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*Published in:*  
European Journal of Pharmaceutics and Biopharmaceutics

*Link to article, DOI:*  
[10.1016/j.ejpb.2016.12.008](https://doi.org/10.1016/j.ejpb.2016.12.008)

*Publication date:*  
2017

*Document Version*  
Peer reviewed version

[Link back to DTU Orbit](#)

*Citation (APA):*  
Nielsen, L. H., Rades, T., Boyd, B., & Boisen, A. (2017). Microcontainers as an oral delivery system for spray dried cubosomes containing ovalbumin. *European Journal of Pharmaceutics and Biopharmaceutics*, 118, 13-20. <https://doi.org/10.1016/j.ejpb.2016.12.008>

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1 **Microcontainers as an oral delivery system for spray dried cubosomes containing ovalbumin**

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3

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12

13

14 **Abstract**

15 The purpose of this study was to prepare cubosomes encapsulating the model antigen ovalbumin (OVA) via  
16 spray drying, and to characterise such cubosomes with a view for their potential application in oral vaccine  
17 delivery. Furthermore the cubosome formulation was loaded into polymeric microcontainers intended as  
18 an oral drug delivery system. The cubosomes consisted of commercial glyceryl monooleate, Dimodan®,  
19 containing OVA and were surrounded with a dextran shell prepared by spray drying. Cryo-TEM was used to  
20 confirm that cubosomes were formed after hydration of the spray dried precursor powder. The precursor  
21 powder had a mean particle size of  $1.3\pm 0.1\ \mu\text{m}$ , whereas the mean diameter of the dispersed cubosomes  
22 was  $282\pm 7\ \text{nm}$  (PDI: 0.18) measured by dynamic light scattering.  $8.5\pm 0.3\ \%$  (w/w) of OVA was present in the  
23 cubosome powder and OVA was found released slowly over the first 70 h, followed by a more rapid  
24 release. Total release of  $47.9\pm 2.8\ \%$  of loaded OVA occurred over 96 h in a buffer at pH 6.8. When the  
25 powder was filled into microcontainers, and the opening covered with the pH sensitive polymer Eudragit  
26 S100, the pH sensitive 'lid' was intact at gastric pH, but release of OVA from the cubosomes and  
27 microcontainers occurred at pH 6.8, releasing  $44.1\pm 5.6\ \%$  of the OVA in 96 h. Small-angle X-ray scattering  
28 (SAXS) revealed that the 'dry' particles possessed an internal ordered lipid structure (lamellar and inverse  
29 micellar phase) by virtue of a small amount of residual water, and after hydration in buffer at pH 6.8, the  
30 particles formed the hexagonal inverse cubic phases, thereby indicating that cubosomes were formed  
31 when released from microcontainers.

32 **Introduction**

33 Vaccination is often regarded as the most significant contribution to public health and disease prevention  
34 and moreover, it is a very cost-effective medical intervention [1,2]. Vaccination has reduced the morbidity  
35 and mortality resulting from diseases such as tuberculosis and smallpox and has thereby saved millions of  
36 lives. In spite of this, many infectious diseases remain endemic in large parts of the world, and therefore  
37 vaccination is an area in continuous development [1,2].

38  
39 Most vaccines are administered by injection and there are only a few oral vaccines on the market such as  
40 rotavirus vaccine (as solution or suspension) and a capsule with vaccine formulation against typhoid fever  
41 [3]. Although, the oral route can be beneficial for vaccine administration [4,5]. Some of the advantages of  
42 oral vaccines are the ease of administration and an increased safety compared to injections. In addition,  
43 there is also a great potential for mass vaccination without the requirements of trained personnel [4,6].  
44 Furthermore, oral vaccines have the ability to induce both mucosal and systemic immune responses [6,7],  
45 as shown in the 1990s with several HIV vaccines [8], and they are therefore considered ideal for combating  
46 infectious diseases. Although, oral vaccines have several attractive features, there are some major  
47 challenges.

48 The target of vaccine formulations in the gastro-intestinal (GI) tract is the M-cells in the intestine [9]. The  
49 antigen might be damaged, when passing through the harsh environment of the GI tract, which in turn will  
50 lead to the need for large doses. In addition, there is a poor transport of the antigen across the intestinal  
51 epithelium [4].

52  
53 Traditional vaccines are mainly composed of heat-inactivated bacteria or viruses resulting in high  
54 immunogenicity. The risk with these types of vaccines is that they, in the body, can change to the active  
55 state and thereby infect the patients with the bacteria or virus and thus, leading to unwanted side effects  
56 [1,10]. Consequently, new generation vaccines are developed with subunit antigens. These subunit  
57 antigens are highly purified components of pathogens and thereby chemically well-defined. Hence, there is  
58 a much higher safety than for traditional antigens, but as the subunit antigens lack most of the features of  
59 the original pathogen they tend to be poorly immunogenic [1,10]. Therefore, to succeed with oral vaccine  
60 delivery, delivery systems need to be developed, in which the antigen can be encapsulated into particles  
61 [11,12]. These particles will assure presentation of the antigen to the antigen-presenting cells, but can also  
62 stabilise and release the antigen over an extended period of time [10]. Some particles will provide an  
63 adjuvant effect in themselves, but potent adjuvants can in addition also be added to the particulates for  
64 inducing an effective immunity [13].

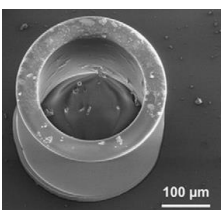
65  
66 There are many possibilities for vaccine delivery systems, and some of the most common ones are:  
67 polymeric micro- and nanoparticles, immunostimulatory complexes and liposomes [14,15]. Cubosomes  
68 have also shown to be an efficient delivery system for vaccines [11]. Cubosomes contain a highly twisted,  
69 continuous lipid bilayer with two congruent, non-intersecting water channels, giving the particles both  
70 hydrophobic and hydrophilic domains [11]. This offers great flexibility with respect to the types of  
71 compounds, that can be incorporated into the particles [16,17]. Rizwan et al. found that significantly higher  
72 amounts of antigen can be encapsulated in cubosomes compared to liposomes due to the larger surface  
73 area of cubosomes, and moreover in cubosomes, the antigen was also retained more efficiently compared  
74 to liposomes [11].

75 Traditionally, cubosomes are produced by mixing monoolein or phytantriol and water and thereby creating  
76 a high-energy dispersion followed by colloidal stabilisation using polymeric stabilisers [11,18,19]. However,  
77 it can be desirable to have the vaccine particles in a powder form (here termed “precursors”), and  
78 precursors of cubosomes have earlier been produced by either freeze drying [20] or spray drying [21–23].  
79 In the spray drying process, dry powder precursors have the active ingredient incorporated, and upon  
80 hydration colloidal stable cubosomes are spontaneously formed. The powder form of the vaccine  
81 formulation can be advantageous in terms of stability of the antigen. Also, there is no need for a cold-chain  
82 storage which is needed for traditional vaccines [4].

83  
84 After oral administration of the vaccine formulations, the antigen needs to be protected in the stomach and  
85 during transportation to the small intestine. In the small intestine, the vaccine particles should be delivered  
86 to the microfold (M) cells of the peyer’s patches as they will present the antigen to the underlying immune  
87 cells and thereby obtain an immune response [24]. The particles, carrying the antigen (and adjuvant), can  
88 give some protection of the antigen through the GI environment, but often the particles will also degrade  
89 on the way to the intestine, and therefore more advanced drug delivery systems can be necessary. An  
90 example of these advanced drug delivery systems is microcontainers. Microcontainers are polymeric,  
91 cylindrical devices in the micrometre size range (Fig. 1) [25–27]. They have the potential for targeted  
92 and/or sustained delivery in the GI tract [28]. Some of the advantages of the microcontainers are that size  
93 and shape can be controlled very precisely. Furthermore, the devices allow for unidirectional release, as  
94 only one side of the microcontainer is open, compared to more conventional microparticles where release  
95 can occur from the whole surface area. This has shown to increase the drug concentration at the  
96 microdevice-cell interface and thereby, allowing for increased permeation of the drug *in vitro* leading to  
97 enlarged oral bioavailability of the drug [27,29,30]. In addition, the antigen can be protected inside the  
98 cavity of the microcontainer from the harsh environment of the stomach until release is desirable [31,32].  
99 The microcontainers have previously shown to interact with the intestinal mucus resulting in prolonged  
100 drug absorption [27]. It is reported in the literature that one way to improve oral vaccine delivery is to  
101 extend the intestinal residence time [13], hence, the microcontainers can be a promising platform for this  
102 purpose. In this paper, SU-8 (an epoxy photoresist) was used as a model polymer for fabrication of the  
103 microcontainers [25,26,31], but microcontainers have also been fabricated using biopolymers such as poly-  
104 L-lactic acid (PLLA) [33,34].

105  
106 The aim of this study was, as a proof-of-concept, to prepare and characterise cubosomes loaded with  
107 ovalbumin (OVA) in a spray dried powder form for future application in oral vaccine delivery. The precursor  
108 powder was filled into microcontainers for protection and release control, and the *in vitro* release was  
109 studied together with small-angle X-ray scattering (SAXS) to confirm whether cubosomes were released  
110 from the microcontainers as internally structured particles.

111



112

113

Fig. 1: SEM image of an SU-8 microcontainer with an inner diameter of 223  $\mu\text{m}$  [26].

114

## 115 **Materials and methods**

### 116 Materials

117 OVA was purchased from TCI Europe (Zwijndrecht, Belgium). Dimodan® D90 was kindly donated by Danisco  
118 (Grindsted, Denmark). Dextran (from Leuconostoc Mesenteroides) and potassium dihydrogen phosphate  
119 were acquired from Sigma-Aldrich (St. Louis, MO, USA). Pierce BCA Protein Assay kit was purchased from  
120 Thermo Fisher Scientific (Rockford, IL, USA). Deionised water was obtained from an SG Ultra Clear water  
121 system (SG Water USA, LLC, Nashua, NH, USA) and was freshly produced in all cases. All other chemicals  
122 used were of analytical grade.

123

### 124 Spray drying of precursors for cubosomes containing OVA

125 Cubosomes were prepared using a commercial source of glyceryl monooleate (GMO), Dimodan® MO 90/D.  
126 The cubosomes were loaded with OVA as a model antigen, and the particles were surrounded by a dextran  
127 shell. The particles were prepared by first dissolving GMO in ethanol (1.78 w/v %), and then mixing with a  
128 solution of OVA in MilliQ water (0.075 final w/w % of OVA). After 1 h of mixing, dextran dissolved in MilliQ  
129 water (1.77 w/v %) was added to the GMO/OVA solution (0.72 w/w % of GMO + OVA), and the final  
130 solution was spray dried using a B 290 Büchi mini spray dryer (Büchi Labortechnik AG, Flawil, Switzerland).  
131 Free OVA was not removed prior to the spray drying process.

132 For the spray drying of the precursors, a 0.7-mm nozzle was used and air was utilised as the drying medium.  
133 Spray drying was performed at an inlet temperature of 200 °C resulting in an outlet temperature of  
134 approximately 85 °C. The drying flow rate was set to 32 m<sup>3</sup>/h and an aspirator capacity of 80 % with a feed  
135 rate of 4 mL/min was used. Particles without OVA were also produced as blank particles and used as  
136 reference.

137

### 138 Cryo-TEM of cubosomes

139 The precursors for the cubosomes with OVA were dispersed in MilliQ water at a concentration of 1 mg/mL.  
140 The samples for the Cryo-TEM studies were prepared in a controlled environment vitrification system  
141 (CEVS). A small amount of the sample (5 µL) was put on a carbon film supported by a copper grid and  
142 blotted with filter paper to obtain a thin liquid film on the grid. The grid was quenched in liquid ethane at  
143 -180 °C and transferred to liquid nitrogen (-196 °C). The samples were then examined using a Tecnai G2  
144 F30 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) operating at a voltage of 300 kV  
145 and a working temperature of -180 °C. Images were recorded using Gatan UltraScan 1000 (2k × 2k) CCD  
146 camera (Gatan, California, USA).

147

### 148 Size of particles

149 The size of the dry particles with the dextran shell was measured using aerosizer particle size analyser  
150 (Model 3321, TSI Incorporated, MN, USA) by setting the pump to 1.37 bar and with a capillary flow of 8  
151 L/min. A small amount of powder was distributed on the plate and the particle size was measured in six  
152 replicates.

153

154 For the particles dispersed in water, the particle size distribution (Z-average), polydispersity (PDI) and zeta  
155 potential were determined using dynamic light scattering (Malvern Zetasizer, NanoZs ZEN 3600, Malvern,  
156 UK). Measurements were performed at 37°C, and the results presented are the mean of three successive

157 measurements of 100 s of at least three independent samples. Samples were diluted with water to adjust  
158 the signal level.

159

#### 160 OVA present in the cubosomes

161 Precursor powder (10 mg) was added to a solution of 20 mM phosphate buffer, pH 6.8 containing 5 %  
162 Triton X-100. After vortex mixing, the cubosomes were dissolved and a sample of 200  $\mu$ L was taken out. A  
163 BCA Protein Assay kit was used to determine how much OVA was present in the cubosome powder by  
164 following the procedures for the standards and samples recommended by the manufacturer. The same  
165 process was performed with the blank cubosomes to check for any cross activity of the formulation. The  
166 absorbance was measured at 562 nm on a plate reader, and the obtained absorbance values were analysed  
167 against the standard curves prepared on the same day as the samples. OVA entrapment was then  
168 determined by calculating the difference between the total OVA added before the spray drying process and  
169 the free fraction of OVA in the solution. The experiments were performed in triplicates.

170

#### 171 Fabrication of SU-8 microcontainers

172 Production of the microcontainers involved two steps of photolithography with the negative epoxy-based  
173 photoresist, SU-8 [26,32]. The microcontainers were structured on a fluorocarbon coating deposited on top  
174 of the supporting silicon wafer by plasma polymerisation. This enabled dry removal of the fabricated SU-8  
175 devices from the support substrate in order to obtain individual microcontainers if needed [27,35]. The  
176 fabricated microcontainers had an inner diameter of  $223\pm 3$   $\mu$ m and a height of  $270\pm 3$   $\mu$ m (mean $\pm$ SD, n=6).  
177 Silicon wafers supporting the microcontainers were finally cut into squares of 12.8 x 12.8 mm<sup>2</sup> using an  
178 Automatic Dicing Saw from DISCO (Kirchheim b. München, Germany). Each chip contained arrays of 25 x 25  
179 containers with a pitch of 450  $\mu$ m.

180

#### 181 Filling of microcontainers with powder precursors

182 Powder precursors were manually distributed on the microcontainer chip. The excess drug in between the  
183 microcontainers was then removed with pressurised air, resulting in powder-filled microcontainers [27].  
184 The chip with microcontainers was weighed before and after filling to determine the amount of drug filled  
185 into the microcontainers.

186

#### 187 Spray coating of the filled microcontainers with Eudragit S100

188 A spray coating system (ExactaCoat, Sono Tek, USA) equipped with an ultrasonic nozzle actuated at 120 kHz  
189 [36] was used to deposit Eudragit S100 (dissolved to a 2 % (w/w) solution in isopropyl alcohol) on the cavity  
190 of the drug-filled microcontainers in a set-up similar to previously described [33]. The generator power was  
191 set to 1.5 W, and the polymer solution was pumped through the nozzle at a flow rate of 100  $\mu$ L/min.  
192 Nitrogen gas at a pressure of 10 mbar was used to direct the beam of droplets onto the microcontainers,  
193 and the distance between nozzle and substrate was 40 mm with the beam diameter on the substrate being  
194 approximately 4 mm. The lateral movements of the nozzle were controlled by an x-y stage and the nozzle  
195 path was defined in the equipment software. The nozzle was moved line-by-line at a speed of 25 mm/s, and  
196 the coating was repeated 60 times to obtain a coating thickness in the  $\mu$ m range.

197

#### 198 Release of OVA from the cubosomes

199 *In vitro* release of OVA from the cubosomes unconfined (bulk powder) and confined in microcontainers  
200 coated with Eudragit S100 was investigated on a  $\mu$ DISS profiler (*p*ION INC, Woburn, MA). In both release  
201 studies, each channel was calibrated with its own OVA standard curve prior to the experiments. For the  
202 calibration curves, aliquots of OVA in water stock solution were repeatedly added to 10 mL of either a HCl  
203 solution or a phosphate buffer in order to achieve a range of defined standard concentrations, and the UV  
204 spectrum of each standard was recorded. The release experiments were performed at  $37\pm 0.5^\circ\text{C}$  using a  
205 stirring rate of  $200\pm 5$  rpm using 20 mm path length *in situ* UV probes on a  $\mu$ DISS profiler. The absorbance  
206 data was evaluated using 280 nm on the standard curve and utilising the 2<sup>nd</sup> derivative function in the Au  
207 Pro software affiliated with the  $\mu$ DISS profiler.

208

209 The release of OVA from the precursor powder was studied in 20 mM phosphate buffer, pH 6.8 for 96 h.  
210 The *in situ* UV probes were situated in each sample vial containing 10 mg of powder and 10 mL of  
211 phosphate buffer was added. The probes scanned and detected the absorbance of released OVA.

212 The release studies from the microcontainers were performed in a set-up similar to one previously  
213 described [25,27,33]. The chips with microcontainers were attached to cylindrical magnetic stirring bars  
214 (using carbon pads) and placed in the bottom of sample vials. The chips were covered with 10 mL of 0.1 M  
215 HCl pH 1.6 for 2 h and subsequently, the medium was changed to 10 mL of 20 mM phosphate buffer, pH  
216 6.8 for 96 h, and the *in situ* UV probes detected the absorbance.

217 Both sets of experiments were performed in 3 replicates.

218

#### 219 Scanning electron microscopy of the microcontainers

220 SEM was utilised to examine the microcontainers after filling, after spray coating of the lid of Eudragit S-  
221 100, and after release in phosphate buffer at pH 6.8. The examinations were carried out using a Phenom  
222 Pro scanning electron microscope (Phenom World, Eindhoven, the Netherlands). Prior to the investigations,  
223 the microcontainer chip was mounted onto metal stubs, and imaging was performed at an operation  
224 voltage of 10kV with a 600x magnification.

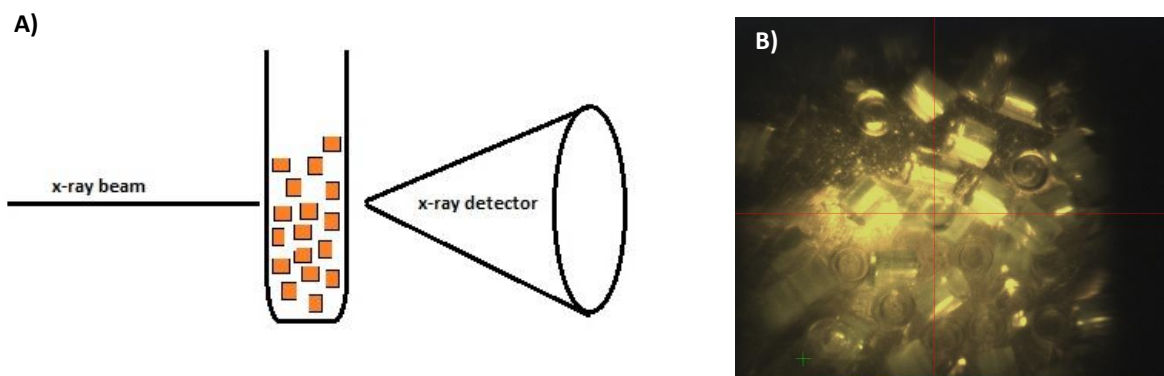
225

#### 226 SAXS determination of the structure of cubosomes loaded into microcontainers

227 The SAXS/WAXS beamline at the Australian Synchrotron, Clayton, Australia [37] was used to determine the  
228 internal structure of the spray dried particles, when the cubosomes were confined in microcontainers and  
229 released from the devices. The X-ray beam had an energy of 11 keV, and the 2D SAXS patterns were  
230 collected using a Pilatus 1M camera (active area  $169 \times 179 \text{ mm}^2$  with a pixel size of  $172 \times 172 \mu\text{m}$ ), which  
231 was located 900 mm from the sample position. The total  $q$  range for the instrument configuration outlined  
232 above was  $0.02 < q < 1.06 \text{ \AA}^{-1}$ , and 2D SAXS patterns were collected for 1 sec. The in-house designed  
233 computer software 'ScatterBrain' was used to acquire and reduce these 2D patterns to 1D intensity versus  
234  $q$  profiles. The powder-filled microcontainers were separated from the base using a scalpel, and filled into a  
235 1.5 mL capillary and SAXS patterns were acquired in dry state followed by addition of 50  $\mu\text{L}$  of MilliQ water,  
236 where after patterns were acquired for a time period of 80 min. The set-up with empty microcontainers as  
237 an example can be seen in Fig. 2A, with an image of the microcontainers in a capillary in the X-ray beam  
238 shown in Fig. 2B.

239





240  
 241 Fig. 2: A) Schematic of the SU-8 microcontainers filled into a capillary to be used in the SAXS/WAXS  
 242 synchrotron. B) Micrograph showing the set-up with the microcontainers in the x-ray beam.

243

244 Statistics

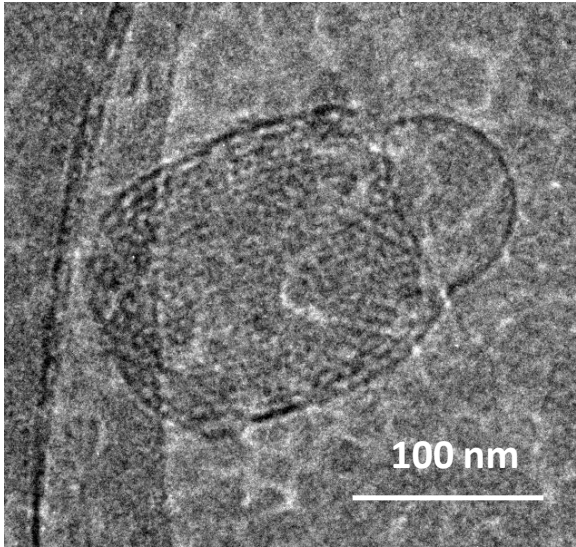
245 The data are expressed as mean  $\pm$  standard deviation (SD). Where appropriate, statistical analysis was  
 246 carried out using Student t-tests using GraphPad Prism version 7.00 (GraphPad Software Inc., CA, USA). P-  
 247 values below 5 % ( $p < 0.05$ ) were considered statistically significant.

248

249 **Results and discussion**

250 For the production of the powder precursors of cubosomes, spray drying was chosen as this is a simple  
 251 technique converting a solution to powder in a one-step process [38]. GMO has for many years been one of  
 252 the lipids of choice for producing cubosomes, as it is non-toxic, biocompatible and biodegradable [39], and  
 253 therefore it was decided to produce GMO particles in this study. The spray drying technique is convenient  
 254 for producing the powder precursors, but GMO can be challenging to spray dry as it immediately forms the  
 255 cubic phase upon hydration. Spicer et al. studied the effect of applying ethanol as a hydrotrope and this  
 256 resulted in the formation of a low-viscous emulsion that was easily spray dried [21]. For this reason, in this  
 257 study, GMO was first dissolved in ethanol and then added to the aqueous dextran solution. It has been  
 258 reported that GMO itself produces sticky agglomerates after spray drying, and to obtain a more flowable  
 259 powder an aqueous starch or a dextran solution can be added prior to spray drying resulting in the GMO  
 260 being encapsulated in a dry starch or dextran shell [21–23]. In this work, it was chosen to add dextran as  
 261 the anti-cohesion agent, and the produced powder was flowable and easy to hydrate. After production of  
 262 the GMO powder precursors, the powder was hydrated and cryo-TEM was performed to identify whether  
 263 cubosomes were obtained. It can be observed in Fig. 3 that cubic structures were found after hydration of  
 264 the powder.

265



266  
 267 Fig. 3: Cryo-TEM image of a representative hydrated particle with a distinct cubic liquid crystalline  
 268 structure. The resolution in cryo-TEM images is limited by the presence of dissolved dextran.

269  
 270 *In vitro* characterisation of the particulates

271 The size, shape and surface charge of a particulate vaccine carrier will influence its performance as a  
 272 vaccine [40]. The dry powder with OVA and the dextran shell had a size of  $1.3 \pm 0.1 \mu\text{m}$ , whereas the dry  
 273 blank particles without OVA had a size of  $1.6 \pm 0.1 \mu\text{m}$ . After hydration, self-assembled, close to neutrally  
 274 charged nanoparticles were formed, with mean size of  $146.1 \pm 1.3 \text{ nm}$  and  $281.7 \pm 7.4 \text{ nm}$  for the blank and  
 275 OVA-loaded particles, respectively (Table 1). There was a significant size difference between the blank and  
 276 OVA-loaded particles ( $p\text{-value} < 0.0001$ ), and the PDI for both formulations was low, indicating  
 277 homogeneous formulations. The particles were much smaller than those reported by Spicer et al., where  
 278 the dry particles had a diameter of  $24 \mu\text{m}$ , and in the hydrated form the cubosomes were in average  $0.6 \mu\text{m}$   
 279 with a size distribution from  $0.1$  to  $5 \mu\text{m}$  [22]. In general, it is reported that the particle size should be  
 280 between  $20 \text{ nm}$  to  $10 \mu\text{m}$  to be well recognised by the immune system [11], but more specifically for oral  
 281 vaccine formulations, a size between  $200\text{-}500 \text{ nm}$  can be advantageous for uptake into the antigen-  
 282 presenting cells after oral administration [40,41]. In relation to this, it can be observed that the size of the  
 283 cubosomes with OVA is in this size range, and the cubosomes should therefore be able to be taken up by  
 284 the antigen-presenting cells.

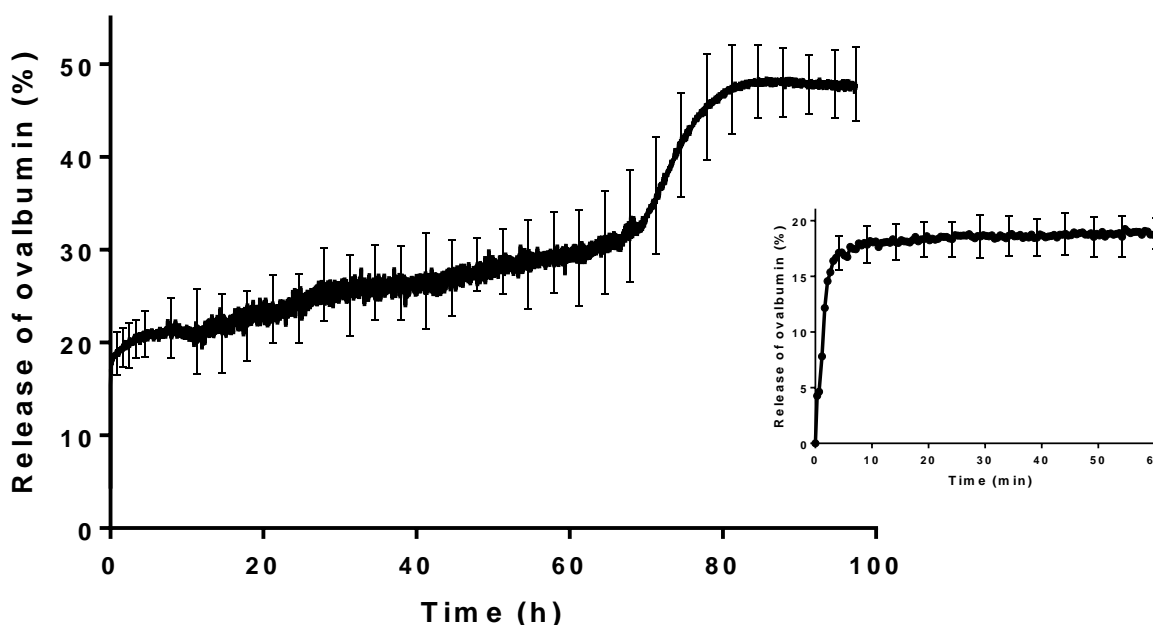
285  
 286 Table 1: Size measurements of the hydrated cubosomes with and without OVA dispersed in MilliQ water.  
 287 The measurements were performed using dynamic light scattering in triplicates, and data are represented  
 288 as mean $\pm$ SD

	Z-average (nm)	PDI	Zeta potential (mV)
<b>Blank particles</b>	$146.1 \pm 1.3$	$0.15 \pm 0.02$	$-0.43 \pm 0.077$
<b>Particles with OVA</b>	$281.7 \pm 7.4$	$0.18 \pm 0.11$	$-0.18 \pm 0.042$

289  
 290 Presence and release of OVA in and from the particles

291 Before the release measurements, it was initially determined that  $8.5 \pm 0.3 \%$  (w/w) OVA was present in the  
 292 cubosome powder. It is also well-known that cubosomes often provide a sustained release of a drug [39],  
 293 and this is also observed in this study, where release studies in buffer at pH 6.8 showed that during the first

294 70 h, OVA was slowly released, followed by a more rapid release from 70-80 h. A total release of  $47.9 \pm 2.8\%$   
 295 was observed in relation to the total loading of OVA in the cubosomes over a 96 h period (Fig. 4). It can be  
 296 seen that there is a significant burst release of OVA from the cubosomes (insert in Fig. 4) of  $18.2 \pm 1.6\%$   
 297 in the first 10 min. This is probably caused by the release of OVA from the powder just when the powder  
 298 precursors are dispersed in the aqueous solution, and thereafter the OVA entrapped in the channels of the  
 299 cubosomes is released. In the literature, it is reported that OVA was released during 168 h from cubosomes  
 300 resulting in a complete release [18]. A study preparing precursors of cubosomes by spray drying, but  
 301 encapsulating the highly lipophilic drug, efavirenz, also is reporting on a burst release of the drug of up to  
 302 16 %, with a total release in 12 h of up to 56 %, again indicating that when dispersing powder precursors in  
 303 aqueous solution a burst release is occurring [23]. A sustained release of OVA is also observed in this study  
 304 and this could be beneficial when developing vaccine formulations [42].  
 305



306  
 307 Fig. 4: Release of OVA from the cubosomes in 20 mM phosphate buffer pH 6.8, expressed as % of the total  
 308 content of OVA. The insert is showing the release over the first 60 min. The release study was performed in  
 309 triplicates, and the data represent mean  $\pm$  SD.  
 310

### 311 Internal structure of particles formed upon hydration from microcontainers

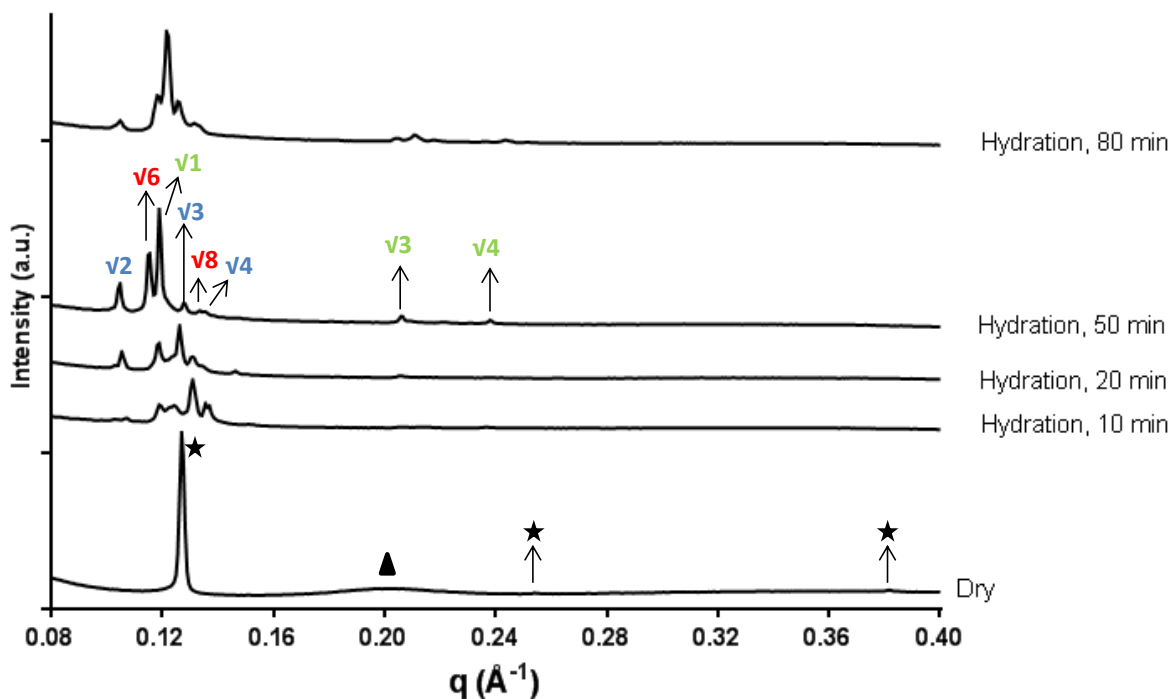
312 SAXS/WAXS can be used to detect phase transformations in self-assembled lipid systems, and this was  
 313 utilised to identify whether particles released from the microcontainers contained internal nanostructures  
 314 consistent with cubosomes. Fig. 5 shows the plot of intensity versus the scattering vector  $q$  obtained from  
 315 the release of GMO particles in dry form and when the microcontainers containing the particles were  
 316 dispersed in water for a period of 80 min. For the dry particles, it can be observed that there are three  
 317 equally spaced peaks in the diffractogram (Fig. 5), indicating that the dry particles are in a lamellar phase  
 318 with the lattice parameter of  $49.5 \text{ \AA}$  (Table 2). There is also an inverse micellar phase present, indicated by  
 319 the broad peak at  $q \sim 0,2 \text{ \AA}^{-1}$  in the diffractogram) with a D-spacing of  $31.1 \text{ \AA}$ . This can be explained by the

320 presence of residual moisture in the spray dried powder. According to the phase diagram of GMO in water,  
 321 the inverse micellar phase and lamellar phase coexist at approximately 5 % water [43,44], consistent with  
 322 Spicer et al. reporting approximately 5 % (w/w) of moisture content in their spray dried cubosome powders  
 323 [22].

324

325 After hydration (here exemplified by the diffractogram at 50 min), the liquid crystalline nanostructured  
 326 particles showed a mix of phases, with peak indexing indicating coexisting inverse hexagonal ( $H_2$ ) phase  
 327 (peaks at  $v1 : v3 : v4$ ), . Pn3m cubic phase (peaks at  $v2 : v3 : v4$ ), and Ia3d cubic phase (peaks at  $v6 : v8$ ).  
 328 These three phases appear in the GMO + water phase diagram [44], and the calculated lattice parameters  
 329 are listed in Table 2. The presence of  $H_2$  and Pn3m cubic phases for commercial GMO samples in water  
 330 might be expected at full hydration, however the Ia3d cubic phase is only expected at less than full  
 331 hydration o the lipid. Therefore it is proposed that the particles were not completely hydrated after 50 min,  
 332 which is also supported by the fact that after 80 min the Ia3d phase appears even less prominent.

333



334

335 Fig. 5: 2D SAXS patterns were collected from cubosomes confined in microcontainers and followed while  
 336 the cubosomes were released from the microcontainers in MilliQ water. The cubosome filled  
 337 microcontainers were enclosed in a glass capillary during hydration for up to 80 min. After 50 min of  
 338 hydration the particles show a mix of phases with inverse hexagonal ( $H_2$ ) phase (peaks at  $v1 : v3 : v4$ ),  
 339 Pn3m cubic phase (peaks at  $v2 : v3 : v4$ ), and Ia3d cubic phase (peaks at  $v6 : v8$ ).

340

341

342

343 Table 2: Phase structure and lattice parameters obtained from SAXS measurements of dry particles and  
 344 particles released from microcontainers after hydration, here with an example after 50 min of hydration.

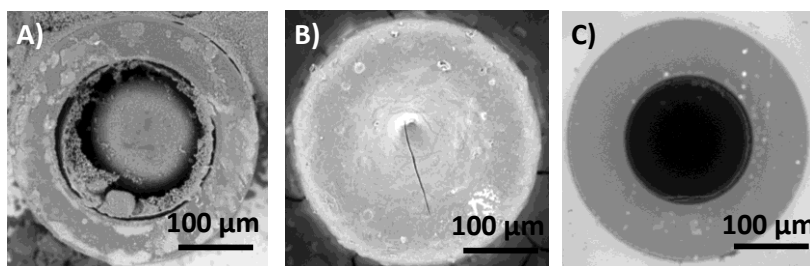
	Lattice parameters (Å)	
	Dry particles	Hydration for 50 min
Lamellar phase, $L_{\alpha}$	49.5	
Inverse micellar phase, $L_2$	31.1	
Inverse hexagonal phase, $H_2$		61.0
Inverse bicontinuous cubic phase, $Pn3m$		84.6
Inverse bicontinuous cubic phase, $Ia3d$		132.7

345

346 Loading of precursors into the microcontainers and coating of the pH sensitive lid

347 After successfully loading the cubosomes into the microcontainers (Fig. 6A), the cavity of the  
 348 microcontainers was coated with Eudragit S100 (Fig. 6B) as this polymer will dissolve at a pH value of  
 349 approximately 7 corresponding to the pH found in the small intestine around the M cells [9].

350



351

352 Fig. 6: SEM images of A) a cubosome-filled microcontainer, B) a filled microcontainers with a lid of Eudragit  
 353 S100, and C) an empty microcontainer after release study in phosphate buffer pH 6.8.

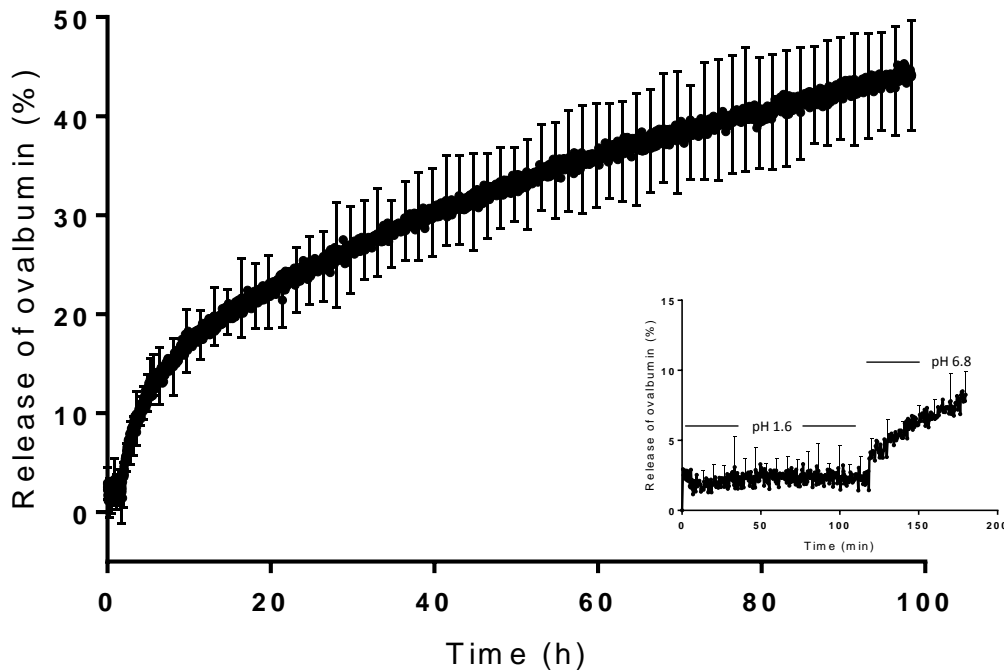
354

355 Release of OVA loaded cubosomes from coated microcontainers

356 The coating on the cavity of the microcontainers can prevent the release until the intestine [33], and a large  
 357 dose (approximately 2  $\mu\text{g}$  of powder) of the vaccine formulation can be loaded into the cavity of the  
 358 microcontainers [27]. In the study, the cavity of the microcontainers was coated with the pH sensitive  
 359 polymer Eudragit S100. The release of OVA from the cubosomes and microcontainers was first measured  
 360 for 2 h in a pH value corresponding to the pH of the stomach (pH 1.6), and here, as expected, no release  
 361 was observed due to the intact layer of the Eudragit lid (Fig. 7). After 2 h, the pH of the medium was  
 362 changed to reflect that of the small intestine (pH 6.8). Fig. 7 shows that the release of OVA is occurring,  
 363 and this indicate that the cubosomes are also released from the microcontainers as these are empty after the  
 364 release studies (Fig. 6C). The release is appearing in a more controlled fashion than observed from the  
 365 unconfined powder cubosomes (Fig. 4). The OVA release in pH 6.8 is  $44.1 \pm 5.6$  % in relation to the amount  
 366 of OVA in the particles. This is comparable to the release from the bulk powder being 47.9 % after 96 h (p-  
 367 value: 0.4311).

368 In the literature, a rice-based oral vaccine has shown to be efficient as a delivery system as it can protect  
 369 the antigen from enzymes in the stomach [7]. The microcontainers have the same feature and therefore,  
 370 there is a promise for the microcontainers to work as an oral vaccine system as well. When delivering  
 371 vaccines by the oral route, the delivery system should be able to present the vaccine formulation to the M  
 372 cells followed by transport to the immune cells to create a response. It has been shown to be effective to

373 keep the vaccine formulation inside a particle for a significant period of time [10,12], securing a slow  
374 release. Therefore the slow release that the microcontainers and the cubosomes provide can be a great  
375 advantage when delivering vaccines.  
376



377  
378 Fig. 7: Release of OVA from the cubosomes, when the vaccine formulation was confined in microcontainers.  
379 The release of OVA is expressed as a % of the loaded OVA into the cubosomes. For the first 2 h the release  
380 was measured in pH 1.6 followed by pH of 6.8 for up to 98 h. The data is presented in triplicates as a  
381 mean±SD.  
382

### 383 Conclusion

384 Powder precursors of cubosomes loaded with OVA have been produced by spray drying, and it was  
385 concluded that the precursors contained cubic structure in bulk as well as when released from  
386 microcontainers. The microcontainers coated with an Eudragit S100 lid can serve as an oral vaccine delivery  
387 system protecting the cubosomes through the GI tract until release occurs in the small intestine. For these  
388 produced cubosomes to be completely developed as an oral vaccine system, an adjuvant needs to be  
389 added to the particles to obtain the optimal effect of this system and further investigations are therefore  
390 also needed for fully develop this oral vaccine delivery system.  
391

### 392 Acknowledgement

393 Assoc. Prof. Stephan Sylvest Keller is acknowledged for the fabrication of the SU-8 microcontainers, and the  
394 SAXS/WAXS beamline at the Australian Synchrotron is thanked as parts of these studies were performed  
395 there. Furthermore, the authors would like to thank the Danish Research Council for Technology and  
396 Production (FTP), Project DFF-4004-00120B for financial support, and in addition, the Danmarks

397 Grundforskningsfonds (project DNRF122) and Villum Fondens Center for Intelligent Drug Delivery and  
398 Sensing Using Microcontainers and Nanomechanics (IDUN) is acknowledged.

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