Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 352 (2008) 115–122
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Cryo-scanning electron microscopy (cryo-SEM) as a tool for studying the ultrastructure during bead formation by ionotropic gelation of calcium pectinate

Pornsak Sriamornsak [a,b,*], Nartaya Thirawong [a,b], Kamonrak Cheewatanakornkool [b], Kanokporn Burapapadh [a,b], Witoon Sae-Ngow [c]

[a] *Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand*
[b] *Pharmaceutical Biopolymer Group (PBiG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand*
[c] *Scientific and Technological Equipment Centre, Faculty of Sciences, Silpakorn University, Nakhon Pathom 73000, Thailand*

Received 25 June 2007; received in revised form 16 October 2007; accepted 20 October 2007
Available online 4 November 2007

Abstract

Understanding the gel ultrastructure is of great importance for process and product development having great effects on the product characteristics. The samples containing high amount of water could not be directly observed using scanning electron microscope (SEM) without removing of water. However, cryo-SEM can be used to study the ultrastructure of hydrated samples. In this study, ultrastructural information of internal structure was obtained by imaging the cryo-fractured beads in a cryo-SEM. This technique was found to be excellent for studying the detailed morphology of structural development and showed better images than normal SEM procedures using freeze-drying for sample preparation. Also, the studies illustrated a morphological change, e.g. from net-like structure to membranous structure caused by syneresis, accompanied by a significant increase in mechanical properties, when the beads are formed by ionotropic gelation. The gelation time of 20 min was found to be the minimum for a complete bead formation, based on the mechanical and SEM data. The results demonstrate the advantageous of cryo-SEM for examining the ultrastructure during bead formation of calcium pectinate.

Keywords: Scanning electron microscopy (SEM); Cryo-scanning electron microscopy (cryo-SEM); Morphology; Bead formation; Gelation kinetics; Calcium pectinate; Pectin

1. Introduction

Pectin, a natural polysaccharide, is an inexpensive, non-toxic product extracted from citrus peels or apple pomaces. Pectin consists mainly of linearly connected α-(1-4)-D-galacturonic acid residues which have carboxyl groups. The degree of esterification (DE), which is expressed as a percentage of carboxyl groups (esterified), is an important means to classify pectin. Low-methoxyl pectin (with DE <50%) form rigid gels by the action of calcium, which cross-links the galacturonic acid chains (Rolin, 1993). Pectin has been used in a wide range of applications, particularly in food, industrial and pharmaceutical fields, because their capacity to hold water, form gels, and stabilize emulsion (May, 2000). Since low-methoxyl pectin can react with calcium ions, it is being investigated, in pharmaceutical application, as a carrier material for different controlled release systems. Recently, calcium pectinate gel (CaPG) beads have been used in various ways in the gastrointestinal tract because it is a gentle and simple encapsulation technique, for example, for sustained release of drugs (e.g. Munjeri et al., 1998; Sriamornsak and Nunthanid, 1998, 1999), and for targeting drugs to the colon (e.g. Sriamornsak, 1998, 1999; Chambin et al., 2006) or stomach for gastroretentive purpose (e.g. Sriamornsak et al., 2005, 2007).

Until now, most experiments have studied only the physicochemical properties of the beads and their drug release properties. The morphology of the CaPG beads formed, as a final product, has also been revealed mostly by scanning electron microscopy (SEM), e.g. back-scattered electron imaging

* Corresponding author at: Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand. Tel.: +66 34 255800; fax: +66 34 255801.
E-mail address: pornsak@email.pharm.su.ac.th (P. Sriamornsak).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.10.038

(Sriamornsak and Thirawong, 2003). Obtaining information on their ultrastructures is interesting from a scientific as well as from a technological point of view. Unfortunately, none of these studies are able to provide insights about the bead formation kinetics that could influence the performance of the beads formed, e.g. swelling behavior and drug release.

Recent advances in SEM technology allow the observation, at the pore scale, of liquid containing samples under a frozen state by cryo-scanning electron microscopy (cryo-SEM) (Echlin, 1992). Cryo-SEM has also been employed to study changes in the ultrastructures of calcium alginate matrices as a result of thermal treatment (Serp et al., 2002). Any attempt to directly observe hydrated samples using other SEM techniques would result in an instantaneous vaporization of water due to the high vacuum. In the cryo-SEM technique, the sample is frozen and maintained within the microscope at low temperature. This enables the sample to remain hydrated in the high vacuum. The freezing step permits the preservation of the original structure and the technique is relatively fast and requiring few preparation steps. In addition, the technique of cryo-SEM was found to be excellent for studying the detailed morphology of structural development and offers many advantages over normal SEM procedures using freeze-drying for sample preparation (Echlin, 1992; Fujikawa and Kuroda, 2000).

In order to understand the structural development of CaPG beads those prepared by ionotropic gelation as previously reported (e.g. Sriamornsak and Thirawong, 2003), the beads (2–3-mm in size) were freshly prepared and investigated. The cryo-SEM was used for the *in situ* study of hydrated beads those already formed, and compared with the normal SEM of freeze-dried samples. The ultrastructural information as well as the morphological and mechanical changes that occur during bead formation by ionotropic gelation (i.e. bead formation kinetics) was also investigated.

2. Materials and methods

2.1. Materials

Low-methoxyl pectin with DE of 28% (GENUpectin type LM-104 AS-FS) was the generous gift of CP Kelco (Denmark). All other chemicals were standard pharmaceutical grade or analytical grade.

2.2. Preparation of CaPG beads

The CaPG beads were prepared by ionotropic gelation method that was previously described (Sriamornsak and Thirawong, 2003). Briefly, pectin was dissolved in water with agitation. The solutions (5%, w/w) were extruded using a nozzle of 0.80-mm inner diameter into 0.3 M calcium chloride with gentle agitation at room temperature. After the predetermined time (e.g. 1, 2, 5, 10, 20, 30, 60, and 120 min), the gel beads were separated and washed with distilled water. The beads were then used for cryo-SEM or subjected to freeze-dry before SEM observation.

2.3. Mechanical properties of the gel beads

The mechanical properties of the CaPG beads were determined using a Texture Analyzer (Model TA.XT plus, Stable Micro Systems, UK) equipped with a stainless steel cylindrical probe (6-mm diameter). The bead sample, after gentle drying on filter paper, was placed centrally under cylindrical probe and the compression test was commenced. The maximum force required to break the bead structure was taken. All analyses were performed on 10 replicate samples under identical conditions.

2.4. SEM and cryo-SEM observations

Morphological examination of the internal structure of the beads were carried out using a scanning electron microscope (Model Maxim-2000, CamScan Analytical, England) equipped with back-scattered electron detector. The freshly prepared CaPG beads were subjected to freeze-dry (Model FreeZone 2.5 L, Labconco, USA). All samples were cut with a razor blade for cross-section observation and then mounted on SEM stubs using double-sided carbonized adhesive tape. The accelerating voltage was 20 kV.

For the cryo-SEM, the samples were loaded on the cryo-specimen holder and cryo-fixed in slush nitrogen (−210 °C), then quickly transferred to the cryo-unit in the frozen state. The frozen bead samples were then fractured by striking them with a pre-cooled razor blade, at the point on the bead surface where the fracture plane was required. The revealed fracture plane was sublimed at −85 °C in a vacuum SEM chamber and examined in the cryo-stage SEM (Model C1002) with temperature controller (Model ITC502, Oxford Instruments, England) under an accelerating voltage of 15–20 keV. Using this technique, the fractured surface of the frozen sample was viewed directly while being maintained at −135 °C. Imaging was performed by collecting the back-scattered electron (BSE) signal with a sensitive crystal detector.

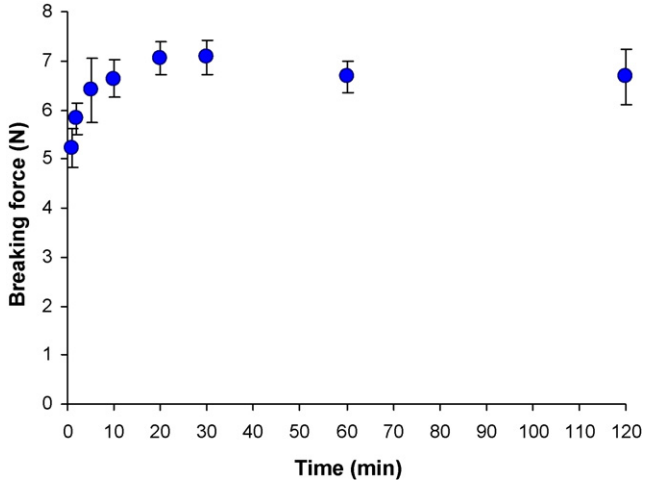

Fig. 1. Increase in mechanical strength (i.e. force required to break the bead sample) of calcium pectinate gel beads as a function of time. The means and standard deviation of 10 replicate data are plotted.

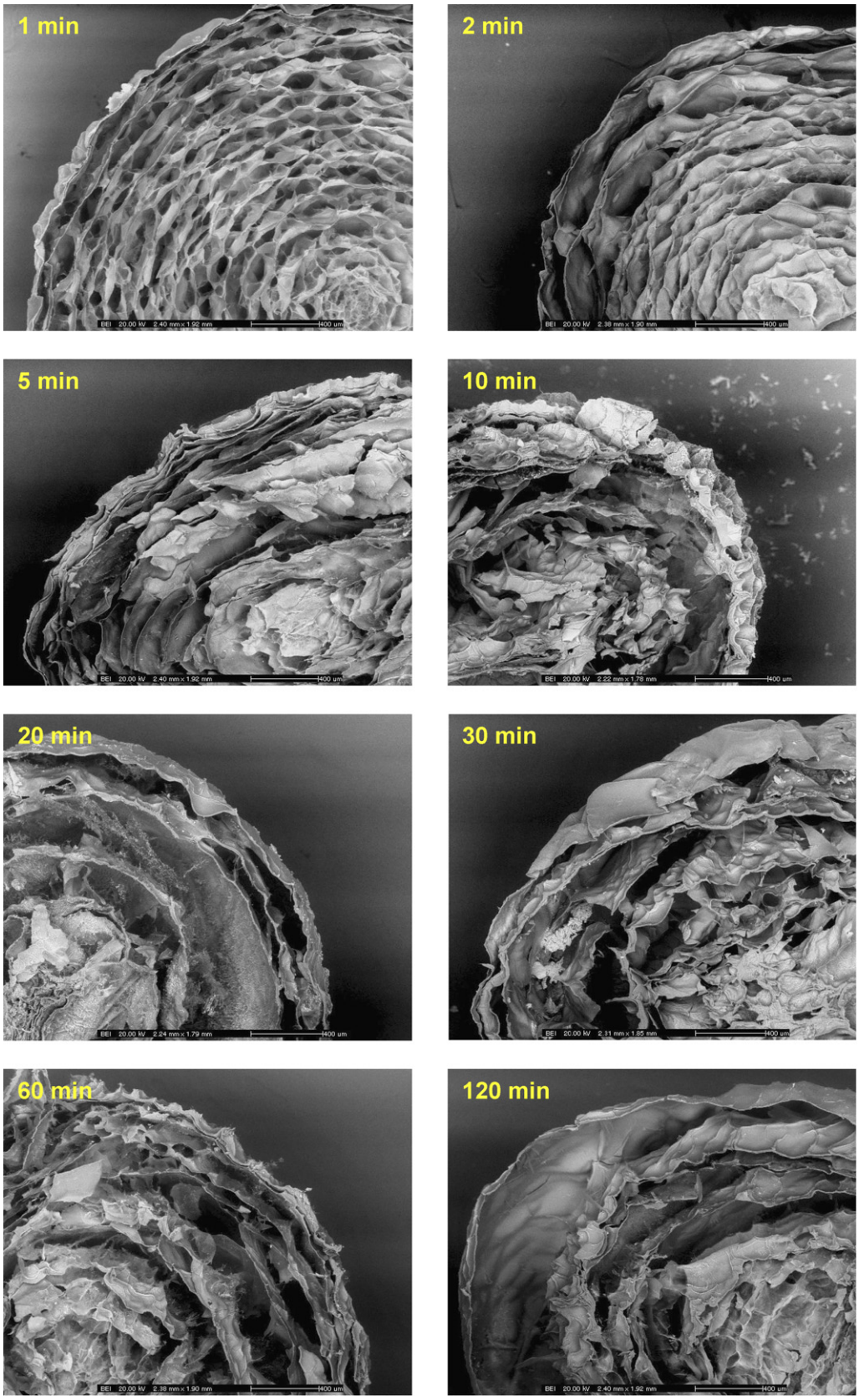

Fig. 2. SEM micrographs of freeze-dried calcium pectinate gel beads at various times after gelation in 0.3 M calcium chloride.

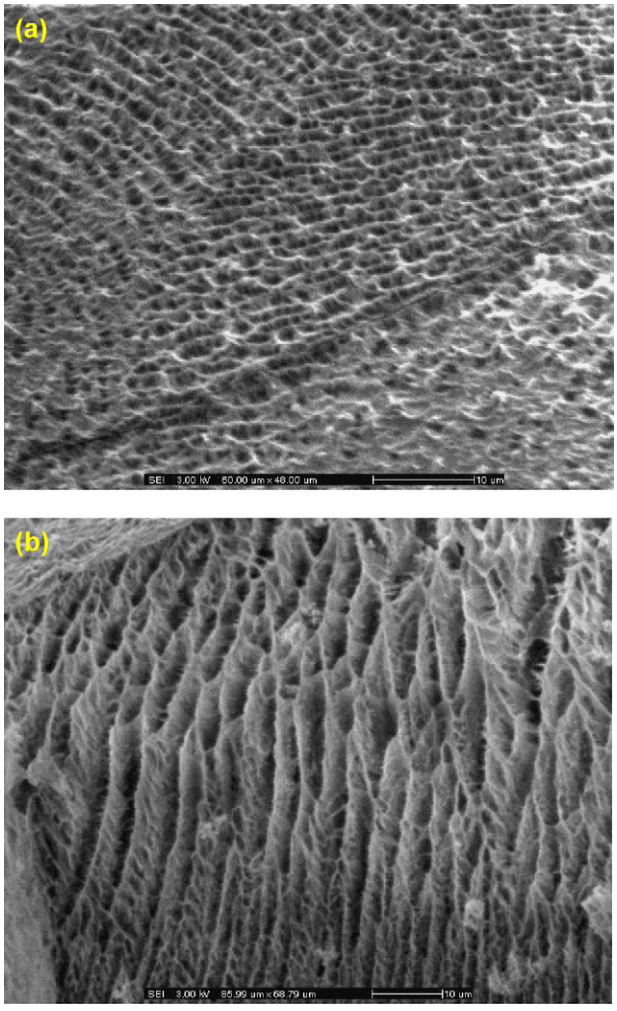

Fig. 3. Cryo-SEM micrographs showing pectin solution after freezing and vacuum drying in the chamber: (a) upper surface and (b) later view of cross-section.

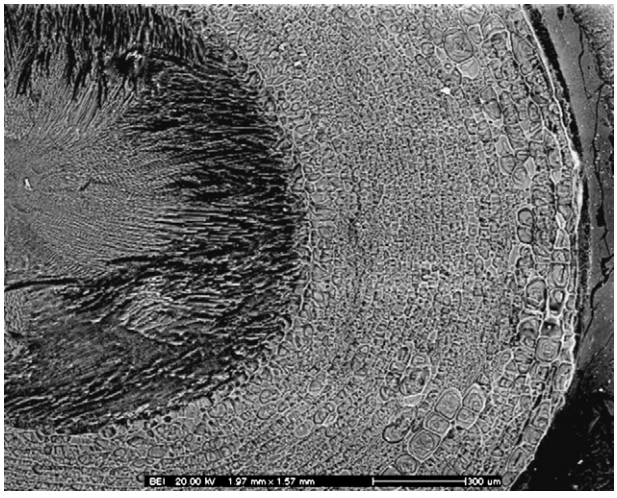

Fig. 4. Cryo-SEM micrographs showing ice crystals presented in the calcium pectinate structure and in the core. The photo was taken after 1 min of bead formation.

3. Results and discussion

3.1. Bead formation and mechanical properties

An aqueous solution of pectin was dropped into calcium chloride solution and gelled spheres were formed instantaneously by ionotropic gelation in which intermolecular cross-links were formed between the divalent calcium ions and the negatively charged carboxyl groups of the pectin molecules as previously described in many reports (e.g. Munjeri et al., 1998; Sriamornsak and Nunthanid, 1998; Sriamornsak and Thirawong, 2003). Fig. 1 demonstrates the mechanical strength (e.g. maximum force required to break the bead sample) of calcium pectinate gel beads as a function of time, to reflect the structure development during bead formation, i.e. bead formation kinetics. The calcium ions, as a cross-linker, induced an interfacial cross-linking reaction of pectin. Therefore, an insoluble calcium pectinate membrane was formed around the pectin droplets. At the very early stage of bead formation, the calcium pectinate shell/membrane with the liquid core was obtained. Besides, the internal liquid core containing pectin might be complexed with the calcium ions that migrated into the core, providing the relatively strong beads with a breaking force of about 5–6 N after 2 min of gelation (Fig. 1). The rate of further membrane formation (especially in a first few minutes) is probably controlled by the rate of diffusion of calcium through the calcium pectinate membrane, which may be very rapid. This is akin to the calcium pectinate gel coated pellets in which the cross-linking reaction occurred after cores containing calcium salts were immersed in pectin solution (Sriamornsak et al., 2006). As the gelation time increased, the thickness of the calcium pectinate shell/membrane may increase according to the formation of cross-linked pectin structure and be accompanied by an increase in mechanical strength of the beads formed (Fig. 1). It appeared that the mechanical strength reached the plateau after about 20 min of cross-linking. This is probably due to molecular re-arrangements and saturation of the carboxyl groups with calcium ions.

3.2. Structural analysis of the CaPG beads by SEM

The information about the ultrastructure of the pectin network during bead formation was gained from cryo-SEM. This technique differs from conventional SEM in the sense that the water within the sample is only partially removed in a controlled way by sublimation, and thus distortion of the sample due to the dehydration process is significantly reduced (Echlin, 1992). Therefore, after cryo-preparation, no reduction of the bead volume or size was observed (the freshly prepared CaPG beads were spherical in shape with a mean size of about 2.6–2.8 mm). However, the bead samples prepared by freeze-drying appeared to shrink slightly while those prepared by high-temperature drying produced a collapsed structure or considerably shrink (Sriamornsak, 1999). This observation is also in agreement with alginate-filler beads those reported by Zohar-Perez et al. (2004).

Fig. 2 shows the SEM images of CaPG beads which were freeze-dried after gelation in calcium chloride at various times. Freeze-drying created artifacts by collapsing the walls of beads,

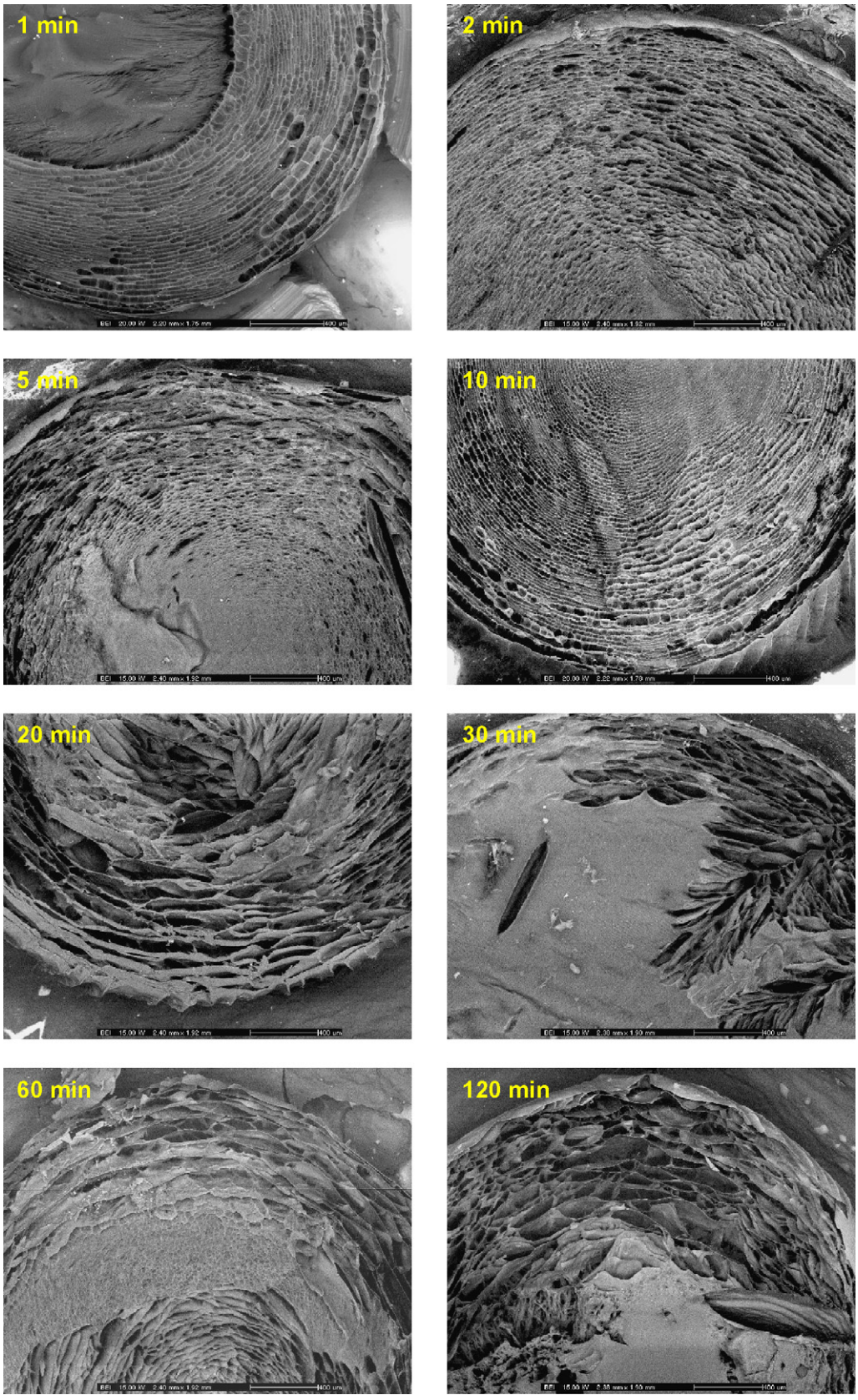

Fig. 5. Cryo-SEM micrographs of calcium pectinate gel beads formed at various times after gelation in 0.3 M calcium chloride.

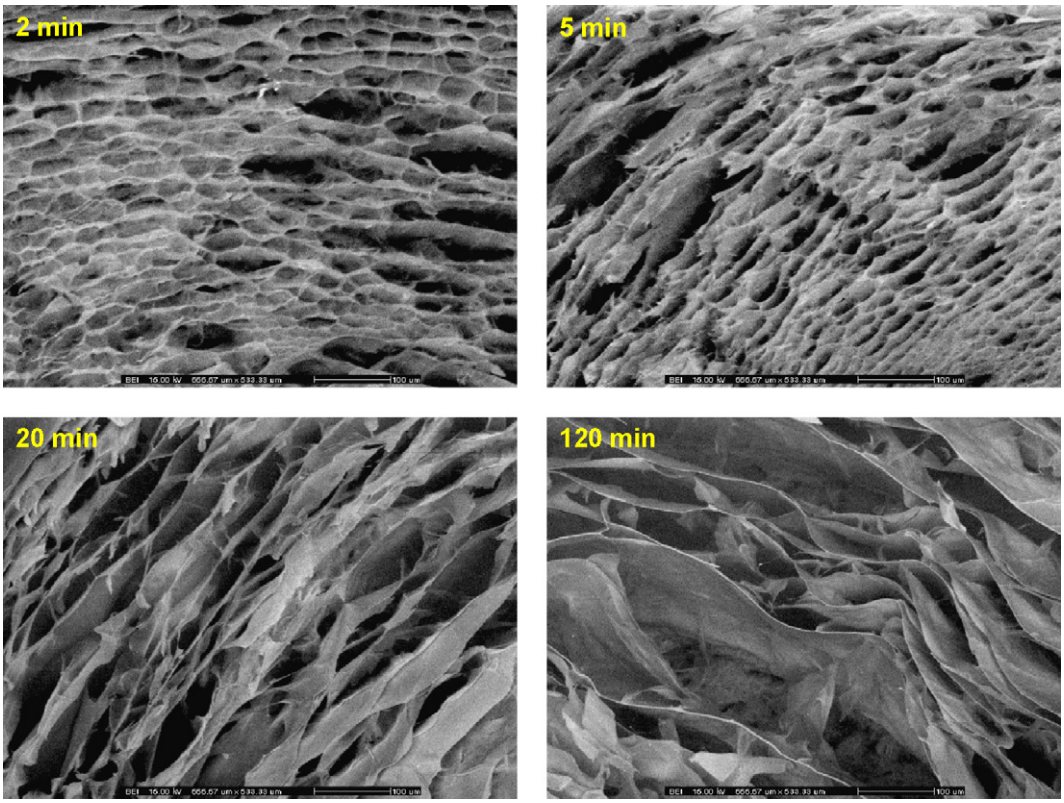

Fig. 6. Cryo-SEM micrographs of close-up of the cross-sectioned calcium pectinate gel beads that formed at various times after gelation in 0.3 M calcium chloride.

especially when they were cut. Although, the collapsed and fragile structure was seen, the structures at various gelation times can be distinguished, as shown in Fig. 2. During a first few minutes, the network-like structure of pectin was observed. This is comparable to the structure of pectin solution after cryo-preparation (Fig. 3), in which characteristic features in the range of 2–3 μm in diameter are shown. After further gelation (i.e. ≥5 min), the microscopic results show that the ultrastructural changes induced in the gel beads upon cross-linking are realized at a macroscopic level, e.g. the increase in mechanical strength. The multi-layered, cabbage-like, structure with many fused open and closed pores was observed, indicating that the calcium ions diffused into the pectin droplets and formed insoluble calcium pectinate shell or membrane. Further diffusion of calcium ions may be controlled by the insoluble membrane already formed. In other words, the rate of further membrane formation is controlled by the rate of diffusion of calcium through the calcium pectinate membrane, as discussed above (Section 3.1). It appears that the ultrastructures of freeze-dried beads at the gelation time of more than 20 min are relatively similar. This is supported by the mechanical data of the beads formed, as shown in Fig. 1.

Cryo-SEM technique has been used not only for observation of the ultrastructure in hydrated materials but also for observation of water distribution within the structure as well as of ice crystal distributions following the freezing of hydrated materials (Echlin, 1992; Fujikawa and Kuroda, 2000). Fig. 4 demonstrates the ice crystals presented in the CaPG beads after 1 min of bead formation. There are two distinct regions of different ice crystal structures, the outer region of the calcium pectinate structure that already formed and the inner core, at the center, of the beads. The ice structure in the outer region of the calcium pectinate structure is coarser and similar to the intracellular ice crystals presented in the xylem ray parenchyma cells in plant tissues (Fujikawa and Kuroda, 2000). Ice crystals were then sublimed, under microscope vacuum starts at −85 °C, from the surface of the sample leaving only void spaces and the underlying structural features.

Fig. 5 shows the cryo-SEM micrographs of the CaPG beads after various gelation times those corresponded to Fig. 2 but which had not been dried before being observed. Obviously, the ultrastructure of the cryo-prepared beads was fairly different from those of freeze-dried beads. The cryo-SEM images of CaPG beads clearly showed an outer region or external shell and a core with the incomplete gelled pectin network at 1 min after bead formation. The outer region shows pores where ice crystals were formed and sublimed off during the cryo-preparation, though the ice layers (i.e. artifacts) were still seen in some photos (Fig. 5). This differed from the SEM images of freeze-dried samples which the network-like structure was observed. It is probable that the cross-linking reaction (i.e. calcium ions diffused inward and cross-linked with pectin molecules) may still occur during the initial phase of freeze-drying process (without snap-freezing by liquid nitrogen), resulting in a morphologically homogenous structure in the freeze-dried beads (Fig. 2). Very likely, for the cryo-preparation, calcium ions could not reach the inner part of the bead within 1 min and remained confined in

the outer region or external shell because of strong cross-linking with pectin molecules.

As time progressed, the calcium ions diffused inwardly and cross-linked with pectin molecules in the inner core of the beads. Thus, the ultrastructural changes were observed, i.e. the proportion of pores with small size shifted to a population with both small and large pores. In addition, the shape of pores in the bead formed at longer gelation times was more elongated, while those at 1 or 2 min were more rounded. This agreed with the work on the microstructure of middle lamella of carrot tissues (i.e. containing pectin as a main component) treated with calcium lactate (Rico et al., 2007). The loss in rounded shape could result from a loss in turgor due to a higher water loss or syneresis caused by the stress produced by a high-ionic strength of calcium solution.

Dentini et al. (2007) also revealed that high levels of calcium ions in solution promoted the lateral association with alginate and a somewhat remarkable degree of syneresis may occur. This suggested the existence of higher calcium concentration in the outer shell of CaPG beads. The suggested results here are similar in trend to those found in the calcium-carrageenan system by MacArtain et al. (2003). The formation of thicker strands and more open nature of network (i.e. larger pores) at higher counterion concentrations supported the observation that added cations increased to the localized aggregation of the strands. Close-up views of the ultrastructure of CaPG beads at some gelation time points are shown in Fig. 6.

The cryo-SEM images in Figs. 5 and 6 also demonstrate that the gelation time of 20 min was found to be a minimum for a complete bead formation, being consistent with the mechanical and SEM data (Figs. 1 and 2). The shape of the pores exhibited in the beads gelled for more than 20 min was somewhat unchanged, although the pores in the 120-min gelled beads were rougher and larger. This is strongly in agreement with the mechanical properties of the beads at various gelation times.

4. Conclusions

In the study of the ultrastructure during bead formation by ionotropic gelation of calcium pectinate, a clear optimal gelation time (i.e. 20 min) is suggested based on the mechanical data as well as the structural analysis by SEM study. A difference in morphological structure, using SEM, has been observed between freeze-dried CaPG beads and the beads prepared by cryo-preparation. The SEM images suggested that cryo-preparation seem to be less destructive for the beads and give a more realistic image of the network structure. Moreover, cryo-preparation offered a simple and rapid approach for preparing the beads for SEM study. This technique avoided many artifacts associated with drying. However, this is a comparative study and the results are limited to the large beads employed. The extrapolation may not be possible for smaller polymeric beads (e.g. micro- or nanoparticles), or beads with other compositions.

Cryo-SEM of CaPG beads has been shown to provide detailed information about the structure and arrangement of the polymer network. Using this technique, a change in gel structure during bead formation, i.e. bead formation kinetics, could be identified among various gelation times. This provided direct proof that the evolved gelation time for ionotropic gelation leads to a rearrangement of polysaccharide network, resulting in a stronger gel structure which corresponds exactly to the mechanical data. Therefore, the inappropriate gelation time chosen owing to the lack of structural information which resulted in suboptimal processing ensuring could be prevented. Additionally, cryo-SEM may be used to follow the drug loading process and drug release mechanism, allowing for the development of controlled release drug delivery applications.

Acknowledgements

Financial support from Silpakorn University Research and Development Institute, Thailand is gratefully acknowledged. The authors would also like to thank Food & Cosmetic System Co. Ltd. (Thailand) for supplying pectin samples, and Scientific and Technological Equipment Centre for allowing access to the Scanning Electron Microscope.

References

Chambin, O., Dupuis, G., Champion, D., Voilley, A., Pourcelot, Y., 2006. Colon-specific drug delivery: influence of solution reticulation properties upon pectin beads performance. Int. J. Pharm. 321, 86–93.

Dentini, M., Rinaldi, G., Barbettal, A., Risica, D., Anselmi, C., Skjak-Braek, G., 2007. Ionic gel formation of a (pseudo)alginate characterised by an alternating MG sequence produced by epimerising mannuronan with AlgE4. Carbohydr. Polym. 67, 465–473.

Echlin, P., 1992. Low Temperature Microscopy and Analysis. Plenum Press, New York.

Fujikawa, S., Kuroda, K., 2000. Cryo-scanning electron microscopic study on freezing behavior of xylem ray parenchyma cells in hardwood species. Micron 31, 669–686.

MacArtain, P., Jacquier, J.C., Dawson, K.A., 2003. Physical characteristics of calcium induced κ-carrageenan networks. Carbohydr. Polym. 53, 395–400.

May, C.D., 2000. Pectins. In: Phillips, G.O., Williams, P.A. (Eds.), Handbook of Hydrocolloids. CRC Press, Boca Raton, pp. 169–188.

Munjeri, O., Hodza, P., Osim, E.E., Musabayane, C.T., 1998. An investigation into the suitability of amidated pectin hydrogel beads as a delivery matrix for chloroquine. J. Pharm. Sci. 87, 905–908.

Rico, D., Martin-Diana, A.B., Frias, J.M., Barat, J.M., Henehan, G.T.M., Barry-Ryan, C., 2007. Improvement in texture using calcium lactate and heat-shock treatments for stored ready-to-eat carrots. J. Food Eng. 79, 1196–1206.

Rolin, C., 1993. Pectin. In: Whistler, R.L., Bemiller, J.N. (Eds.), Industrial Gums: Polysaccharides and Their Derivatives. Academic Press, New York, pp. 257–293.

Serp, D., Mueller, M., von Stockar, U., Marison, I.W., 2002. Low-temperature electron microscopy for the study of polysaccharide ultrastructures in hydrogels. II. Effect of temperature in the structure of Ca^{2+}-alginate beads. Biotechnol. Bioeng. 79, 253–259.

Sriamornsak, P., 1998. Investigation of pectin as a carrier for oral delivery of proteins: using calcium pectinate gel beads. Int. J. Pharm. 169, 213–220.

Sriamornsak, P., 1999. Effect of calcium concentration, hardening agent and drying condition on release characteristics of oral proteins from calcium pectinate gel beads. Eur. J. Pharm. Sci. 8, 221–227.

Sriamornsak, P., Nunthanid, J., 1998. Calcium pectinate gel beads for controlled release drug delivery. I. Preparation and in vitro release studies. Int. J. Pharm. 160, 207–212.

Sriamornsak, P., Nunthanid, J., 1999. Calcium pectinate gel beads for controlled release drug delivery. II. Effect of formulation and processing variables on drug release. J. Microencapsul. 16, 303–313.

Sriamornsak, P., Thirawong, N., 2003. Use of back-scattered electron imaging as a tool for examining matrix structure of calcium pectinate. Int. J. Pharm. 267, 151–156.

Sriamornsak, P., Thirawong, N., Puttipipatkhachorn, S., 2005. Emulsion gel beads of calcium pectinate capable of floating on the gastric fluid: effect of some additives, hardening agent or coating on release behavior of metronidazole. Eur. J. Pharm. Sci. 24, 363–373.

Sriamornsak, P., Burton, M.A., Kennedy, R.A., 2006. Development of polysaccharide gel coated pellets. I. Physico-mechanical properties. Int. J. Pharm. 326, 80–88.

Sriamornsak, P., Sungthogjeen, S., Puttipipatkhachorn, S., 2007. Use of pectin as a carrier for intragastric floating drug delivery: carbonate salt contained beads. Carbohydr. Polym. 67, 436–445.

Zohar-Perez, C., Chet, I., Nussinovitch, A., 2004. Irregular textural features of dried alginate-filler beads. Food Hydrocoll. 18, 249–258.