and ionic strength conditions. Mechanical properties of the beads similarly reflect the changes in the gel microstructure with the beads becoming stronger in gastric conditions and weaker in intestinal conditions. In a study of lactoferrin encapsulation in alginate microbeads, its digestion in gastric conditions was shown to

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take place within 2 h, whereas digestion of all forms of lactoferrin in the intestinal conditions was very rapid, i.e. within 10 min [13].

The problem of vitamins deficiency is relevant during whole year since it is related mainly with inadequate nutrition. According to statistics, from 60% to 80% of population is experiencing a steady lack of vitamin B complex, whereas vitamin C deficiency was observed in almost 100% of people. In a view of the actual problem of modern nutrition, various functional foods enriched with micro-nutrients have been developed over the years. The worldwide research interest remains in improvement of stability and quality of processed foods, where chemical reaction pathways and enzymatic processes are critical points.

It has long been known that ascorbic acid has a protective role as an antioxidant in various foods and dietary supplements. However, it is unstable in air, light, oxygen, moisture, basic pH conditions, and high temperature, being easily degradable from the biologically active substance (L-ascorbic acid and dehydroascorbic acid) to 2,3-diketogulonic acid, which has no biological activity in the human body [14, 15]. The biological activity of vitamin C is preserved to a good extent when delivered within a polymeric matrix using, for example, spray-drying techniques [16, 17]. The purpose of this study was to provide understanding on kinetics of the release of ascorbic acid from capsules containing alginate during in vitro digestion, taking into account different methods of their preparation. This can provide information whether the specific preparation procedure of alginate beads with encapsulated ascorbic acid can protect the bioactive compound from the harsh gastric conditions. The work utilizes rheological profile of alginate in combination with ascorbic acid as well as the mechanical properties of alginate beads at different stages of in vitro digestion complemented with the changes in weight and size of the capsules. Further, the rate of transport of ascorbic acid from alginate beads prepared by different procedures was determined, thus confirming the controlled delivery of the bioactive compound in model small intestine of a human digestive system.

MATERIALS AND METHODS

Materials

Sodium alginate (form of a powder) was from Sigma Aldrich (St. Louis, Missouri, USA). According to the specification sheet, viscosity of 1% (w/w) sodium alginate in distilled water was

0.02 Pa·s. Ascorbic acid was obtained from Hebei Welcome Pharmaceutical (Shijiazhuang, China); the chemical was in the form of white crystals of analytical grade (purity more than 99%). Analytical reagents sodium tetraborate and 40 mmol·l⁻¹ sodium dodecyl sulfate, calcium chloride, sodium chloride, hydrochloric acid, monobasic potassium phosphate were obtained from BDH Chemicals (Poole, United Kingdom). All reagents were used without further purification. Bile salts, pancreatin (6000 U) and pepsin (3600 U) were purchased from Aventis Farma (Mumbai, India).

Sample preparation

For rheological investigations, 1.5% (w/w) sodium alginate solution was prepared by gradually sprinkling the biopolymer powder into deionized water whilst stirring at 22 °C. The solution was stirred for approx. 2 h to ensure complete hydration and left to stand for a further 30 min to allow any bubbles to surface before preparing the alginate beads. Then, to 40 ml of alginate solution, 10 ml of calcium chloride solution was added in a concentration of 3, 9, 12 or 15 mmol·l⁻¹. The sample containing 1.5% alginate (w/w), 1.0% ascorbic acid (w/w), 12 mmol·l⁻¹ calcium chloride (pH 3.21) was used to study the structural behaviour of alginate gels depending on pH of the system, which was adjusted with 1 mol·l⁻¹ HCl and 0.5 mol·l⁻¹ NaOH to 2, 4, 6, 8.

For alginate beads preparation, all beads were prepared at room temperature. Three different ways to prepare the alginate beads were used:

Procedure 1

1.5% (w/w) sodium alginate dissolved in deionized water with continuous stirring for 2 h at 22 °C. The solution was left to stand for a further 30 min to allow any bubbles to surface before preparing the alginate beads. A volume of 100 ml of the alginate solution was dripped into 200 ml solution containing 12 mmol·l⁻¹ calcium chloride and 1.0% ascorbic acid using a 250 ml funnel to create a stream of droplets. Once all the alginate solution had dripped into the calcium chloride – ascorbic acid solution, the beads were left there for 30 min and then sieved from the solution and rinsed using deionized water. The beads were stored in a container with fresh 12 mmol·l⁻¹ calcium chloride solution at 5 °C for 22 h until characterization.

Procedure 2

1.5% (w/w) sodium alginate and 1.0% (w/w) ascorbic acid were dissolved in deionized water with continuous stirring. Same as for Procedure 1, the solution was dripped into 50 ml of 12 mmol·l⁻¹

calcium chloride solution until the beads were formed. The beads were left in calcium chloride bath for 30 min, then sieved and rinsed with deionized water. The beads were stored as described previously.

Procedure 3

1.5% (w/w) sodium alginate was dissolved in deionized water with continuous stirring. The solution was dripped into 50 ml of 12 mmol·l⁻¹ calcium chloride solution until the beads were formed. The beads were left in calcium chloride bath for 30 min, then sieved and rinsed with deionized water. To make multilayer capsules, the beads were then placed into 50 ml of 1.0% (w/w) ascorbic acid solution and left there for 30 min, then transferred into calcium chloride bath and left to stand for 30 min, and finally rinsed with deionized water. The beads were stored as described previously.

Rheological analysis

Small deformation dynamic oscillation in shear was used to provide readings of the elastic (G' ; storage modulus) and viscous (G'' , loss modulus) components of the network. These two parameters are part of the complex shear modulus (Eq. 1) and variations of the relative liquid-like and solid-like structure of the material with time and temperature.

$$G^* = G' + G'' \quad (1)$$

where G^* is a complex modulus (in pascals); G' is a storage modulus and G'' is a loss modulus.

Measurements were carried out on MCR 102 (Anton Paar, Wundschuh, Austria) with magnetic trust bearing technology.

Samples of sodium alginate in the presence of 3, 9, 12 and 15 mmol·l⁻¹ of calcium chloride at a total level of solids of 1.5% (w/w), and samples containing sodium alginate (1.5%), ascorbic acid (1.0% w/w) in the presence of 12 mmol·l⁻¹ calcium chloride at different pH (2, 4, 6, 8) that was adjusted with addition of 1 mol·l⁻¹ HCl and 0.5 mol·l⁻¹ NaOH, were loaded on the pre-heated Peltier plate at 40 °C. A measuring geometry of 50 mm diameter was used within the experimental temperature range. The preparations of alginate and ascorbic acid were cooled from 40 °C to 5 °C at 1 °C·min⁻¹ with a frequency of 1 rad·s⁻¹ and strain of 0.1%, then they were kept at 5 °C for 15 min and finally heated up to 20 °C at the same rate. All experiments were performed within the linear viscoelastic region of the material.

In vitro digestion

A digestion protocol that simulates the gastric

and intestinal digestion in the gastrointestinal tract was designed and adapted to the systems studied in order to take into account the structural characteristics of the alginate beads and release kinetics of model antioxidant. In the gastric stage, the simulated gastric fluid (SGF) was used, which contained 2 g·kg⁻¹ NaCl solution in Millipore water (Millipore, Billerica, Massachusetts, USA) and pH adjusted to 2 with 1 mol·l⁻¹ HCl and pepsin 3600 U·ml⁻¹. To 25 g of gel beads, 500 ml of pre-warmed (37 °C) SGF was added. Samples were incubated in a water bath under constant horizontal shaking (37 °C, 1.67 Hz) for a set time interval. After 15, 30, 45, 60, 75, 90, 105 and 120 min the samples were washed with deionized water. An amount of 200 mg of alginate beads were taken at each stage of simulated gastric digestion and analysed for ascorbic acid concentration.

For in vitro intestinal digestion, initial digestion of the encapsulated micro-gel particles in SGF was conducted for 2 h at 37 °C. Then, to (20 ± 2) g of micro-gel particles, being digested in simulated gastric conditions, 400 ml of pre-warmed (37 °C) simulated intestinal fluid (0.7% monobasic potassium phosphate; 0.1% bile salts; 0.4% pancreatin) was added. The pH was adjusted to 7.5 with 0.5 mol·l⁻¹ NaOH (approx. 40 ml). The entire sample was incubated at 37 °C under constant horizontal shaking (1.67 Hz) for a set time interval (up to 20 min). At the end of the set time interval, 200 mg of digested sample were washed with deionized water and analysed for ascorbic acid concentration.

Determination of ascorbic acid concentration

An amount of 500 mg of digested and washed with deionized water alginate beads were crushed and dissolved in deionized water. Samples were kept within 30 min with continuous stirring at 22 °C. Then, an aliquot of 1 ml was taken, filtered, centrifuged for 3 min at 8000 ×g and the supernatant was used for ascorbic acid determination. To determine ascorbic acid, capillary electrophoresis was utilized. To carry out this method, the buffer which contained 0.02 mol·l⁻¹ sodium tetraborate and 0.04 mol·l⁻¹ sodium dodecyl sulfate was placed in capillaries for performing high efficiency separations of both large and small molecules at 20 °C. Concentration of ascorbic acid in the sample was determined using a special software and a calibration curve, which was previously constructed with a range of ascorbic acid concentrations from 0.01 ml·l⁻¹ to 10 ml·l⁻¹ in distilled water.

Swell ratio determination

The beads were tested for their swelling cha-

acteristics in gastric and intestinal conditions in vitro. The swell ratio (S) was determined using the following equation:

$$S = \frac{m_f - m_i}{m_i} \times 100 \quad (2)$$

where m_f is final bead mass (in grams); m_i is initial bead mass (in grams) [12].

Texture profile analysis

Uniaxial compressing of single alginate beads digested within certain time interval was made by using Brookfield CT3 Texture Analyzer with a load cell of 5 kg (Brookfield Engineering Laboratories, Middleboro, Massachusetts, USA). Beads were immersed in water and placed onto a flat platform. The measuring geometry consisted of a cylindrical aluminium probe (6 mm diameter), which was driven to compress the sample. Tests were carried out at $1.5 \text{ mm} \cdot \text{s}^{-1}$ with a trigger load of 0.067 N and a compressive deformation of up to 30% of the original height. The probe was set to return to its original position immediately after compression. Thirty beads from each sample were compressed in order to give statistically representative results. All experiments were conducted at room temperature ($22^\circ\text{C} \pm 1^\circ\text{C}$).

Young's modulus E (in pascals) was calculated using the following equation:

$$E = \frac{3(1-\nu^2) \times F}{\sqrt{d} \times H^3} \quad (3)$$

where d is the diameter of a bead (in metres), F is the force applied to bead (in newtons), H is the displacement (in metres) and ν is the Poisson ratio.

Optical microscopy

Light microscopy of beads was employed to provide tangible evidence of the network topology of alginate beads. Images were obtained using MBS-10 Microscope (Litkarsk, Litkarino, Russia) with an attached digital camera Digital Camera DCM 35 (Hitachi, Tokyo, Japan). Samples were prepared for microscopic examination by placing the beads on a glass slide, ensuring that their structure was not destroyed, and obtaining a flat imaging plane.

Encapsulation yield

Encapsulation yield (EY) of alginate beads with ascorbic acid was calculated as the ratio between the weight of microcapsules (m_e , in grams), obtained at the end of the process, and the initial weight of ascorbic acid (m_{iAA} , in grams) and polymers (m_{iP} , in grams), according to Eq. 4 and ex-

pressed in percent [18]:

$$EY = \frac{m_e}{m_{iAA} + m_{iP}} \times 100 \quad (4)$$

Encapsulation efficiency

Encapsulation ratio was calculated as the ratio between the weight of ascorbic acid in the microcapsule and its initial weight to be encapsulated [18]. An amount of 200 mg of washed with deionized water alginate beads were crushed and homogenized with the addition of phosphate buffer (pH 7.4), and the concentration of ascorbic acid was determined as described earlier following the calculation of ascorbic acid concentration in one capsule. Each sample was analysed in triplicate, and average results for the encapsulation efficiency (EE) were calculated according to the Eq. 5:

$$EE = \frac{m_{AA}}{m_{tAA}} \times 100 \quad (5)$$

where m_{AA} is weight of ascorbic acid in the microcapsule (in grams), and m_{tAA} is a theoretical weight of ascorbic acid to be encapsulated (in grams).

Moisture analysis

Moisture content of alginate beads was determined using a moisture analyser Evlas 2M (Siga-gropribor, Novosibirsk, Russia). Three grams of sample was loaded on an aluminium plate and heated to 103°C for 40 min. Average of three replicate measurements was reported.

Water activity analysis

Water activity of capsules was determined using AVK R9 water activity meter (AVK, Saratov, Russia). The experiment was performed at room temperature (22°C) using 10 g of sample. Average of three replicate measurements was reported.

Colour measurement

The colour of alginate beads was measured using a NR 100 colorimeter (3NH Technology, Shanghai, China). In the analysis, three different colour parameters were recorded, namely, L^* (degree of whiteness/darkness), a^* (degree of redness and greenness) and b^* (degree of yellowness and blueness). Multiple sets of readings ($n \geq 10$) were performed in all samples randomly, and average values were reported.

Determination of calcium

To determine Ca, capillary electrophoresis was utilized (Kapel 105M, Lumex, Saint-Petersburg,

Russia) at a voltage of 25 kV. The electrophoretogram was obtained at excitation wavelength of 254 nm. To carry out this method, 0.02 mol·l⁻¹ benzimidazole containing 8 mmol·l⁻¹ tartaric acid and 2 mmol·l⁻¹ 18-Crown-6 (Sigma Aldrich) were placed in capillaries for performing high efficiency separations of both large and small molecules at 20 °C. An amount of 100 mg of washed with deionized water alginate beads were crushed and dissolved in hydrochloric acid, which was previously prepared by dilution of concentrated hydrochloric acid with distilled water in a ratio of 1:1. Samples were kept for 16 h with continuous stirring at 110 °C. Then, an aliquot of 0.5 ml was taken and dried for 30 min at 60 °C. After drying, the rest of the dried component was dissolved in 0.5 ml of distilled water, filtered, centrifuged for 3 min at 8000 ×g, and supernatant was used for analysis of Ca. Concentration of Ca in the sample was defined using a Elforun ver. 3.2.3 software (Lumex) and a calibration curve, which was constructed with Ca²⁺ from 0.1 ml·l⁻¹ to 0.5 ml·l⁻¹ in distilled water. All experiments were performed in triplicate with data statistical testing by ANOVA (one way, $p > 0.05$).

RESULTS AND DISCUSSION

Small deformation dynamic oscillation measurements of alginate gels with the presence of ascorbic acid

Detailed investigation on the effect of pH and addition of divalent cations to alginate matrices at concentration levels of industrial interest de-

monstrated that there was a sol-gel transition depending on the nature of the cation, the polymer and cation concentration, and the ionic strength [19]. In investigation of physicochemical changes in alginate systems depending on concentration of calcium and pH, we made a start by considering cooling profiles of the sodium alginate plus ascorbic acid mixtures at a standard frequency of oscillation (1 rad·s⁻¹) and strain amplitude (0.1%).

As shown in Fig.1A, controlled cooling at 1 °C·min⁻¹ from 40 °C to 0 °C of 1.5% sodium alginate plus 1.0% ascorbic acid at increased amounts of ions (3–15 mmol·l⁻¹) exhibited a trend of enhanced network strength. The “egg-box” model is generally invoked to explain how the divalent cations, bound in the interchain cavities (essentially polyguluronate sequences) giving a rise to a rod-like cross-linked complex [19]. However, the maximum gel strength occurs at a calcium ion concentration of about 15 mmol·l⁻¹, which is a 40% stoichiometric equivalent. Reduction in G' values at lower concentrations of calcium chloride is an expected consequence of formation of fewer intermolecular junction zones. PAPAGEORGIOU, KASAPIS and GOTHARD [20] showed that further increase in salt content up to stoichiometric 80% led to a reduction of the gel network due to excessive, calcium-induced aggregation of egg-box dimers, driving the network towards a precipitate. However, this was not an interest in the current investigation. Having investigated the behaviour of alginate systems at different salt addition, the concentration of 12 mmol·l⁻¹ of calcium chloride was chosen for further studies on the effect of pH on the sodium alginate-ascorbic acid preparations

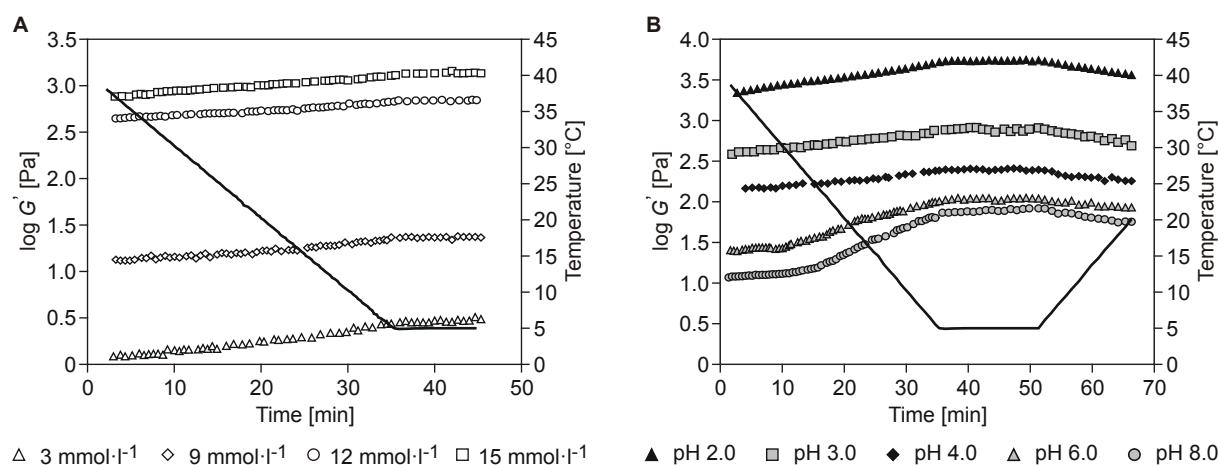


Fig. 1. Changes in storage modulus of alginate gels containing ascorbic acid.

A – dependence of storage modulus on concentration of CaCl_2 , B – dependence of storage modulus on pH. Systems contained sodium alginate (1.5% w/w) and ascorbic acid (1% w/w). G' – storage modulus.

Tab. 1. Physico-chemical properties of different alginate beads after preparation.

Alginate beads	Procedure 1	Procedure 2	Procedure 3
Encapsulation yield [%]	53.4 ± 1.8	52.7 ± 2.1	52.7 ± 2.2
Encapsulation efficiency [%]	36.1 ± 3.7	38.0 ± 4.5	35.6 ± 3.7
Moisture [%]	98.6 ± 0.3	98.4 ± 0.1	98.5 ± 0.3
Ascorbic acid [ml·l ⁻¹]	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.1
Calcium [mg·ml ⁻¹]	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Water activity	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01
L* colour	27.54 ± 1.21	29.50 ± 0.91	28.85 ± 1.13
a* colour	1.95 ± 0.16	2.71 ± 0.14	2.13 ± 0.16
b* colour	5.74 ± 0.18	5.67 ± 0.24	5.86 ± 0.43
Diameter [mm]	4.6 ± 0.3	4.2 ± 0.4	4.5 ± 0.6

n = 3.

and on formation of single and multilayer capsules, on the basis of evidence for smooth gel-like character at these concentrations.

Fig. 1B depicts the time-temperature profile of gelation for a 1.5% sodium alginate with 1.0% ascorbic acid (12 mmol·l⁻¹ CaCl₂). As expected, the gel-like character of the sample could be seen during cooling, where the initial monotonic increase in the shear moduli was recorded until a second wave of structure formation was observed at about 20 °C, marking an increase in G' . On holding at 5 °C for 15 min, the network strength was increased, giving a distinct sigmoidal time-temperature dependence of G' . It is depicted that values of G' of the biopolymer system at pH 2 are greater (approx. 43 Pa) as compared to the preparations at pH 3, 4 and 6 (approx. 13 Pa), reflecting the behaviour of the capsules at human gastrointestinal conditions. With the progressive decrease of pH value from 5.0 to 3.0, the system exhibited a marked viscosity enhancement, suggesting that the electrostatic repulsion was suppressed, intermolecular hydrogen bonds were enhanced, possible entanglements emerged and association structures were formed. It is shown that, with the decrease of pH, the number of dissociated carboxylic groups in alginate chains decreased, which made alginate lose its hydrophilicity to some extent. From Fig. 1B, as the pH value decreased, formation of hydrophobic segments in the alginate chains increased, while the number of hydrophilic segments decreased [21]. Consequently, at pH 8, weakening of the network strength of alginate preparations appeared, which was attributed to the increased amounts of hydrophilic segments in alginate chains [22].

The results on the thermomechanical behaviour of sodium alginate-ascorbic acid prepa-

rations at different ionic strengths and pH values provided evidence on how the preparations behaved in simulated gastrointestinal conditions. It appeared that the strong network of sodium alginate with incorporated ascorbic acid at acidic pH will be able to withstand the harsh conditions, thus facilitating delivery of biomaterials later in gastrointestinal conditions. Hence, further work dealt with formation and characterization of single and multilayer alginate beads during gastric and intestinal phase of in vitro digestion.

Physicochemical properties of alginate beads in in vitro digestion model system

Tab. 1 shows the physicochemical characteristics of single and multilayer alginate beads prepared according by Procedures 1–3. The encapsulation yield and efficiency, moisture and Ca concentration, water activity and colour parameters of the samples prepared by different procedures were not significantly different ($p > 0.05$). Results from Tab. 1 also show that size and other physicochemical parameters of all capsules were very similar to those reported earlier in the literature [12, 23, 24]. All samples of beads showed comparable concentrations of encapsulated ascorbic acid, which was also confirmed by similar values of encapsulation efficiency.

The swell behaviour of the alginate beads in simulated gastrointestinal fluid is displayed in Fig. 2. Three types of beads followed the same trend of shrinking in gastric solution, most notably when pH was reduced to 2. Here, the beads prepared according to Procedure 3 shrank more significantly (by approx. 40%) than the beads prepared by Procedure 1 (by approx. 30%) and Procedure 2 (by approx. 20%). It has been proposed that shrinking occurs due to a decrease

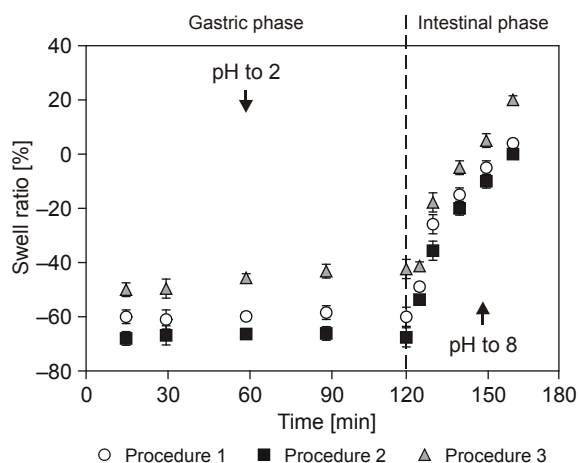


Fig. 2. Swell ratio for alginate beads in simulated gastro-intestinal conditions.

in the repulsive charge due to protonation of free carboxylate groups on alginate. Furthermore, due to dissociation of calcium ions at low pH, an acid gel can be formed, where COO^- groups become protonated, which allows the alginate chains to come closer together and form hydrogen bonds [25]. In respect to the type of beads, it appeared that beads prepared by Procedure 2 swelled less as compared to other samples. This could be probably attributed to low pH values due to addition of ascorbic acid to sodium alginate at initial stage of preparation, which resulted in formation of hydrogen bonds and, therefore, shrinking of the beads in the beginning of experiment. The finding has been also confirmed by data in Tab. 1 depicting the lower diameter of beads prepared by Procedure 2. Once placed in the intestinal solution, all beads began to swell to

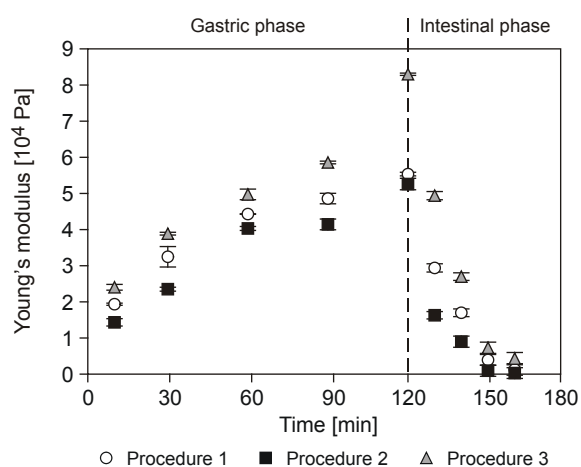


Fig. 3. Young's modulus data for alginate beads in simulated gastro-intestinal conditions.

varying degrees presumably due to an increase in the electrostatic repulsive forces at pH above the acidity (pK_a) of the uronic acid groups on the alginate [12]. Over time, disintegration of the beads was observed, presumably as a result of the increased concentration of monovalent ions, which occurs in intestinal conditions.

Mechanical properties of the beads were determined as a Young's modulus and are displayed in Fig. 3. The strength of the beads under control conditions differed considerably depending on the type of preparation. Thus, alginate beads prepared by Procedure 3 displayed the highest network strength having values of Young's modulus approximately three and two times higher than preparations of alginate beads prepared by Procedure 1 and Procedure 2. This was due to a coherent encapsulation of the bioactive compound, which involved subsequent addition of sodium alginate solution to calcium chloride followed by placing the beads in ascorbic acid solution and final storage in calcium chloride. Presumably, this resulted in enhanced formation of the capsule with ascorbic acid inside, with increased protective role of the core material against the harsh gastro-intestinal conditions.

During the gastric phase, the weak beads increased in strength on shrinking, which correlated with the denser gel network at acidic pH shown using small deformation oscillation (Fig. 1B). The initial decrease in modulus, when the beads were first acidified, might have been due to dissociation of calcium ions from the gel network and formation of an acid gel stabilized by weaker hydrogen bonding [25]. All types of beads exhibited a decrease in network strength during the intestinal phase, which correlated with formation of a more open, porous gel network as the beads swelled during this period (Fig. 2). During the intestinal phase, Young's modulus of all beads, prepared by different procedures, was not statistically different due to swelling and disintegration of the polymer network.

The effect of gastric conditions on morphology of the beads was also considered by optical microscopy and it was found that, although the shape of the beads was slightly altered, their integrity was largely maintained (Fig. 4). Observations of structural changes of beads before and during the *in vitro* digestion showed that beads were as discrete particles displaying no stickiness or tendency to aggregate and coalesce. It could be seen that beads shrank in acidic conditions due to occurrence of syneresis and due to an increase in polymer concentration. Fig. 4F provides clear evidence of the increase in the bead size of preparation under-

going intestinal phase of in vitro digestion, which is about three times greater as compared to the initial size of the bead (Fig. 4A). It appears that although lowering pH could cause some shrinkage of the alginate beads, the extent and duration of this process was not sufficient to irreversibly disrupt the beads. However, over time, disintegration of the beads was observed as a result of the increased concentration of monovalent ions at intestinal conditions (data not shown).

The formulation and processing variables involved in preparing ascorbic acid-loaded alginate beads by different procedures, incorporating ascorbic acid at different stages, were investigated. Using image and mechanical analysis, it was found that the structure of the beads at the end of the gastric phase had denser gel network than the beads in intestinal conditions. This was due to pH-responsive changes in alginate resulting in shrinking and swelling in gastro-intestinal conditions. The aim of this work was to prepare the beads with ascorbic acid in a reproducible way, for their use as functional carriers for the release of the antioxidant in the gastro-intestinal tract, where ascorbic acid is protected by the gel carrier. It became clear that, in intestinal phase, the beads eventually disintegrated, which resulted in a controlled delivery of the bioactive compound. So, at the next stage, the rate of ascorbic acid transport from the beads prepared by different procedures was characterized.

Kinetics of ascorbic acid transport from alginate beads

As discussed, alginate is readily processable for applicable three-dimensional scaffolding materials such as hydrogels, microspheres, microcapsules, sponges, foams and fibres. Alginate-based biomaterials can be utilized as drug delivery systems and cell carriers for tissue engineering. The choice of encapsulation of antioxidants is governed by criteria of advanced application, economics and safety [26]. Thus, for example, PENICHE et al. [27] showed that it is possible to utilize Ca-alginate beads coated with chitosan as carrier matrices for the delivery of bioactive compounds. However, behaviour of such beads in simulated gastro-intestinal conditions remained insufficiently characterized.

Kinetics of the release of ascorbic acid from alginate beads were monitored using a capillary electrophoresis system, which provided a sensitive quantitative analysis. This method is based on migration of the anionic forms of the analyte (ascorbic acid) under the influence of electric field in a quartz capillary in a phosphate or borate electro-

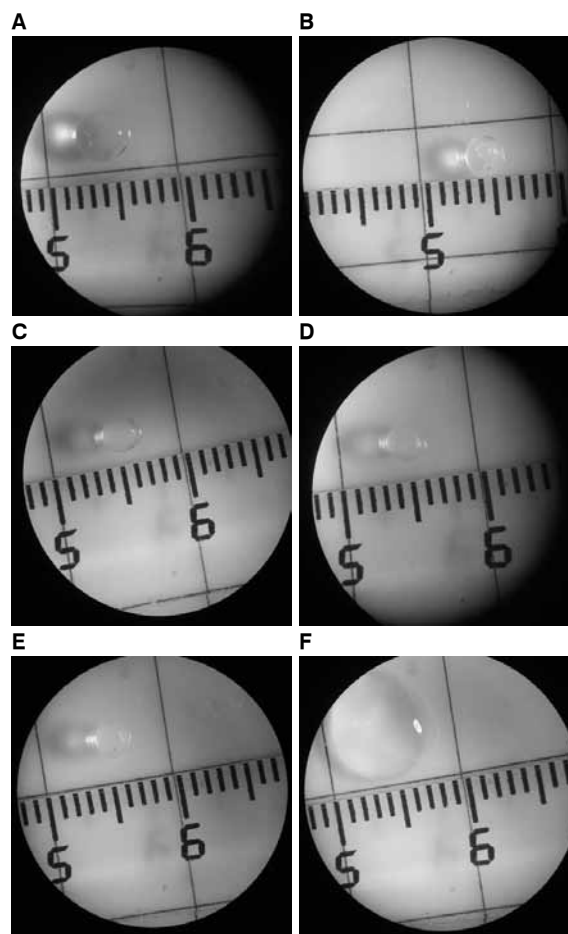


Fig. 4. Optical microscopy images for alginate beads in simulated gastro-intestinal conditions.

A – initial beads, B – beads after 30 min of in vitro digestion, C – beads after 60 min of in vitro digestion, D – beads after 90 min of in vitro digestion, E – beads after 120 min of in vitro digestion, F – beads after 135 min of in vitro digestion. One division on the ruler represents one millimeter.

lyte. Observation of ascorbic acid release was carried out for 180 min in simulated gastric and intestinal conditions. Smooth absorbance curves were obtained, with maximum release in intestinal conditions, i.e. after 120 min. As presented in Fig. 5, maximum release of ascorbic acid was observed from the beads prepared by Procedure 2. This means that these preparations were less stable to keep the antioxidant inside the matrix due to a partially destroyed nature of the carrier during the addition of ascorbic acid to sodium alginate at the initial stage of preparation. However, diffusion of the bioactive compound from alginate beads prepared by Procedure 3 was less pronounced due to a multilayer nature of the capsule.

Fig. 5 shows that almost 90% of the residual amount of ascorbic acid was released from the alginate beads in intestinal phase. Thus, the results

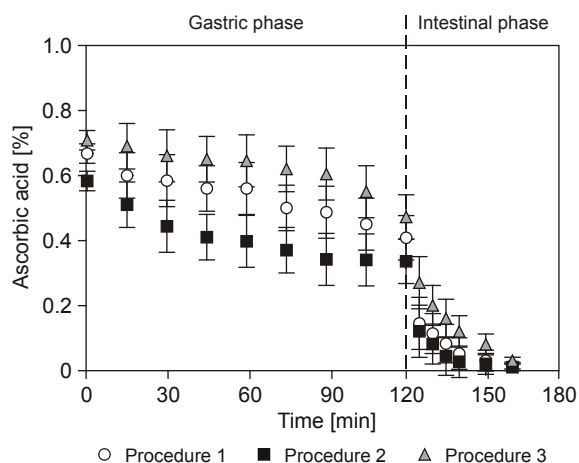


Fig. 5. Concentration of ascorbic acid in alginate beads in simulated gastro-intestinal conditions as a function of time.

revealed that alginate beads with approx. 0.7% of ascorbic acid at the beginning of *in vitro* digestion, had a loss of about 0.2% by the end of gastric phase, but still retained 0.5% of the original amount of the antioxidant. Further, after exposure to simulated intestinal conditions, alginate beads released all encapsulated bioactive compound (Fig. 5).

Fig. 6 depicts changes in Young's modulus (the ratio of Young's modulus at particular time of measurement and initial Young's modulus) and changes in the release profile (Eq. 6) of ascorbic acid from alginate beads prepared by Procedure 2

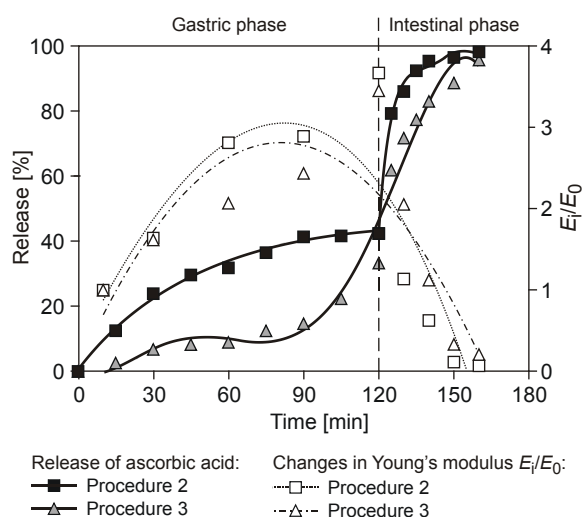


Fig. 6. Changes in Young's modulus and in release profile of ascorbic acid in simulated gastro-intestinal conditions.

E_t – Young's modulus at particular time of measurement, E_0 – initial Young's modulus.

and Procedure 3 in simulated gastro-intestinal conditions, as a function of time.

$$C = 100 - \left(\frac{c_i}{c_0} \times 100 \right) \quad (6)$$

where C is release of ascorbic acid (in percent), c_i is the concentration of ascorbic acid at particular time of measurement and c_0 is an initial concentration of ascorbic acid (expressed as millilitres per litre).

These results indicate a significant increase in values of Young's modulus in simulated gastric conditions and their considerable decrease in intestinal conditions. It is shown that release kinetics remained relatively constant up to 120 min with their sharp rise further during the experiment, arguing for delivery of the bioactive compound in a simulated small intestine. It has been proven that release of bioactive compounds is governed by the nature of capsules and the environment conditions, where mechanical properties of capsules are considered to be a basis of optimal utilization of delivery kinetics with maximum rates in the intestinal phase.

We were interested also in the preparation routine of alginate beads that brings understanding of the release profile of the bioactive compound. Thus, the multilayer beads prepared by Procedure 3 had the most attractive function to keep the bioactive compound inside, with its slow and controlled delivery. It is known that, in addition to physico-chemical factors studied here, absorption of ascorbic acid *in vivo* is also affected by physiological and metabolic factors such as the ascorbic acid status of the individual and cellular uptake. This investigation, which established the basic physico-chemical factors, should be complemented by studies in realistic conditions of human studies [28], which are going to be undertaken in the near future.

CONCLUSIONS

This study developed a systematic protocol of experimentation to rationalize the release patterns of ascorbic acid in alginate beads prepared by different procedures. Industrial application may derive from the use of alginate as microcontainers to deliver bioactive compounds through the harsh gastric environment, which was well characterized in the past and confirmed currently in their techno-functionality. It has been documented that behaviour of vitamin encapsulated in alginate matrices can be reliably studied by

simulated in vitro hydrolysis within 180 min. Using instrumental techniques it is shown that alginate beads display shrinkage in gastric conditions as a result of reduction of electrostatic repulsive forces at low pH and dissociation of calcium ions, whereas in intestinal phase all beads increasingly swell. Optical microscopy and mechanical observations showed that all alginate beads had an increasingly porous nature in intestinal conditions, providing a controlled release of the bioactive substance from multilayer capsules, which protected at the best from changes of pH and ionic strength. A comprehensive relationship between Young's modulus and release rates of vitamin, in relation to simulated gastro-intestinal conditions with time, was identified, which confirmed controlled delivery of the bioactive compound. In this regard, utilizing biopolymeric membranes based on plant components, which allow to carry out direct and controlled transport of substances, is the most promising solution in this regard. This investigation is significant in relation to the formulation of "functional foods", such as infant and sports nutrition formulae, health bars, breakfast cereals, dairy products as well as in ingredient powders and concentrates. Future work will extend the present findings in *in vivo* models to characterize controlled delivery.

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