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# Changes in starch structure during manufacturing of starch microspheres for use in parenteral drug formulations: Effects of temperature treatment

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ABSTRACT

Starch microspheres were produced by emulsification of a starch dispersion in an aqueous polyethylene glycol (PEG) solution. Crystalline/ordered structure was formed within these starch droplets during incubation at 6 °C for 25 h followed by incubation at 37 °C for 28 h. After incubation at 37 °C the crystalline structure in the samples was of type B. The crystallization process of microspheres was compared with crystallization in a model system. The crystalline structure of the microspheres melted at temperatures almost 20 °C lower than in the model system incubated under the same conditions, as determined by differential scanning calorimetry. It was thus concluded that the crystallization process within microspheres was different than that of bulk starch and the ability of the starch molecules to reorganize themselves within the dispersed starch phase of an aqueous two-phase system at the higher incubation temperature was limited. It was also observed that the presence of PEG or carbonate buffer protected the molecular order formed by the starch molecules during incubation from breakdown during freeze-drying.



## 1. Introduction

The use of microspheres as a dosage form for the administration of active substances is attracting growing interest, especially as a means of delivering proteins (Harris, Gauden, Fraser, Williams, & Parker, 2002; Huang, Mehta, & DeLuca, 1997; Morise et al., 2006; Teder, Johansson, d'Argy, Lundin, & Gunnarsson, 1995). Starch is one of the polymers that is suitable for the production of microspheres. It is biodegradable and has a long tradition as an excipient in drug formulations. Starch microspheres have been used for nasal delivery of drugs (Björk & Edman, 1988; Illum, Farraj, Critchley, & Davis, 1988; Illum, Fisher, Jabbal-Gill, & Davis, 2001; Shirui, Jianming, Huan, Zhenping, & Dianzhou, 2004; Vivien, Buri, Balant, & Lacroix, 1994), and for the delivery of vaccines given orally and intramuscularly (Heritage et al., 1996; Mundargi, Shelke, Rokhade, Patil, & Aminabhavi, 2008; Rydell, Stertman, & Sjöholm, 2005; Sturesson & Degling Wikingsson, 2000). Many of the studies on starch microspheres during recent years have been carried out on Spherex® microspheres, prepared by cross-linking soluble starch with epichlorohydrin (Hamdi, Ponchel, & Duchene, 2001; Lindberg, Lote, & Teder, 1984), or on starch particles produced by the polymerization of acrylated starch in a water-in-oil emulsion (Artursson, Edman, Laakso, & Sjöholm, 1984; Rydell et al., 2005; Sturesson & Degling Wikingsson, 2000).

In the work presented in this article, the production method used is similar to the one for Biosphere® microspheres (SkyePharma AB, Sweden). These are starch microspheres intended for subcutaneous injection (Reslow, Jönsson, & Laakso, 2002b). The microspheres were prepared using an aqueous two-phase system consisting of two structurally different polymers (polyethylene glycol (PEG) and starch) utilizing the ability of starch to crystallize as a method of cross-linkage. These starch particles can be coated with poly(lactic-co-glycolic acid) (PLGA) in order to achieve sustained release of active substances for periods of a few weeks up to several months (Reslow, Gustafsson, Jönsson, & Laakso, 2002a). A human growth hormone formulated in PLGA-coated starch microspheres has been tested in a Phase I/II study (Jostel, Mukherjee, Alenfall, Smethurst, & Shalet, 2005).

Preparation of the starch matrix of the Biosphere® microspheres includes heating the starch in excess solvent, during which most of the original structure of the starch is destroyed. The starch dispersion obtained is emulsified in PEG solution and subjected to incubation at a lower temperature followed by incubation at a higher temperature. In this way, hardened starch microparticles can be obtained. The formation of a crystalline structure is thus critical for the production of these starch microspheres.

In an earlier study on starch microspheres we found that the onset and the melting peak temperatures of the microspheres were considerably lower (by almost 15 °C) than those of starch/water dispersions subjected to the same incubation conditions as the microspheres (Elfstrand, Eliasson, Jönsson, Reslow, & Wahlgren,

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2006). The use of a carbonate buffer and the addition of PEG and then bovine serum albumin (BSA) to starch dispersions to mimic the microsphere composition (Elfstrand et al., 2007b), led to even higher melting temperatures than those of the starch/water dispersion, raising the question of why the melting temperatures of the crystalline structure formed in the microspheres were so low. X-ray powder diffraction (XRPD) showed the crystalline structure of the freeze-dried starch microspheres to be type A (Elfstrand et al., 2006), while recrystallized starch gel usually exhibits a type B structure (Radley, 1953; Ring, 1985).

The aim of this study was to elucidate the reason for the formation of a crystalline structure in microspheres with melting parameters different from those in the model system reported in our earlier studies (Elfstrand et al., 2007a, 2007b). The crystallization of starch during various stages of the manufacture of starch microspheres was studied and compared with crystallization in the model system and in an unstirred starch/PEG/BSA mixture with the same composition as the microspheres. The crystalline structures exhibited by these three crystallized starch samples after various kinds of treatment were investigated by X-ray diffraction (XRD) and differential scanning calorimetry (DSC). Scanning electron microscopy (SEM) was employed to study the morphology of the samples after freeze-drying. The effects of the first incubation step (at 6 °C), the second incubation step (at 37 °C) and freeze-drying on the crystalline structures formed in these three systems were investigated.

## 2. Materials and methods

### 2.1. Materials

The material used in this study was produced from a waxy maize starch (Cerestar SF 04201) by SkyePharma AB (Malmö, Sweden) by acid hydrolysis and high-pressure homogenization. The starch material had a molecular weight of $5.1 \times 10^5$ g mol$^{-1}$and a polydispersity as calculated by Mw/Mn of 4 (Elfstrand et al., 2004). It had a similar degree of polymerization, degree of branching and unit chain length distribution as the original maize starch but did not have any granular or crystalline order. PEG (20,000 g/mol) was provided by VWR International Ltd. and BSA (A7906) was purchased from Sigma–Aldrich. Sodium carbonate buffer (50 mM, pH 8.0) was used for the preparation of the dispersions. Sodium phosphate buffer (5 mM, pH 5.0) was used as a washing buffer. All chemicals used for the buffers were of extra-pure grade and were obtained from VWR International Ltd. (Germany).

### 2.2. Sample preparation

A PEG solution (38% polymer by weight) was prepared by dissolving an appropriate amount of PEG in carbonate buffer while stirring. A BSA solution (3.7% by weight) was prepared by dissolving the desired amount of BSA in carbonate buffer. The starch/buffer dispersion was prepared by dispersing the starch in the carbonate buffer and heating 10 g of the mixture in a microwave oven (800 W; MS-194A, LG Electronics Inc., PRC) for three periods of 6 s. The heated dispersions were transparent and visually homogeneous. The preparation of the samples studied is illustrated in Fig. 1. The concentration of starch in the starch phase and other preparation conditions are given in Table 1.

The starch microspheres were prepared by an emulsification technique. This process, described in detail by (Reslow et al., 2002b), can be summarized as follows. The starch/buffer dispersion (30/70% by weight), prepared as described above, was cooled to 50–55 °C and 2.9 g was mixed with 1 g of the BSA solution (28 °C). The starch/BSA mixture was then added to a PEG solution (29 g, 28 °C) while being stirred (200 rpm). The mixture was stirred for an additional 15 min. The emulsion was then incubated, while being stirred at 50 rpm, for 25 h at a temperature of 6 °C, followed by 28 h of incubation at 37 °C. These time/temperature conditions have been found to give the optimal crystalline structure in model systems (Elfstrand et al., 2007b). After these two periods of incubation the samples were centrifuged (10,000 rpm, 10 min) (Optima™ LE-80K Ultracentrifuge, USA) and the particles were recovered. The microspheres were then washed with phosphate buffer (5 mM, pH 5.0) and centrifuged three times (3000 rpm, 5 min) and, finally freeze-dried (Hetosicc, Heto Birkerød, Denmark).

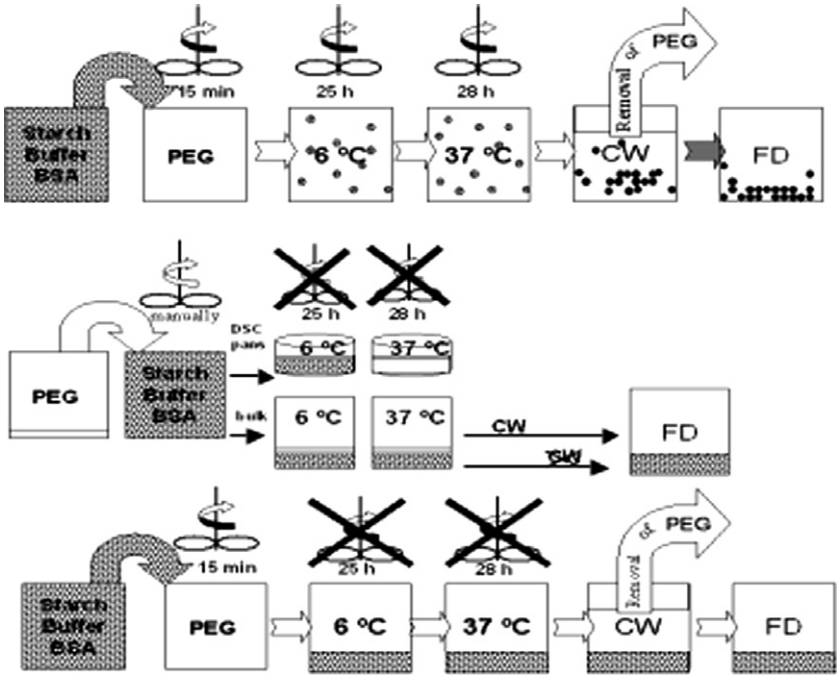


**Fig. 1.** The preparation of microspheres (I), model system. (II) and unstirred microspheres (III). CW is centrifugation/washing and FD is freeze-drying.

**Table 1**
Differences in the preparation of the microspheres, unstirred microspheres and the model system

| Samples | PEG content | Stirring first 15 min before incubation, 200 rpm | Stirring during incubation, 50 rpm | Centrifugation/ washing procedure applied | Dry matter (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Before PEG addition | After 25 h at 6 °C | After 25 h at 6 °C and 28 h at 37 °C |
| Microspheres | In excess | + | + | + | 22 | 24.2 (2.4) | 30.8 (1.7) |
| Unstirred microspheres | In excess | + | – | + | 22 | 29.9 (2.9) | 36.8 (4.2) |
| Model system | Limited | – | – | – | 30 | 30.9 (0.3) | 32.3 (1.2) |
| | | | | + | 30 | n.m. | 22.2 (0.2) |

n.m., not measured.

The model system was prepared as described previously for the starch/buffer/PEG/BSA system (Elfstrand et al., 2007b). Briefly, the BSA solution (1 g, 28 °C) was added to the starch/buffer dispersion (45 wt.%, cooled to 50 °C) to give a BSA concentration of 1.3 wt.%. The starch/buffer dispersion for this system was prepared to give a starch-to-buffer ratio in the final mixture of 30/70% by weight. The PEG solution was added to the starch/buffer/BSA mixture at a ratio of 0.063:1. The mixture was stirred slowly for 15 min and kept at room temperature for the following 30 min. Samples taken during the next 15 min were weighed into DSC pans, and the pans were sealed and incubated at 6 °C for 25 h followed by incubation at 37 °C for 28 h. Samples of the model system used for XRD analysis were incubated in bulk.

Unstirred microspheres were prepared as described above for the microspheres, but incubation at 6 and 37 °C was performed without stirring. Differences in the preparation conditions of the systems studied can be seen in Table 1.

Apart from the systems described above, a starch/buffer dispersion (30/70 wt.%) was prepared as described above and incubated under the same conditions as the other systems in this study: 6 °C for 25 h followed by 37 °C for 28 h. The sample was freeze-dried and investigated using SEM, XRD and DSC.

### 2.3. DSC

DSC was used to investigate the formation of ordered structure in the starch gel. The samples were characterized by the endothermic heat required to melt the ordered structure of the samples ($\Delta H$) and the three temperatures: onset temperature ($T_{on}$), melting temperature of the dominating peak ($T_m$) and completion temperature ($T_c$).

Samples of freeze-dried materials were prepared for DSC in the following way. Approximately 3 mg of the sample was weighed into coated aluminium DSC pans, after which water was added to give a final concentration of 30% (w/w) dry substance. The samples were left for 3–5 h before heating in the calorimeter, and were scanned at a rate of 5 °C/min with a temperature sweep from 20 to 100 °C.

Preparation of wet samples of the microspheres and unstirred microspheres for DSC scanning after the first incubation step (25 h at 6 °C) and after the second incubation step (28 h at 37 °C) included the removal of PEG by centrifugation, washing with phosphate buffer. Approximately 10 mg of the sample was placed in DSC pans and scanned immediately. The samples were scanned at a rate of 5 °C/min with a temperature sweep up to 100 °C, starting at 6 °C for samples incubated at 6 °C, and at 30 °C for samples investigated after the second incubation step (at 37 °C).

In all the experiments, the aluminium pans (TA Instruments, New Castle, DE, USA, Ref no. 900790.901 and 900796.901) were hermetically sealed and the analysis was carried out with a DSC 6200 calorimeter (Seiko Instruments Inc., Shizouka, Japan) with an empty pan as a reference. All measurements were carried out at least in duplicate. The dry substance was determined by drying punctured pans at 105 °C overnight. The starch content was calculated by subtracting the amount of BSA in the sample, and $\Delta H$ was calculated based on the dry weight of starch.

### 2.4. XRD

Samples were analyzed using synchrotron radiation on beamline I711 ($\lambda$ = 1.083 Å) or I911 ($\lambda$ = 0.907 Å) at the MAX II synchrotron facility (Lund, Sweden), with low-divergence, high-intensity X-rays, reduced by slits down to a beam size of 0.3 × 0.3 mm. The beamline has been described previously by (Cerenius et al., 2000). The samples, in powder form, were placed in glass capillaries with diameters of 0.3–0.5 mm. The wet samples for X-ray diffraction analysis were prepared by fixing small amounts (2 × 2 mm) on the top of a thin needle. The time for analyse were at no time longer than 5 min and the sample where visibly wet also after the X-ray analysis. The wet samples of microspheres and unstirred microspheres were washed and centrifuged before X-ray analysis, as described above for the DSC samples. The model system samples incubated in bulk were analyzed without centrifugation/washing, except in an additional experiment in which this procedure was included.

Data were collected during 5 min of exposure using a Mar165 area CCD detector (Mar Research GmbH, Norderstedt, Germany). The scanning regions of the diffraction angle ($2\theta$) included the region 3°–40°, which covers all the significant diffraction peaks of starch crystallites. The 2D raw image was integrated to produce a $2\theta$ scan using Fit2D software (Hammersley, Svensson, Hanfland, Fitch, & Häusermann, 1996).

The collected integrated data were transformed to the $2\theta$ scale based on a wavelength ($\lambda$) of 1.54 Å. This is the most common wavelength found in the literature for performing X-ray analyses on starch and for the presentation of X-ray diffraction results.

$$\theta_{1.54} = \arcsin\left[\frac{1.54x \sin\theta_\lambda}{\lambda}\right],$$

where: $\theta_{1.54}$ is the diffraction angle corresponding to a wavelength of 1.54 Å
$\lambda$ is the X-ray wavelength and
$\theta_\lambda$ is the diffraction angle when using X-rays of wavelength $\lambda$.

### 2.5. SEM

Scanning electron microscopy was performed with a JEOL JSM-5600 scanning electron microscope (JEOL, Tokio, Japan). The samples were attached to circular stubs with double-sided adhesive tape and coated with gold-palladium using a Sputter Coater Polaron SC7640 (Thermo VG Microtech, East Grinstead, West Sussex, UK). Samples were viewed by scanning the whole specimen, and an area deemed to be representative of the sample was photographed at magnifications up to 6000×.

## 3. Results and discussion

### 3.1. Effect of incubation time and temperature on crystal patterns in the wet systems

#### 3.1.1. Microspheres

X-ray diffraction and DSC analyses were performed after incubation at 6 °C and after incubation at 37 °C. The results of these analyses are shown in Fig. 2 and Table 2. The dry matter of the samples was determined and is presented in Table 1. It is clear from the DSC and X-ray data that an ordered/crystalline structure was formed during the incubation of the microspheres at 6 °C, and that this structure was further affected by incubation at 37 °C.

Typical B-type crystallinity diffraction peaks at spacings of 15.5, 5.8, 5.1 and 3.9 Å were observed for both samples as seen from X-ray diffraction results (Fig. 2). However, the structure obtained after the first incubation step, at 6 °C for 25 h, also had two other peaks at 3.75 Å ($2\theta \sim 23.7°$) and 4.59 Å ($2\theta \sim 19.3°$), which are not related to the B-type crystalline form. The diffractograms revealed a reflection of 3.75 Å that normally is related to the A-form, and a reflection of 4.59 Å. During the second incubation step (at 37 °C) the crystalline array positions at the larger spacings (15.5, 5.8 and 5.1 Å) remained unchanged, while the non-B-type peaks disappeared, and a pure B-type crystalline structure was observed in the microspheres. It is known that crystallization in starch dispersions stored at low temperatures usually results in the B-type polymorph, a kinetic product consisting of a more open packing of helices with a correspondingly greater amount of inter-helical water than the thermodynamically more stable A-pattern (Gidley, 1987). The A type is the more stable form, and it is known that additives (Hizukuri, Fujii, & Nikuni, 1960) and incubation at higher temperature favor the formation of this type (Gidley, 1987). It is noteworthy that, in the case of microspheres the non-B-type pattern (C-pattern) was seen after the low-temperature incubation step (at 6 °C) and that A-pattern disappeared after high temperature treatment.

The area under the peak recorded at 15.5 Å (or $2\theta = 5.7°$, see Table 3) is often used as an index of crystallinity of B-starches (Biliaderis, 1998). The level of crystallinity did not change essentially during the second incubation step, judging from the height of the 15.5 Å peak. However, the amount of ordered structure measured by DSC did increase during incubation at the higher temperature, as can be seen by the increase in the melting enthalpy of around 30% (Table 2). This discrepancy could be due to changes in water content affecting the DSC and X-ray analyses. As can be seen in Table 1, the dry matter content of the microspheres increases during incubation at both temperatures. It is known that the intensity of the diffraction reflections varies with water content in the sample, becoming sharper as hydration increases (Cleven, van den Berg, & van der Plas, 1978). However, for the samples studied here the crystallinity might still be compared as the water contents (70–75 wt.%) are much higher than that reported by Cleven. It is also known that the water content of starch samples can affect the melting enthalpy. However, also in this case, the samples are in a region where water content has little effect on the analyses. A more likely explanation is that the DSC and X-ray methods do not fully measure the same types of structures, and that the increase in enthalpy which followed incubation at 37 °C reflects the growth of ordered structure but not in crystalline regions. Thus, it appears that within the droplets surrounded by PEG molecules the mobility of starch was too limited to allow increased crystalline perfection, but the molecular mobility was high enough to increase the overall amount of double helices.

Recrystallised starch usually melts at lower temperatures than its native counterpart at the same water content, and the crystallization process depends on the molecular properties, and also on the temperature and duration of incubation (Eliasson & Gudmundsson, 1996; Whistler, 1953). Generally, the crystallites obtained at lower temperatures, such as 4–6 °C, will melt at lower temperatures and will be more heterogeneous than crystallites obtained at temperatures closer to the melting temperature (Baik, Kim, Cheon, Ha, & Kim, 1997; Durrani & Donald, 1995; Eliasson &

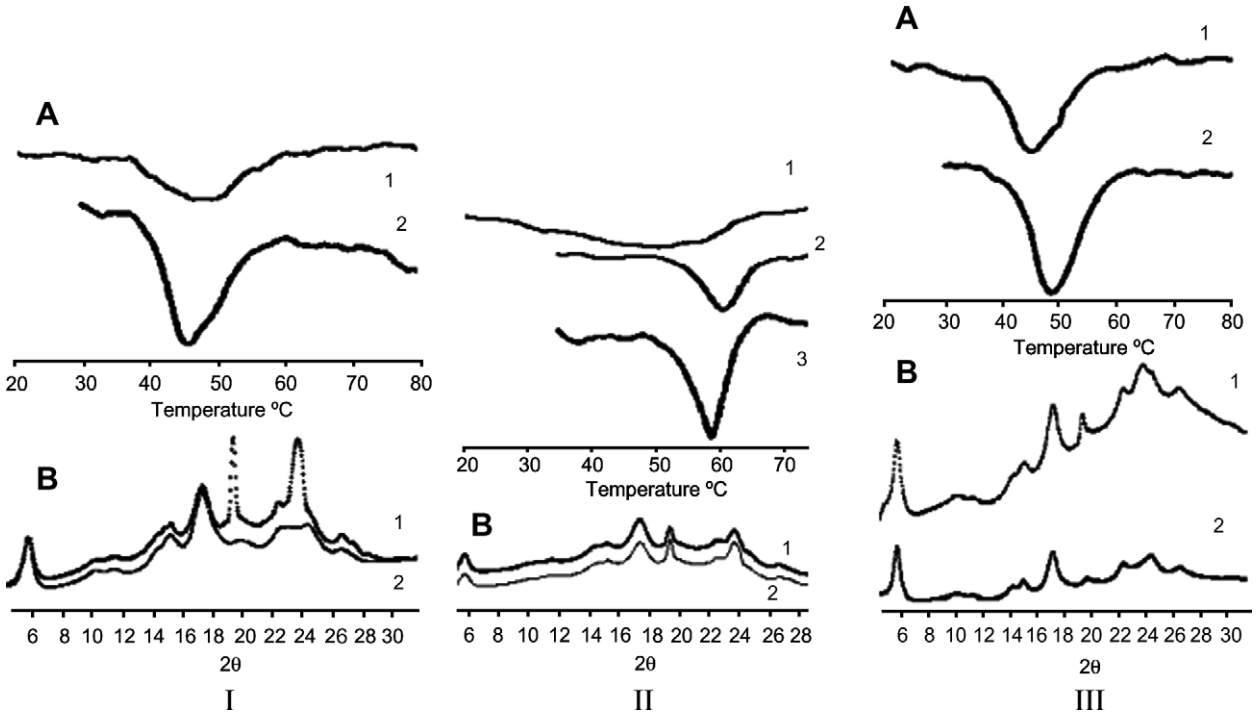


**Fig. 2.** DSC thermograms (A) and X-ray diffractograms: (B) for microspheres (I), model system (II) and unstirred microspheres (III). (1) After incubation at 6 °C for 25 h; (2) after incubation at 6 °C for 25 h and at 37 °C for 28 h and for system II. (3) DSC thermogram for model system that has been washed after incubation at 6 °C for 25 h, at 37 °C for 28 h.

**Table 2**
Summary of the DSC results for the systems studied

| Indication of water presence | Processing history | ΔH, J/g | $T_{on}$,°C | $T_m$,°C | $T_c$,°C | $T_{range}$,°C |
|---|---|---|---|---|---|---|
| *Microspheres* | | | | | | |
| Microspheres, wet | 25 h at 6 °C, centrifuged/washed | 7.3 (0.5) | 32.9 (3.3) | 47.3 (0.8) | 60.2 (0.1) | 27.3 (3.3) |
| | 25 h at 6 °C, 28 h at 37 °C, centrifuged/washed | 9.9 (0.8) | 37.2 (0.5) | 46.2 (0.5) | 58.3 (1.2) | 21.1 (1.3) |
| Microspheres, dry | 25 h at 6 °C, 28 h at 37 °C, centrifuged/washed, freeze-drying. Analyzed directly after freeze-drying | 6.2 (0.4) | 34.8 (1.9) | 44.6 (0.5) | 58.1 (1.8) | 23.3 (3.7) |
| | 25 h at 6 °C, 28 h at 37 °C, centrifuged/washed, freeze-dried. Analyzed 2 months after freeze-drying | 5.9 (0.9) | 38.2 (0.4) | 47.4 (0.7) | 57.8 (2.1) | 19.6 (2.5) |
| *Model system* | | | | | | |
| Model system, wet | 25 h at 6 °C | 10.1 (0.8) | 32.9 (2.1) | 52.1 (0.5) | 64.9 (0.4) | 32.0 (2.3) |
| | 25 h at 6 °C, 28 h at 37 °C | 8.5 (0.6) | 51.8 (0.5) | 60.8 (0.3) | 68.0 (0.9) | 16.2 (1.2) |
| | 25 h at 6 °C, 28 h at 37 °C, centrifuged/washed | 13.1 (0.3) | 48.8 (0.4) | 59.1 (0.4) | 66.3 (0.1) | 17.5 (0.5) |
| Model system, dry | 25 h at 6 °C, 28 h at 37 °C, freeze-dried | 8.7 (1.6) | 48.4 (2.6) | 58.8 (2.8) | 65.6 (2.3) | 17.2 (0.3) |
| | 25 h at 6 °C, 28 h at 37 °C, centrifuged/washed, freeze-dried. | 9.6 (0.4) | 39.3 (0.5) | 53.6 (0.6) | 62.1 (1.0) | 22.8 (1.1) |
| *Unstirred microspheres* | | | | | | |
| Unstirred | 25 h at 6 °C, centrifuged/washed | 9.7 (0.8) | 34.5 (1.3) | 43.4 (1.0) | 57.3 (1.2) | 22.8 (1.9) |
| microspheres, wet | 25 h at 6 °C, 28 h at 37 °C, centrifuged/washed | 11.4 (0.5) | 37.7 (1.4) | 48.2 (0.2) | 60.3 (0.3) | 22.7 (1.5) |
| Unstirred microspheres, dry | 25 h at 6 °C, 28 h at 37 °C, centrifuged/washed, freeze-dried | 8.4 (1.1) | 37.4 (0.5) | 48.6 (0.5) | 59.2 (1.6) | 21.8 (1.1) |
| *Starch/water* (adapted from Elfstrand et al., 2004) | | | | | | |
| Starch/water, wet | 20 h at 6 °C | 3.1 (0.3) | 39.8 (3.5) | 54.4 (3.5) | 65.4 (0.8) | ~25 |
| | 14 days at 6 °C | 6.9 (0.3) | 34.9 (2.4) | 49.0 (1.2) | 62.7 (1.4) | ~27 |

The onset temperature ($T_{on}$), peak melting temperature ($T_m$), melting interval ($T_{range}$) and the endothermic heat (ΔH) of melting, obtained from DSC analysis are given within brackets.

**Table 3**
Diffraction angle and spacing of X-ray diffraction peak of the wet starch samples

| Peak number | Microspheres | | | | Model system | | | | Unstirred microspheres | | | | Waxy maize starch | | Potato starch | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | After 25 h at 6 °C | | After 25 h at 6 °C and 28 h at 37 °C | | After 25 h at 6 °C | | After 25 h at 6 °C and 28 h at 37 °C | | After 25 h at 6 °C | | After 25 h at 6 °C and 28 h at 37 °C | | | | | |
| | 2θ, ° | d, Å | 2θ, ° | d, Å | 2θ, ° | d, Å | 2θ, ° | d, Å | 2θ, ° | d, Å | 2θ, ° | d, Å | | | | |
| 1 | 5.7 | 15.49 | 5.7 | 15.49 | 5.8 | 15.22 | 5.8 | 15.22 | 5.6 | 15.76 | 5.6 | 15.76 | | | 5.8 | 15.22 |
| 2 | 15.2 | 5.82 | 15.3 | 5.78 | 15.4 s | 5.75 | 15.3 s | 5.78 | 15.1 | 5.86 | 15.1 | 5.86 | 15.3 | 5.78 | 15.3 | 5.78 |
| 3 | 17.4 | 5.09 | 17.4 | 5.09 | 17.4 | 5.09 | 17.4 | 5.09 | 17.2 | 5.15 | 17.2 | 5.15 | 17.4 | 5.09 | 17.3 | 5.12 |
| 4 | | | | | | | | | | | | | 18.3 | 4.84 | | |
| 5 | 19.3 | 4.59 | 20.0 s | 4.43 | 19.4 | 4.57 | 19.4 | 4.57 | 19.4 | 4.57 | 19.2 low | 4.62 | | | 20.1 low | 4.41 |
| 6 | 22.5 | 3.95 | 22.7 wide | 3.91 | 22.5 s | 3.95 | 22.5 s | 3.95 | 22.4 | 3.96 | 22.5 | 3.95 | | | 22.7 | 3.91 |
| 7 | 23.7 | 3.75 | | | 23.7 | 3.75 | 23.7 | 3.75 | 23.9 | 3.72 | 23.7 | 3.75 | 23.5 | 3.78 | | |
| 8 | | | 24.5 wide | 3.63 | | | | | 24.5 s | 3.63 | 24.5 | 3.63 | | | 24.5 | 3.63 |
| 9 | 26.7 low | 3.33 | 26.8 low | 3.32 | 26.8 low | 3.32 | 26.7 low | 3.33 | 26.8 | 3.32 | 26.8 low | 3.32 | 26.4 s | 3.37 | 26.9 | 3.31 |

Diffraction data for waxy maize and potato starch in powder form are given as references for A- and B-type polymorphic arrangement.
s, shoulder.

Gudmundsson, 1996). As expected, the microspheres showed a broad melting interval and lower melting temperatures than the native starch (Fig. 2 and Table 2). However, incubation at 37 °C did not affect the melting temperatures with the exception that $T_{on}$ recorded for these microspheres increased moderately (~4 °C) to 37 °C. Thus, only those crystallites that had a melting temperature below the incubation temperature were eliminated. The low melting temperatures of the microspheres seen here have not been observed for any of the model systems investigated in our previous works although they were prepared from the same starch material as the microspheres in the present study (Elfstrand et al., 2007a, 2007b). In order to understand this discrepancy the present results were compared to model systems.

### 3.1.2. Comparison of microspheres and model systems

The preparation of the two model systems is illustrated in Fig. 1. In the first model system no microspheres are formed, as can be seen by SEM (Fig. 3.) and there is no excess PEG in the system. This system will be called the model system. In the second system investigated, starch dispersion is formed by first stirring for 15 min, while stirring is suspended during the two incubation steps. It can be seen that the initial stirring for 15 min is sufficient to produce small microspheres (Fig. 3). However, the sample also includes aggregates that are not formed during microsphere production. This system will be called unstirred microspheres.

The DSC and X-ray data for the systems can be seen in Fig. 2 and in Table 2. Crystalline and ordered structures were formed in both systems during the first incubation period at 6 °C for 25 h, as seen by both DSC and XRD. The DCS thermograms for these two systems differed from that of the microspheres. Both systems showed higher enthalpies than the microspheres, but while the thermogram for the model system was less distinct than that for the microspheres, the unstirred microspheres showed a more distinct melting profile (Fig. 2).

The X-ray diffraction data indicate that both the model systems show a C-pattern, in similarity with the microspheres. The unstirred microspheres show more diffuse scattering than the microspheres and stronger peaks, especially at 15.5 Å, but in general the X-ray profiles are similar for the two systems. The stronger peaks may indicate that more crystalline material has been formed, in line with the DSC data. This is in contrast to the model system, where the X-ray pattern is weaker than for microspheres but the

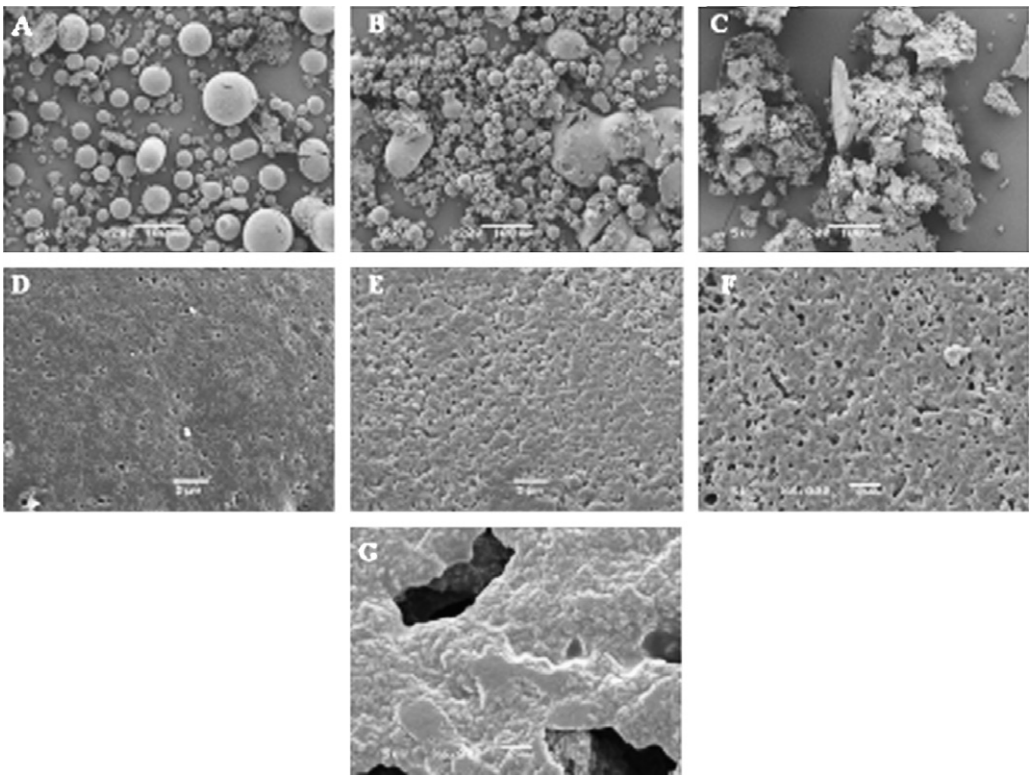


**Fig. 3.** SEM images of freeze-dried samples. General view (the bar is 100 μm) of (A) microspheres, (B) unstirred microspheres, (C) model system. Surface morphology (the bar is 2 μm) of (D) microspheres, (E) unstirred microspheres, (F) model system, (G) starch/buffer.

melting enthalpy was higher (∼27%). The latter in combination with the broad melting peak could be an indication that the higher enthalpy is due to non-crystalline material.

Following the second incubation period, at 37 °C for 28 h, the differences between the microspheres and especially the model system become more pronounced. Most noticeable was that the melting temperatures of the model system shifted to higher values, but not those of the microspheres. The increase in melting temperature for the model system was almost 20 °C, for $T_{on}$, but also $T_m$ (+8°) and $T_c$ (+3°) shifted to higher values, while the melting interval became narrower and the melting curve more distinct than for the sample after the first incubation step. However, the melting enthalpy decreased by 15%. The unstirred microspheres behaved in this aspect more like the microsphere preparation. As for the microspheres, further incubation at 37 °C promoted the growth of the ordered material, which was evident by an increase in the value of ΔH (+15 %), but there was only a minor change in the melting temperature.

To investigate whether the observed differences were due to the washing procedures used to remove PEG from the microspheres and unstirred microspheres, an experiment was performed in which the model system was subjected to the same washing procedure as the microsphere system. The effect of centrifugation/washing was investigated by analysing samples of the model system before and after the procedure. The procedure was performed on the sample incubated in bulk at 6 °C for 25 h and at 37 °C for 28 h, and was then scanned using DSC (Fig. 2). The melting parameters were compared with those of the sample before this procedure (Fig. 2, Table 2). The effect of centrifugation/washing on the melting parameters of the sample was seen as a moderate decrease in the melting temperatures (2–3 °C), while the enthalpy increased by more than 50%. Thus, the difference in the procedures applied to samples of microspheres and unstirred microspheres was not the reason for the formation of crystallites that melted at lower temperatures. However, it might explain the high enthalpies. This experiment also showed that the difference in incubating the samples in DSC pans compared to bulk was not the reason for the low-temperature-melting crystallites, since the melting temperatures of the model system samples incubated in DSC pans and incubated in bulk were similar.

The X-ray data also differed between the model system and the microspheres/unstirred microspheres. The X-ray diffraction pattern changed due to the incubation at 37 °C for the microspheres/unstirred microspheres, while the model system did not show any such changes. This led us to conclude that the organization of the structure in the model system and the microspheres/unstirred microspheres proceeded in different ways. Since the components involved in the systems were the same and the incubation conditions were identical, it was reasonable to assume that excess PEG-solution and/or the fact that crystallization proceeded within the spherical particles created during stirring in PEG solution could be the reason for this difference. One interesting observation is that in the production of the microspheres and the stirred microspheres, the water content of the starch phase changed during the two incubation steps, indicating that water is expelled from the starch phase, while for the model system this ratio was almost unchanged. The transport of water out of the starch phase could be due to a change in the starch/water interaction when an ordered structure is formed. The initial difference in water content and the gradual change in water content seen in the microsphere system could thus partly explain the difference in crystallization kinetic between microspheres and the model system. Furthermore not only water but also low molecular weight species of the starch could be transported out of the microspheres. The molecular weight reduction of the starch material used has lead to an increased polydispersity of the starch and the low molecular fraction could be more soluble in the PEG phase. When studying release from dry starch microspheres it was observed that also in the

absence of amylase carbohydrates where released in up to 20% from the microspheres (unpublished results). If low molecular substances are removed from the microspheres during incubation this could also affect the changes in structure during the incubation at 37 °C. The degree of change could in this case be strongly dependent on the starch material used. However, the melting temperature interval and the x-ray diffraction pattern of starch material where different methods have been used to reduce the molecular weight is quite similar (unpublished results). As microspheres cannot be produced from native starch it can however not be excluded that the reduction in molecular weight is the reason for the observations seen in this investigation.

### 3.2. Effect of freeze-drying on crystal patterns of the different systems

Samples of all three systems studied, microspheres, unstirred microspheres and model system, were freeze-dried. All the samples had been incubated under identical conditions before freeze-drying. Moreover, a sample of the washed/centrifuged model system, and a starch/buffer dispersion incubated at the same time/temperature conditions (i.e. at 6 °C for 25 h and at 37 °C for 28 h) were also freeze-dried. The freeze-dried samples were analyzed by DSC (Fig. 4) and XRD (Fig. 5). The corresponding DSC data are given in Table 2. The morphology of the samples was analyzed using SEM at different magnifications (Fig. 3).

#### 3.2.1. DSC and XRPD

DSC analysis showed the presence of an ordered structure in all freeze-dried samples (Fig. 4). However, the freeze-drying obviously affected the microspheres, as revealed by the decrease in melting enthalpy of microspheres and unstirred microspheres after freeze-drying of 37 and 26%, respectively (Table 2). XRD also showed a loss of structure compared to wet samples. No changes were observed in the melting temperatures of unstirred microspheres while both $T_{on}$ and $T_m$ of the microspheres analyzed directly after freeze-drying seemed to be slightly lower (by 1–2 °C). These temperatures increased to the level before freeze-drying after the microspheres had been stored for 2 months at 6 °C.

In contrast to microspheres and unstirred microspheres, freeze-drying of the model system did not markedly change the enthalpy. The melting temperatures decreased by 2–3 °C. The application of centrifugation/washing prior to freeze-drying changed the effect of freeze-drying on the ordered structure of the model system. The enthalpy decreased by 27% (Table 2), the melting temperatures were reduced (by 9 °C for $T_{on}$ and 4–5 °C for $T_m$ and $T_c$), and the melting interval widened by at least 5 °C. Thus, freeze-drying had a destructive effect on the molecular short-range ordering of microspheres and unstirred microspheres, while the model system was not affected to the same extent until the PEG was removed. After removal of the PEG and changing the carbonate buffer to phosphate buffer the model system seemed to be much more sensitive to freeze-drying than it was in the presence of PEG, and even more sensitive than the microspheres and unstirred microspheres, judging from the shift in melting temperatures. This suggests that the PEG or the carbonate buffer protected the structure during freeze-drying, but also that in microspheres and unstirred microspheres the structure formed during incubation was less sensitive than that formed during the same incubation in the model system.

Diffractograms of freeze-dried microspheres recorded immediately after the drying procedure and after 2 months' storage at 6 °C are presented in Fig. 5A (1 and 2). Storage of freeze-dried microspheres for 2 months affected the melting parameters less, but improved the crystalline long-range ordering, as seen by the development of more distinct peaks, although at the same positions as before. Thus, during storage the crystallization process proceeded slowly. XRD revealed that the long-range order was destroyed to a large extent during freeze-drying. Judging from the model system samples it was clear that the presence of PEG could not prevent this effect. However, the peaks in the diffractograms of microspheres and unstirred microspheres had higher resolution than those in the model system. Thus, it appears that the presence of PEG could prevent the breakdown of the structure to some extent in this case. Common to the microspheres/unstirred microspheres was the absence of the peak at ~15 Å, which is indicative of the type B-pattern, and which was clearly seen in all the samples before freeze-drying. In contrast, this peak could be seen in the freeze-dried sample of the starch/buffer (Fig. 5D).

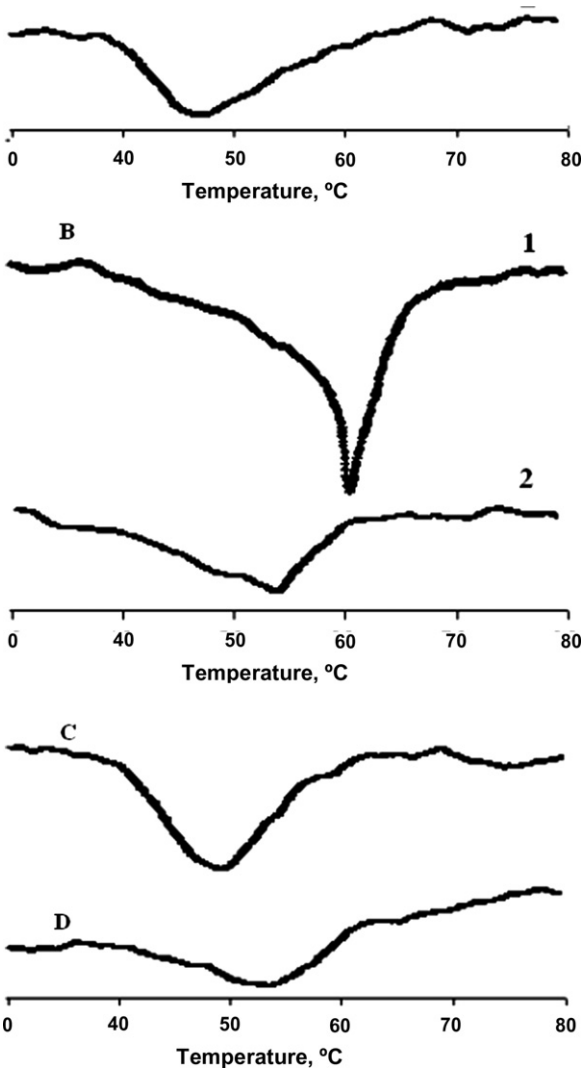


**Fig. 4.** DSC thermograms of freeze-dried powders: (A) microspheres, (1) directly after freeze-drying, (2) 60 days after freeze-drying and storage at 6 °C. (B) model system, (1) freeze-dried after incubation at 6 °C and 37 °C, (2) freeze-dried after centrifuging/washing of the sample incubated at 6 °C and 37 °C. (C) unstirred microspheres; (D) starch/buffer.

#### 3.2.2. SEM

Observations using SEM (200×) revealed considerable differences in appearance between the samples (Fig. 3). The microspheres were mainly spherically shaped, although the population included particles with cracks and fractures (Fig. 3A). The surface of the particles was rough and porous (Fig. 3D).

SEM imaging of unstirred microspheres also revealed the presence of spherical particles in the sample (Fig. 3B), which indicated

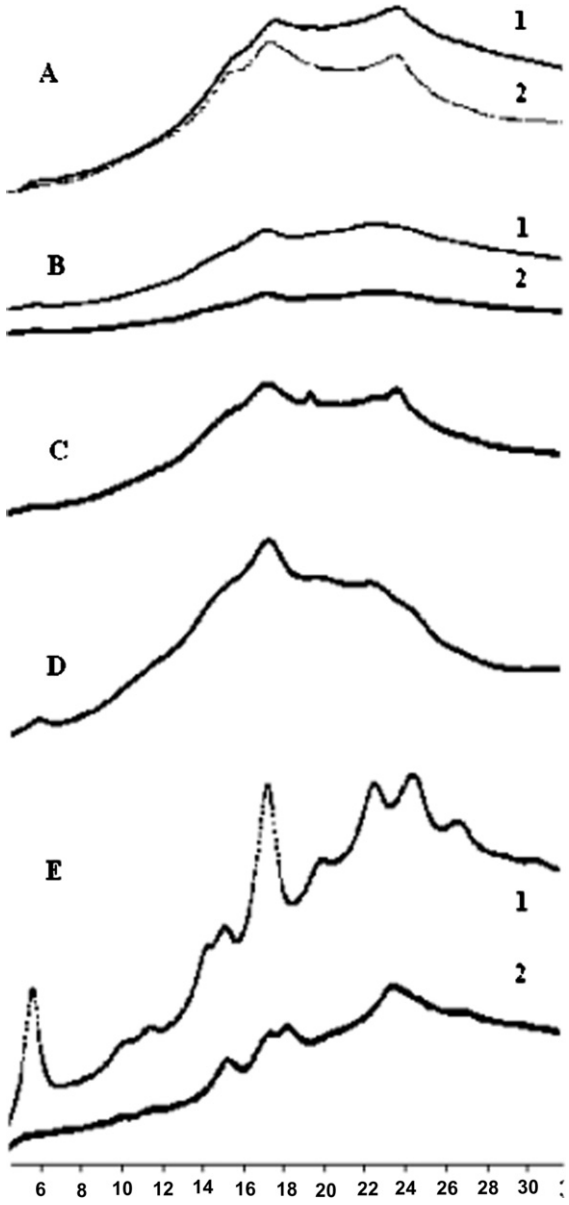


**Fig. 5.** X-ray diffractograms of freeze-dried powders. (A) microspheres, (1) directly after freeze-drying, (2) 60 days after freeze-drying and storage at 6 °C. (B) model system, (1) freeze-dried after incubation at 6 °C and 37 °C, (2) freeze-dried after centrifuging/washing of the sample incubated at 6 °C and 37 °C. (C) unstirred microspheres; (D) starch/buffer and (E) (1) native potato starch and (2) native waxy maize starch.

that the primary stirring for 15 min at 20 °C was sufficient to stabilize some but not all of the starch microparticles. However, the particles seemed to be much smaller than those of the microspheres. In the image of unstirred microspheres these particles can be seen together with larger irregular fragments. The spherical particles of unstirred microspheres were perfectly round, and were seen to have a rough porous surface when studied at a higher magnification (6000×) (Fig. 3E).

The model system samples did not reveal any spherical particles. However, when examining the surface of this material at a magnification of 6000× a rough, porous surface was seen, similar to that observed for unstirred microspheres (Fig. 3F) and that reported for starch microspheres of high quality (Elfstrand et al., 2006). This surface was observed in all the samples that were prepared in the presence of PEG and BSA, while the samples of starch/buffer, for example, did not reveal a porous surface. This confirms our previous results that the presence of PEG and BSA affects the ordering of the starch molecules (Elfstrand et al., 2007b).

Thus, the differences revealed by DSC and XRD between the model system and the two other samples, could be due to the fact that the microspheres and unstirred microspheres contained spherical particles that were formed in a dispersed phase in an aqueous PEG/starch system. It is also interesting to note that the unstirred microspheres formed much smaller spherical particles than the microspheres, but showed the highest enthalpy after the full temperature treatment, and the lowest values of $T_m$ and $T_c$ after incubation at 6 °C.

## 4. Conclusions

The crystallization and ordering of starch occurring within small droplets distributed through the continuous PEG phase during starch microsphere production is different from that seen in a model system that does not contain spherical particles. The ordering of starch molecules increased during incubation at the higher temperature and the structure formed melted at lower temperatures than the corresponding model system. The crystalline/ordered structure obtained during incubation at 6 °C was changed during incubation at 37 °C and this reformation occurred within structural cell units at spacings under 5 Å. The reformation on crystalline level was accompanied by an increase in melting enthalpy, reflecting the increase in crystallinity or number of double helices.

Freeze-drying had a destructive effect on the long-range (crystalline) order in the crystallized starch, but the short-range order (double helical) was less affected. The presence of PEG or carbonate buffer had a cryo-protective effect on the short-range order, preventing it from breaking down.

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