



## Microcontainers for protection of oral vaccines, in vitro and in vivo evaluation

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1 Microcontainers for protection of oral  
2 vaccines, *in vitro* and *in vivo* evaluation  
3

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11 **Abstract**

12 Oral vaccines are highly desirable due to simple logistics, mass vaccination potential and for  
13 mucosal immunity. Subunit vaccines are preferred due to high safety, but are inherently  
14 difficult to deliver orally, thus providing motivation for the use of advanced oral delivery  
15 systems. Polymeric devices in micrometer size (microcontainers) were tested here for this  
16 purpose. Microcontainers were loaded with a vaccine consisting of spray dried cubosomes  
17 with OVA and Quil-A, and coated with a pH-sensitive lid for oral delivery to C57Bl/6 mice.  
18 The microcontainers were explored *in vitro* and *in vivo* for their potential as oral vaccine  
19 delivery system in an oral prime-boost setting and as an oral booster after a subcutaneously  
20 injected prime. The pH of the stomach of C57Bl/6 mice was measured to be < 4.7 and it  
21 ranged from pH 5.8-7.1 in the small intestine, where the residence time of microcontainers  
22 was less than one hour. Eudragit® L100-55 was therefore chosen as lid material on the  
23 microcontainers as it remained stable *in vitro* at pH 4.7 and allowed release of the cubosomes  
24 within 30-60 min at pH 6.6, which simulated the mean pH of the distal half of the small  
25 intestine. *In vitro* small angle x-ray scattering showed that cubosomes dissolved in small  
26 intestinal fluid when not confined in microcontainers but when loaded into microcontainers  
27 they were released as hexosomes. However, while microcontainers could protect and release  
28 particles with OVA and Quil-A within relevant time frames *in vitro*, an immune response was  
29 not elicited *in vivo* after oral administration. Nonetheless, some effect was observed when the  
30 microcontainers were used to deliver oral boosters following a subcutaneous prime. This  
31 work indicates that oral vaccination with subunit vaccines has potential when combined with  
32 a parenteral prime and that oral delivery systems like microcontainers may be used to  
33 increase the potency of vaccines with low oral immunogenicity.

34

35

36 **Keywords**

37 Cubosomes; Ovalbumin; Quil-A; Eudragit® L100-55; Microdevices; C57Bl/6

38

39

## 40 **1. Introduction**

41 Most vaccines are administered by injection, demanding trained health care personnel to  
42 administer the vaccine [1]. This can limit distribution of vaccines due to costs and logistics.  
43 The problem is exacerbated by the need for most vaccines to be injected 2-3 times over  
44 several months in order to stimulate protective immunity [2]. For example, it is recommended  
45 that the diphtheria-tetanus-pertussis vaccine is given three times, yet 14 % of the world's  
46 children did not receive the third immunization in 2016 [3].

47  
48 Self-administrable vaccines hold promise to greatly improve global vaccination coverage  
49 by simplifying logistics and eliminating the need for repeated health-care visits. The most  
50 attractive route for this is oral administration [1,4] with, for example, vaccine provided in a  
51 capsule. Capsules for oral administration could be easily distributed for patients to self-  
52 medicate according to a provided schedule. This additionally eliminates the need for needles,  
53 which are reported to be the main risk associated with vaccination in developing countries  
54 due to improper usage [5]. Furthermore, oral vaccination has the advantage over parenteral  
55 vaccination that it offers the possibility to achieve mucosal immunity in the gastrointestinal  
56 (GI) tract as well as at distant mucosal compartments [6–8]. Zhu et al. reported on a large  
57 intestine targeted oral vaccine against HIV which gave protection against rectal and vaginal  
58 HIV challenge [9]. This indicated that mucosal immunization may be able to give protection  
59 against some targets that have proven elusive to effective immunization through parenteral  
60 immunization.

61  
62 Oral vaccines in routine clinical use are all against enteric pathogens. All are whole  
63 pathogen vaccines with most being live attenuated and only cholera vaccines being an  
64 inactivated vaccine [4,10]. All of them are very potent and live attenuated intestinal  
65 pathogens additionally have their own mechanisms of mucosal entry [10]. However, concerns  
66 over safety have caused an emphasis in modern vaccine research on developing subunit  
67 vaccines, which are based on purified antigenic fragments of pathogens [1,11]. This greatly  
68 improves the safety of the vaccine, but results in reduced immunogenicity, which is why  
69 adjuvants must be co-delivered with these antigens [1,4]. Subunit vaccines often use proteins  
70 or peptides as antigens, which are easily damaged and degraded by chemical and enzymatic  
71 challenges in the GI tract [4,11]. It is therefore important to design oral delivery systems that  
72 can protect the vaccines from these challenges. The ideal oral vaccine delivery system for  
73 subunit vaccines should protect the vaccine from degradation, limit the elimination/dilution  
74 of the vaccine in the GI tract, and facilitate uptake by M-cells in the intestine and by antigen  
75 presenting cells (APCs) to stimulate a strong immune response [10].

76  
77 Microcontainers are a new approach to enable oral vaccination with subunit vaccines.  
78 Microcontainers are reservoir-based cylindrical polymeric microstructures fabricated from  
79 the polymer SU-8 with precisely controllable dimensions and an opening at one end of the  
80 cylinder. They are a versatile delivery system that can be loaded with most powder  
81 formulations [12] as well as other types of formulations [13–15]. After loading,  
82 microcontainers can be sealed with a lid suitable for the application [16]. The use of pH-  
83 sensitive lids gives them the potential for targeted delivery to specific segments of the GI

84 tract [13,14,17]. Microcontainers have been observed to be trapped in the intestinal mucus  
85 after intestinal perfusion in rats, thus aiding mucus penetration of the cargo [13]. They have  
86 furthermore been shown to be a promising oral drug delivery system [13,14,18,19], but have  
87 never been tested as an oral vaccine delivery system. Vaccine studies are normally performed  
88 in mice which do not have the same pH levels in the GI tract as rats [20]. New  
89 microcontainer lids suitable for use in mice are therefore developed in this study. The pH in  
90 both the stomach and small intestine of C57Bl/6 mice has not previously been studied,  
91 although the pH of the ileum and cecum of female C57Bl/6 mice has been reported to be 6.7  
92 and 6.4 respectively [21]. Other studies describe variable values from different strains as well  
93 as variation caused by external inputs (e.g. fasting) [20,22,23]. Since knowledge of the pH in  
94 the stomach and small intestine is crucial for pH controlled vaccine delivery, the pH in male  
95 C57Bl/6 was measured prior to this study (supplementary material).

96

97 Quil-A is an adjuvant that is well tolerated orally [24,25], but needs to be coupled with a  
98 nanoparticle system such as cubosomes to be effective [26]. Cubosomes are composed of a  
99 highly twisted and ordered continuous lipid bilayer that forms two sets of intertwined and  
100 highly tortuous, but nonintersecting, water-channels. This gives cubosomes a large surface  
101 area making them flexible regarding the antigens and adjuvants they can carry [27]. Quil-A  
102 has previously been used for oral vaccination in a water/oil/water emulsion [28] and in  
103 ISCOMs [29–35] with some success indicating that Quil-A is suitable for oral use. We have  
104 previously reported the spray drying of cubosome precursors with ovalbumin (OVA) as  
105 model antigen and Quil-A as adjuvant. The powder formulation retained antigen integrity  
106 during storage at room temperature for at least 6 months and formed cubosomes after  
107 rehydration. The cubosomes elicited strong humoral and cellular immune responses after  
108 subcutaneous (s.c.) administration, but had no effect after oral administration indicating that a  
109 better oral delivery system was required [26]. These spray dried cubosomes are well suited  
110 for testing the efficacy of the microcontainers since they 1) have a high antigen content 2)  
111 appear to be stable during storage at room temperature and 3) are ineffective orally without a  
112 delivery system, although they are made with an adjuvant known to work mucosally. The aim  
113 of this study was to design microcontainers as oral delivery system for spray dried  
114 cubosomes, characterize the system *in vitro* and evaluate it *in vivo* in C57Bl/6 mice.

115

## 116 **2. Materials and Methods**

### 117 *2.1. Materials*

118 Dimodan<sup>®</sup> MO 90/D (monoolein) was kindly donated by Danisco (Grindsted, Denmark).  
119 Dextran (from *Leuconostoc* spp., 40 kDa), ovalbumin (Grade VII, from chicken egg white)  
120 and dibutyl sebacate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quil-A was  
121 obtained from Brenntag Biosector (Frederikssund, Denmark), phosphate buffered saline  
122 (PBS) tablets were acquired from Oxoid limited (Basingstoke, England) and Eudragit<sup>®</sup> L100-  
123 55 (EL100-55) was purchased from Evonik (Darmstadt, Germany). 5,6-Carboxyfluorescein  
124 diacetate succinimidyl ester (CFSE) and CellTrace<sup>™</sup> Violet Cell Proliferation Kit (CTV)  
125 were purchased from Molecular Probes<sup>®</sup> (Eugene, OR, USA). OVA<sub>257–264</sub> peptide

126 (SIINFEKL) was acquired from Mimotopes (Clayton, Australia). PeCy7 anti-CD8,  
127 propidium iodide and HRP Goat anti-mouse IgG were from BioLegend® and APC-H7 anti-  
128 CD4, PE anti-V $\alpha$ 2, biotin anti-V $\beta$ 5 and APC streptavidin from BD Pharmingen™. Complete  
129 mini protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim  
130 Germany) and Mouse Anti-OVA IgA Antibody Assay Kits from Chondrex inc. (WA, USA).  
131 All other chemicals were of analytical grade and used as received. Milli-Q water (Merck  
132 Millipore, Darmstadt, Germany) was used throughout the study.  
133

## 134 2.2. Mice

135 6-8 weeks old male specific pathogen free C57BL/6 mice and male OT-I and OT-II mice  
136 were obtained from the HTRU, University of Otago, Dunedin, New Zealand. Mice had free  
137 access to food and water at all times. All experiments were approved by the Animal Ethics  
138 Committee at the University of Otago (AEC no. 80-16).

## 139 2.3. Spray drying cubosomes

140 Cubosomes were prepared as previously described [26]. Briefly, Dimodan in ethanol (5.33  
141 mg/mL) was mixed 1:3.04 (v/v) with an aqueous solution of dextran, OVA and Quil-A (2.63,  
142 0.52 and 0.035 mg/mL, respectively). The mixture was spray dried on a Büchi B-290 mini  
143 spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) with a pressure nozzle of 1.5 mm  
144 diameter. A feed rate of 4.5 mL/min was used with atomizing airflow rate of 667 L/h, inlet  
145 temperature of 150°C and 100 % aspirator rate. Particles without OVA were produced in the  
146 same way to be used as controls. Collected powders were stored at 86°C for 24 h and then  
147 dried at room temperature until use.

## 148 2.4. Fabrication, loading and sealing of microcontainers

149 Microcontainers were fabricated with the negative epoxy photoresist SU-8 by a two-step  
150 photolithography process as described previously [36]. However, in this study, the design  
151 was modified to achieve a larger internal diameter while preserving the external geometry of  
152 the microcontainers to increase the loading capacity. The microcontainers were produced on  
153 top of a fluorocarbon coated silicon wafer to allow easy mechanical removal from the wafer.  
154 The wafer was then cut into 12.8 by 12.8 mm<sup>2</sup> chips containing 25 by 25 arrays of  
155 microcontainers using a dicing saw (DISCO, Kirchheim bei München, Germany).

156 Microcontainers on chips were loaded with cubosome precursor powder using an embossing  
157 method as described previously [12]. A screen-mask was used to cover the gaps between the  
158 microcontainers thus filling the microcontainers without filling the space between them with  
159 powder. The average powder load in the microcontainers was estimated by weighing 21 sets  
160 of three individual microcontainers before and after loading.

161 After loading, the microcontainers were sealed with the pH-sensitive polymer Eudragit®  
162 L100-55 (EL100-55) through a spray coating process. Isopropanol containing 1 % (v/v)  
163 EL100-55 and 5 % dibutyl sebacate (w/w in relation to EL100-55) was sprayed over the chip  
164 with microcontainers using an ExactaCoat spray coater (Sono Tek, Milton, NY, USA)

165 equipped with an ultrasonic nozzle actuated at 120 kHz (Accumist, Sono Tek, Milton, NY,  
166 USA). Spray coating parameters were as follows: feed flow rate 0.1 mL/min with generator  
167 power of 2.2 W and nebulizing air pressure of 0.02 kPa. The chips were kept at 40°C. The  
168 nozzle was positioned with a nozzle-to-microcontainer distance of 6.5 cm and moved  
169 laterally across the chip by a software controlled pattern to cover the entire chip equally. The  
170 translational speed of the nozzle was 5 mm/s and the coating was repeated to give a total of  
171 36 passages.

## 172 *2.5. Microcontainer characterization and qualitative release study*

173 Microcontainers were visualized empty, loaded with cubosome precursor powder, and sealed  
174 with EL100-55 lids using a table top scanning electron microscope (SEM) (Hitachi  
175 TM3030plus, Tokyo, Japan). Samples were placed on carbon tape on metallic holders prior to  
176 investigation and then imaged using 15 kV acceleration voltage at 60x or 120x magnification.

177 The thickness of the EL100-55 lids was estimated by covering half of an SU-8 coated chip  
178 and then spray coating it as described earlier on four independent samples. The height of the  
179 produced half-lids was measured by contact profilometry (Alpha-Step IQ Stylus profilometer,  
180 KLA-Tencor Corporation, Milpitas, USA) and used as estimate of the thickness of the lids  
181 deposited on the microcontainers. Profilometry was performed at a scan speed of 20  $\mu\text{m/s}$ ,  
182 using a 15.6 mg tip force at a sampling rate of 50 Hz.

183 Release of cubosomes from microcontainers sealed with EL100-55 lids was investigated  
184 qualitatively with SEM. Three full chips of microcontainers were submerged in buffer  
185 simulating the pH of the mouse stomach (2 mM maleic acid at pH 4.7 and 37°C in a water  
186 bath rotating at 120 rpm) for 60 min. One chip was then removed from the buffer and imaged  
187 using SEM as described above while the two other chips were moved into buffer simulating  
188 intestinal pH (10 mM maleic acid at pH 6.6 and 37°C in a water bath rotating at 120 rpm) for  
189 30 or 60 min. The buffer was changed every 15 min to simulate the sink conditions of the  
190 intestine. The microcontainers were imaged by SEM and evaluated visually for the removal  
191 of lids and powder.

192 Another *in vitro* release experiment was performed using gastric and intestinal fluids as  
193 release buffer. Here, individual microcontainers were submerged either into pooled gastric or  
194 pooled intestinal fluids (collected as described above) and placed for 30 min at 37°C in a  
195 water bath rotating at 120 rpm. Microcontainers were then recovered and imaged with SEM.  
196 The gastric and intestinal fluid was not washed off before imaging to avoid affecting the  
197 release with a washing step.

## 198 *2.6. Small angle x-ray scattering of hydrated particles and particles released from* 199 *microcontainers*

200 The internal structure of particles was investigated using small angle x-ray scattering (SAXS)  
201 at the Austrian SAXS/WAXS beamline at the synchrotron light source ELETTRA (Trieste,  
202 Italy). The x-ray beam had an energy of 8 keV (1.54 Å) and the samples were placed 1327  
203 mm from the detector. Diffraction patterns were converted to intensity vs. q-value plots to

204 identify relative peak positions for determination of the space group of the dominant internal  
205 structure of the samples.

206 Powder precursors of cubosomes with OVA and Quil-A were suspended *in vitro* at  
207 approximately 50 mg/mL into stomach or intestinal fluids at 37°C. The structure of the  
208 particles was measured at 3 minute intervals with SAXS within timeframes ranging between  
209 6 and 18 min depending on the sample.

210 Microcontainers were loaded with powder precursors of cubosomes with OVA and Quil-A  
211 and were submerged in PBS (9.5 mM, pH 7.3) for SAXS measurements at 3 min intervals  
212 over 39 min. Powder-filled microcontainers were then sealed with EL100-55 lids and  
213 submerged in PBS or in pooled intestinal fluid at 37°C. SAXS patterns were measured every  
214 3 min for 138 min (PBS) and 21 min (intestinal fluid).

### 215 *2.7. Kinetics of microcontainer transit in the mouse GI tract*

216 Microcontainers filled with powder precursors of cubosomes with OVA and Quil-A and  
217 sealed with EL100-55 lids were filled into oral capsules designed for use in mice (gelatin  
218 capsules size M, Torpac®, USA). The average weight of a microcontainer was estimated by  
219 weighing sets of loaded and coated microcontainers. Each capsule was weighed before and  
220 after filling with microcontainers to estimate the number of microcontainers in each capsule.  
221 Mice were dosed with one capsule and sacrificed by cervical dislocation after 60 or 90 min  
222 followed by collection of their stomach, small intestine, cecum and colon. The small intestine  
223 was divided into a proximal and a distal segment. Segments were examined with optical  
224 microscopy (Olympus IX53 inverted microscope with 4x bright-field optics) to count the  
225 number of microcontainers present in each segment. For each mouse, the recovered numbers  
226 of microcontainers in each segment were normalized to the total number of microcontainers  
227 recovered from all segments. Three mice were used for each time point.

### 228 *2.8. In vivo immunological investigation of microcontainers loaded with cubosomes as* 229 *oral vaccines*

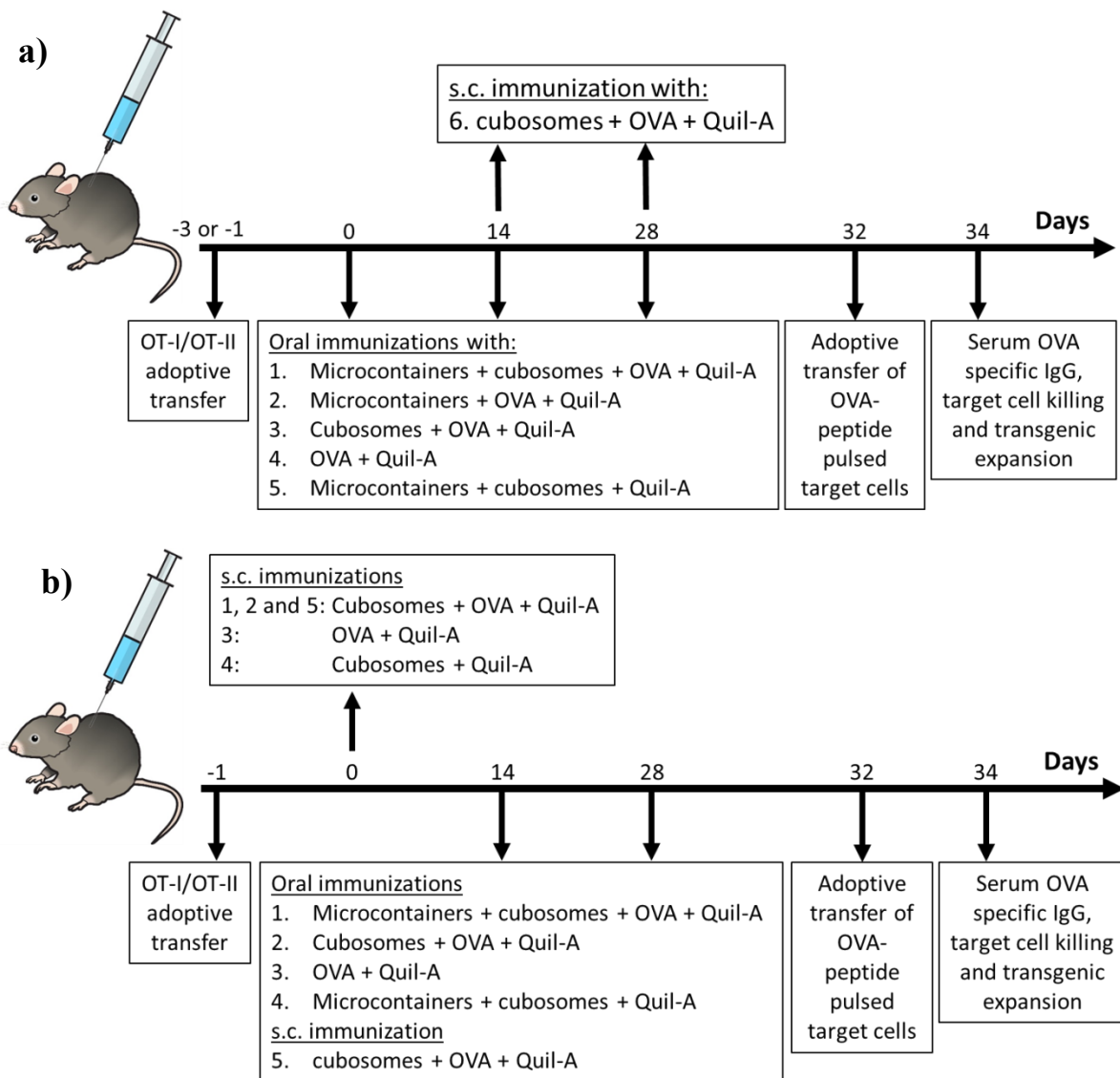
230 Two vaccine studies were performed. In both studies, 200 µL PBS with 2 x 10<sup>6</sup> naïve OT-I  
231 and OT-II lymphocytes (which have T cell receptors for CD8 and CD4 epitopes from OVA  
232 [37]) were injected intravenously (i.v.) into the tail vein 1-3 days prior to the first vaccination.

233 The first study evaluated microcontainers as an oral vaccine. Six groups of five mice were  
234 given three oral immunizations as described in Figure 1a. Further details of the vaccination  
235 regime are included in Table S1. As a positive control, one group of mice was vaccinated  
236 twice s.c. with cubosomes containing 10 µg OVA and 0.67 µg Quil-A, while the oral  
237 vaccines contained 100 µg OVA and 6.7 µg Quil-A.

238 The second study investigated microcontainers as an oral boost. Five groups of six mice  
239 received an s.c. prime followed by two boosts orally or s.c. as described in Figure 1b. Further  
240 details of the vaccination regime are included in Table S2. Doses were 10 µg OVA and 0.67  
241 µg Quil-A.



242 The s.c. vaccines were injected into the flank of the mice, whilst liquid oral vaccines were  
 243 administered by gavage using a soft gavage needle (category #7202K, Fuchigami, Kyoto,  
 244 Japan) and oral capsules were dispensed into the back of the mouth of the mouse using a  
 245 dosing syringe. Four days after the last vaccination, mice were injected i.v. with  $4 \times 10^6$   
 246 C57Bl/6 lymphocytes pulsed with 10  $\mu\text{g}/\text{ml}$  SIINFEKL and labelled with CFSE together with  
 247  $4 \times 10^6$  unpulsed lymphocytes stained with CTV. On day 33, mice were moved to fresh cages  
 248 for collection of fresh fecal pellets from the cage floors on day 34. Fecal pellets were stored  
 249 at  $-20^\circ\text{C}$  until analysis. Mice were sacrificed on day 34 and blood, spleens and lymph nodes  
 250 (mesenteric and inguinal lymph nodes from mice vaccinated orally or s.c., respectively) were  
 251 collected.



252

253

254 **Figure 1.** Schematics of the *in vivo* studies investigating (a) oral prime and boost or (b) s.c.  
 255 prime followed by oral boosts. Positive control groups received s.c. prime and boost in both  
 256 studies. Numbering of groups correspond with those of Tables S1 and S2.

257

## 258 2.9. Flow cytometry

259 Spleens and lymph nodes from individual mice were processed into single cell suspensions  
260 essentially as described previously [38]. Aliquots of cells were stained with anti-CD8, anti-  
261 CD4, anti-V $\alpha$ 2 and anti-V $\beta$ 5 antibodies and the live/dead stain propidium iodide. Data was  
262 acquired on a BD FACSCanto™ II (BD Biosciences) and analyzed using FlowJo version  
263 10.3 (Tree Star, Inc.) with the gating strategy shown in Figure S1. Antigen specific killing of  
264 peptide pulsed target cells was evaluated as described previously [26].

## 265 2.10. Measurement of OVA-specific serum IgG and OVA-specific fecal and serum IgA

266 Sera were separated from whole blood and OVA-specific serum IgG was measured by  
267 ELISA as previously described [26]. Briefly, wells were coated with OVA and then blocked  
268 with 2 % w/v BSA in PBS (9.5 mM, pH 7.3). Sera were diluted 1:100, serially diluted across  
269 well plates (high-binding 96 well plate, Corning inc. Corning, NY, USA) and incubated for 2  
270 h. 225 ng/mL HRP Goat anti-mouse IgG was used as detection antibody and color was  
271 developed using a substrate reagent pack (R&D SYSTEMS®, MN, USA). Color development  
272 was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and absorbance at 450 nm was read using a Polarstar Omega  
273 Microplate Reader (BMG Labtech, Ortenberg, Germany)

274 For IgA measurement, fecal pellets were powdered with mortar and pestle and IgA was  
275 extracted from the solids by mixing at 150 mg/mL in PBS (9.5 mM, pH 7.3) containing  
276 protease inhibitors used according to the manufacturer's protocol. The extraction was run for  
277 45 min and agitated repeatedly by aspirating and ejecting through a 1 mL syringe. Solids  
278 were separated from liquid by centrifugation for 10 min at 2,000 G and OVA-specific IgA in  
279 the supernatants was measured using an ELISA kit according to manufacturer's instructions.  
280 OVA-specific IgA from pooled undiluted sera was measured using the same protocol.

## 281 2.11. Statistics

282 Data are presented as mean  $\pm$  standard deviation (SD), except the pH measurements which  
283 are presented as median with quartiles and extrema together with the mean. Statistical  
284 analysis was performed by one-way ANOVA and Tukey's multiple comparisons test using  
285 Prism 7 (GraphPad, San Diego, USA).

286

## 287 3. Results and discussion

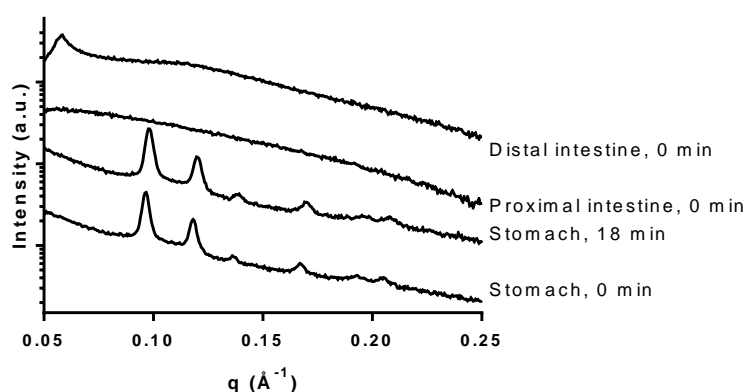
### 288 3.1. Cubosomes retain their structure in stomach fluid but are degraded in intestinal fluid

289 A dry flowable powder of cubosome precursors with OVA and Quil-A was prepared by spray  
290 drying and formed Pn3m cubosomes when dispersed in PBS as previously described [26].  
291 Following oral administration, however, the gastric and intestinal fluids may affect the  
292 structure of the nanoparticles in vivo and this has not previously been investigated. To  
293 investigate if cubosomes form after oral administration to mice, the powder was mixed into  
294 stomach and intestinal fluids from mice *in vitro*. Cubosomes formed immediately in stomach  
295 fluid and remained intact for at least 18 min (Figure 2). Conversely, in intestinal fluid from

296 either intestinal segment, SAXS patterns without peaks (characteristic of vesicles) were  
297 obtained (Figure 2). This indicates that cubosomes are stable for at least 18 min in stomach  
298 fluid, but quickly lose their cubic structure to form vesicles in intestinal fluid.

299 Since cubosomes are lipid-based particles, they may be expected to be emulsified into  
300 vesicles by bile salts. This is consistent with our results as bile salts are present in large  
301 amounts in the intestine while no or only small amounts of bile salts are reported to be  
302 present in the stomach [39]. For oral administration to mice, it was therefore important that  
303 the microcontainers are able to not only protect the antigen from chemical and enzymatic  
304 degradation in the stomach, but also to protect the cubosomes from emulsification by bile  
305 salts in the intestine. Because microcontainers are known to be trapped in the mucus close to  
306 the intestinal wall [13], emulsification would be reduced by the release of cubosomes near the  
307 epithelium. As will be shown below, the chemistry of the lid may also provide protection of  
308 the particles even after release.

309



310

311 **Figure 2.** q vs. intensity patterns obtained from SAXS measurements of cubosomes with  
312 OVA and Quil-A mixed *in vitro* with fluids from segments of the GI tract of mice at 37°C.  
313 Measurements were performed at 0 and 18 min after mixing cubosomes into stomach fluid  
314 and 0 min after mixing cubosomes into fluid from the proximal or distal half of the small  
315 intestine.

316

### 317 3.2. Fabrication, loading and coating of microcontainers

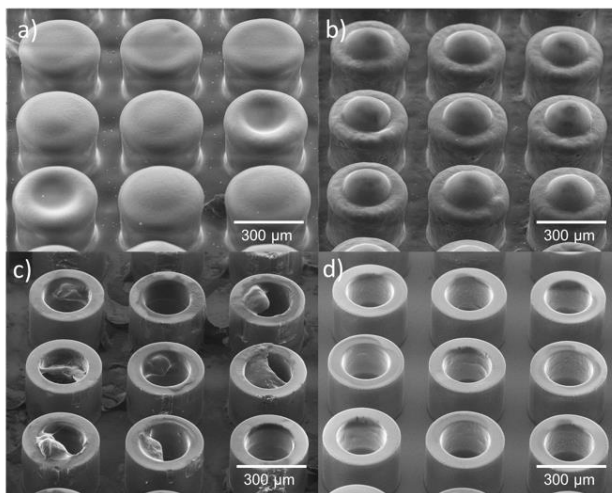
318 Microcontainers were fabricated with an outer diameter of  $326.3 \pm 1.0 \mu\text{m}$  and height of  
319  $255.4 \pm 6.0 \mu\text{m}$ , and with an inner diameter of  $231.5 \pm 0.9 \mu\text{m}$  and height of  $216.8 \pm 6.4 \mu\text{m}$ ,  
320 giving a reservoir volume of  $9.1 \pm 0.3 \text{ nL}$  ( $n = 4$ , mean  $\pm$  SD). Microcontainers were loaded  
321 with  $6.6 \pm 4.6 \mu\text{g/microcontainer}$  of powder and then sealed with EL100-55 lids. The average  
322 thickness of the lid coating was measured by contact profilometry to be  $29.7 \pm 3.5 \mu\text{m}$  with a  
323 surface roughness (SD of the thickness of the individual chip)  $\leq 1.2 \mu\text{m}$ .

324

### 325 3.3. Release of OVA and Quil-A cubosomes from microcontainers

326 The pH-activated release of cubosomes from coated microcontainers was tested *in vitro*. For  
327 simulation of gastric pH, the highest pH that may be expected in the stomach was chosen to  
328 ensure that biological variance would not cause unexpected lid disintegration in the  
329 subsequent *in vivo* evaluation. Microcontainers (Figure 3a) were therefore submerged in  
330 buffer at pH 4.7 and 37°C to simulate stomach pH of C57Bl/6 mice (Supplementary  
331 material). After 60 min, the EL100-55 lids were completely intact (Figure 3b) and the  
332 microcontainers were moved to buffer at pH 6.6 to simulate the pH of the distal segment of  
333 the small intestine (Supplementary material). Here, the lids dissolved and most of the  
334 cubosomes were released within 30 min (Figure 3c), although a small amount was still left at  
335 the top of most microcontainers (but not deep into the reservoir). After 60 min, most  
336 microcontainers were empty (Figure 3d).

337 The experiment was repeated *in vitro* using fluids from the stomach, and the proximal- and  
338 distal segments of the small intestines of mice. Once again, lids were intact after 30 min in  
339 the stomach, but dissolved within 30 min in the proximal segment of the intestine (Figure  
340 S2). The microcontainers generally appeared empty after 30 min in fluid from either segment  
341 of the intestine but the degree of emptying was difficult to evaluate due to the presence of  
342 sticky solids in the intestinal fluids (Figure S2). Lids made from Eudragit L100-55 thus  
343 appear to have the same function in mice as we have previously observed for rats with lids  
344 made from Eudragit L100 [13].



345

346 **Figure 3.** SEM images showing dry microcontainers on a chip after loading with cubosomes  
347 and coating with EL100-55 (a). Microcontainers were soaked for 60 min in 2 mM maleic acid  
348 at pH 4.7 simulating the pH of the mouse stomach (b) and then in 10 mM maleic acid at pH  
349 6.6 simulating pH of the mouse intestine for 30 min (c) and 60 min (d).

350

### 351 3.4. Effect of microcontainers on particle morphology

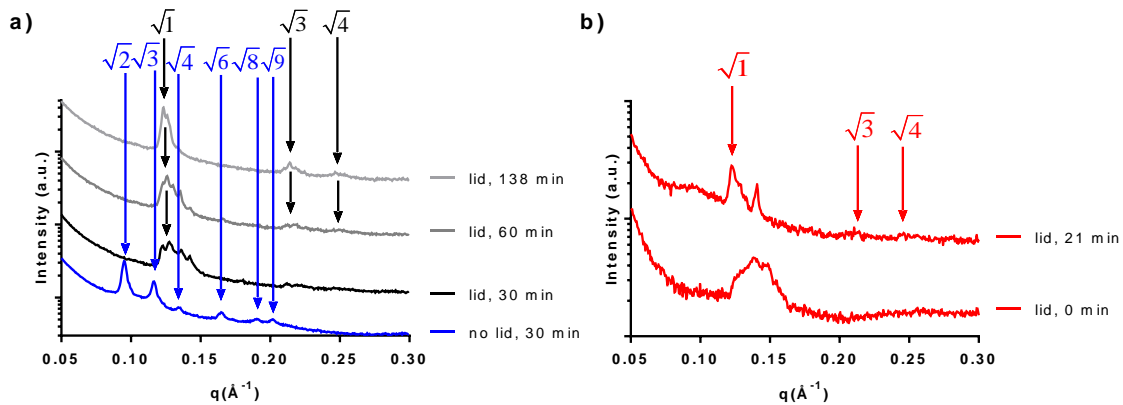
352 The entrapment of monoolein-based cubosome precursors in microcontainers sealed with  
353 Eudragit<sup>®</sup> S100 lids has previously been reported to result in hexosomes being formed after  
354 release of the precursors into water [17]. However, it was not tested whether it was the  
355 microcontainers or the lids that cause the change in particle morphology. Here, the effect on  
356 particle morphology of sealing the microcontainers with EL100-55 lids was investigated in  
357 addition to the effect of microcontainers on their own. Powder precursors loaded into  
358 microcontainers and released into PBS were investigated with SAXS. Release of cubosome  
359 precursors from microcontainers without lids resulted in Bragg peaks at relative positions of  
360  $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{8}:\sqrt{9}$  (Figure 4a) characteristic of Pn3m cubic phase [40,41] similar to  
361 those from cubosomes without microcontainers [26]. The peaks were evident after 6 min and  
362 persisted thereafter (shown at 30 min, the measurement was stopped after 39 min). This  
363 indicates that microcontainers do not affect particle morphology, as was expected since the  
364 cubosomes only form after rehydration [26] and thus after release.

365 Particles released from microcontainers with EL100-55 lids resulted in Bragg peaks at  
366 relative positions of  $\sqrt{1}:\sqrt{3}:\sqrt{4}$  (Figure 4a) characteristic of hexagonal phase [40]. SAXS  
367 patterns from the hexosomes became increasingly sharper even 60 min after submersion in  
368 PBS. Since microcontainers do not affect particle morphology, this indicates that after  
369 dissolution of the lids, the components of the lids interact with the lipid changing the particle  
370 morphology from cubosomes to hexosomes.

371 In the pure monoolein/water phase diagram, the hexagonal phase exists only at temperatures  
372 above 80°C [42]. However, the addition of other amphiphiles with a higher hydrophobicity  
373 than monoolein, or lipophilic additives, can transform the cubic phase to a hexagonal phase  
374 [42,43]. At pH 6.6, EL100-55 will be mostly deprotonated and thus have a hydrophilic  
375 structure that allows its dissolution in water. The addition of EL100-55 at pH 6.6 is therefore  
376 not an obvious driving factor for the observed phase change. The only other component of the  
377 lid is the dibutyl sebacate used as plasticizer for the EL100-55 lids. Dibutyl sebacate was  
378 chosen for its hydrophobic nature, which provides more water tight lids than when using a  
379 hydrophilic alternative [44]. It is therefore likely to become incorporated into the monoolein  
380 bilayers where it might affect the packing of the lipids increasing curvature and consequently  
381 driving the phase change from the inverse cubic to the inverse hexagonal structure [45,46].  
382 However, no plasticizer was used in the study by Nielsen et al., where a mixture of  
383 cubosomes and hexosomes were released from Eudragit<sup>®</sup> S100 coated microcontainers [17].  
384 Since the two Eudragit types are copolymers composed of the same monomers at different  
385 ratios, it is likely that both EL100-55 and dibutyl sebacate have influence on the observed  
386 complete phase change observed in this study. The explanation might be that their  
387 incorporation reduces the packing frustration of the lipid bilayer inherent to the hexagonal  
388 structure [46].

389 Interestingly, hexosomes were also formed when the precursor powder was released from  
390 microcontainers with lids into intestinal fluid from mice (Figure 4b). They take some time to  
391 form the crystal structure though as evident from the time-dependent change of diffraction  
392 pattern from a soft hump to clear peaks. Since release of cubosomes from the microcontainers

393 is expected to happen in the mucus, it is likely that hexosomes with OVA and Quil-A will  
 394 form *in vivo* in the mucus in close proximity to the intestinal wall, where the antigen-  
 395 sampling M-cells and dendritic cells are present. Any type of particle generally facilitates the  
 396 recognition and uptake of antigen by antigen presenting cells [47]. This morphology change  
 397 is therefore likely not of substantial importance to the stimulation of an immune response.  
 398 However, release of actives from the bulk hexagonal phase is generally slower than from the  
 399 bulk cubic phase [48]. It could therefore be speculated that hexosomes retain the antigen  
 400 better than cubosomes, and thereby might stimulate a better immune response [11].



401

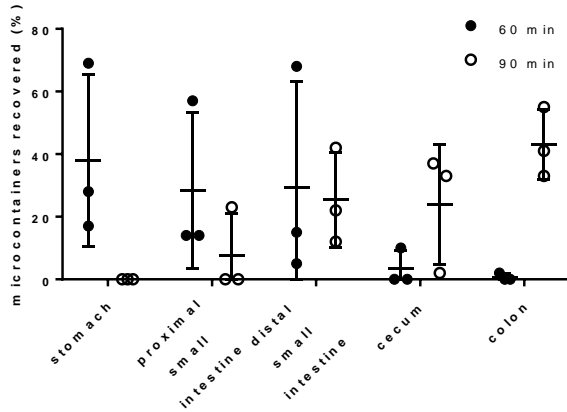
402 **Figure 4.**  $q$  vs. intensity patterns obtained from SAXS measurements of particles with OVA  
 403 and Quil-A released from microcontainers at (a) 37°C in PBS and (b) in mouse intestinal  
 404 fluid. SAXS patterns shown in (a) are from particles released from microcontainers without  
 405 lid after 30 min and with lid after 30, 60 and 138 min. SAXS patterns shown in (b) are from  
 406 particles released from microcontainers with lid just after submersion in mouse intestinal  
 407 fluid and after 21 min.

### 408 3.5. Kinetics of microcontainer transit in the mouse GI tract

409 The transit time of microcontainers or similarly sized particles through the GI tract of mice  
 410 has never been evaluated, but is important for interpretation of *in vitro* studies of vaccine  
 411 release from the microcontainers. To investigate whether the microcontainers stay long  
 412 enough in the intestine to allow release of the vaccine before the microcontainers are expelled  
 413 with the feces, the transit time through the GI tract was evaluated. Mice were administered  
 414 one capsule loaded with microcontainers and were sacrificed after 60 or 90 min.  
 415 Microcontainers were quantified by microscopy and  $86 \pm 11$  % of the estimated number of  
 416 administered microcontainers were found in each mouse (Figure 5). The microcontainers  
 417 were generally spread widely within each mouse indicating a large variability in how fast  
 418 individual microcontainers move through the GI tract. However, after 60 minutes more than  
 419 60 % of the microcontainers had passed the stomach, and 58 % of the microcontainers were  
 420 distributed along the small intestine. After 90 min, the majority of microcontainers recovered  
 421 were present in the cecum and colon. The time available for release in the small intestine is  
 422 therefore short and some of the vaccine might be released in the large intestine. Both the

423 cecum and the colon contain lymphoid tissue [20] and are good mucosal vaccine targets to  
424 obtain immunity at rectal and vaginal mucus surfaces [9].

425



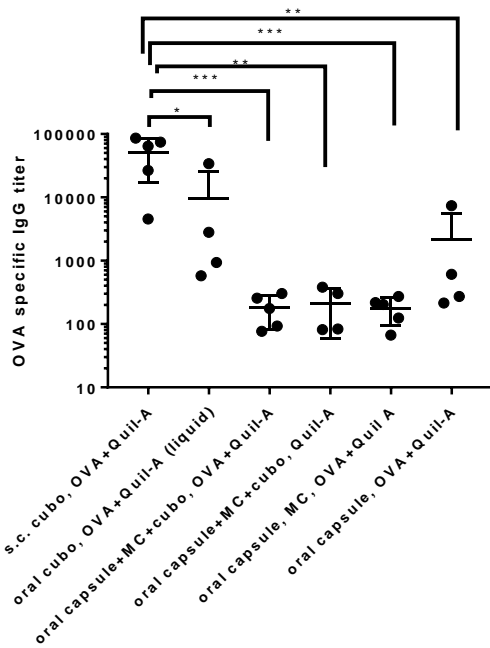
426

427 **Figure 5.** Relative numbers of recovered microcontainers found in the stomach, proximal-  
428 and distal half of the small intestine, cecum and colon in mice killed 60 or 90 min after oral  
429 administration. Error bars represent mean  $\pm$  SD (n = 3).

430

431 *3.6. Oral cubosomes elicit a weak humoral immune response but no response when*  
432 *delivered in microcontainers*

433 Microcontainers appear to have promise as an oral delivery system and were therefore  
434 evaluated *in vivo*. Microcontainers were administered in capsules because their pH-sensitive  
435 lids would dissolve in buffer at neutral pH. OVA-specific serum IgG titers were measured by  
436 ELISA to evaluate the humoral immune response to the vaccines. A strong anti-OVA IgG  
437 response was seen after s.c. administration as previously reported [26]. Oral cubosomes  
438 resulted in slightly increased IgG response in one experiment (Figure 6), but not in the  
439 replicate of the experiment (not shown). OVA and Quil-A delivered in capsules or in  
440 microcontainers (in capsules) with or without cubosomes had no effect.



441

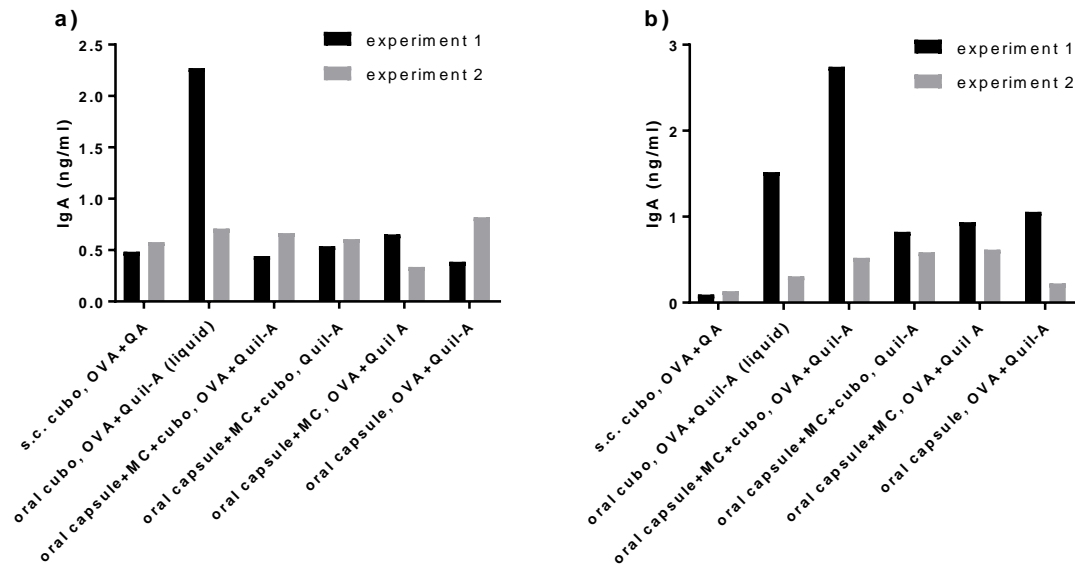
442 **Figure 6.** OVA-specific serum IgG antibody titers. Mice were given vaccines three times  
 443 orally or two times s.c. as indicated with 14 days between each vaccination. Data shown are  
 444 from individual mice plus the average and SD (data is from 1 of 2 independent experiments,  
 445 n = 4-5 mice/experiment). \* p < 0.5, \*\* p < 0.1, \*\*\* p < 0.001, cubo = cubosomes, MC =  
 446 microcontainers.

447

448 Fecal and serum OVA-specific IgA titers were measured by ELISA to evaluate the mucosal  
 449 immune response. Low and inconsistent levels of IgA were seen in pooled fecal and serum  
 450 samples from oral cubosomes (Figure 7). Mice treated orally with cubosomes in  
 451 microcontainers had low levels of IgA in serum in one experiment.

452





453

454 **Figure 7.** OVA-specific IgA from (a) fecal samples collected at the time of sacrifice and (b)  
 455 serum samples. Mice were given vaccines three times orally or two times s.c. as indicated  
 456 with 14 days between each vaccination. Data are from pooled mice with 4-5 mice/group.  
 457 cubo = cubosomes, MC = microcontainers.

458

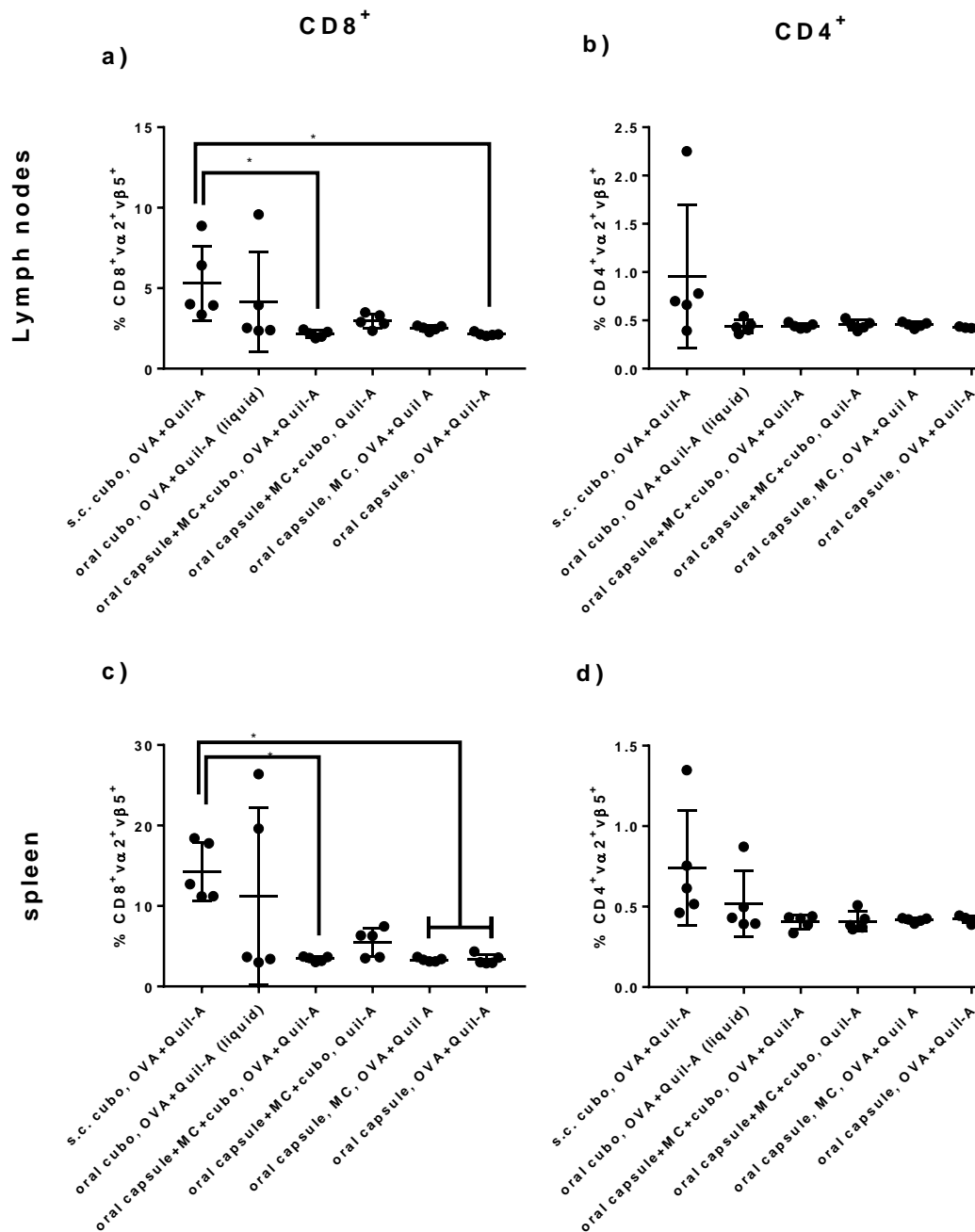
459 *3.7. Oral cubosomes inconsistently elicit a cellular immune response but no response*  
 460 *when delivered in microcontainers*

461 Expansion of OVA-specific transgenic cells was used to evaluate the cellular immune  
 462 response to the vaccines (Figure 8) along with an *in vivo* cytotoxicity assay to measure  
 463 cytotoxic T cell responses (Figure 9). For the *in vivo* cytotoxicity assay, killing of OVA-  
 464 peptide labelled lymphocytes injected i.v. two days before sacrifice was measured.

465 Subcutaneous injection of cubosomes elicited strong OVA-specific CD8<sup>+</sup> expansion in lymph  
 466 nodes and spleens (Figure 8a and c) as well as target cell killing (Figure 9) as observed  
 467 previously [26]. Oral cubosomes with OVA and Quil-A were able to elicit a strong CD8<sup>+</sup>  
 468 response in two mice, but had no effect in the remaining three mice (Figure 8a and c). All  
 469 other oral treatments had no effect. The CD4<sup>+</sup> responses were generally weak (Figure 8b and  
 470 d). A slight CD4<sup>+</sup> response was observed in the same mice treated with oral cubosomes with  
 471 OVA and Quil-A that also expressed strong CD8<sup>+</sup> responses and in some of the mice treated  
 472 with cubosomes s.c. Oral cubosomes caused variable target cell killing, but this was not  
 473 comparable to the effect of s.c. cubosomes (Figure 9). All other oral groups did not stimulate  
 474 target cell killing.

475 We have previously observed that these cubosomes do not work orally when administered in  
 476 powder form in a capsule [26]. In this study, the powder form of the cubosomes in  
 477 microcontainers was also ineffective, whereas cubosomes that had been rehydrated prior to  
 478 gavage had a small effect. It therefore seems that when this vaccine is administered in powder

479 form, it is not suitable for oral delivery, even if using an oral delivery system. However, both  
 480 cubosomes and microcontainers have the potential to be further developed and

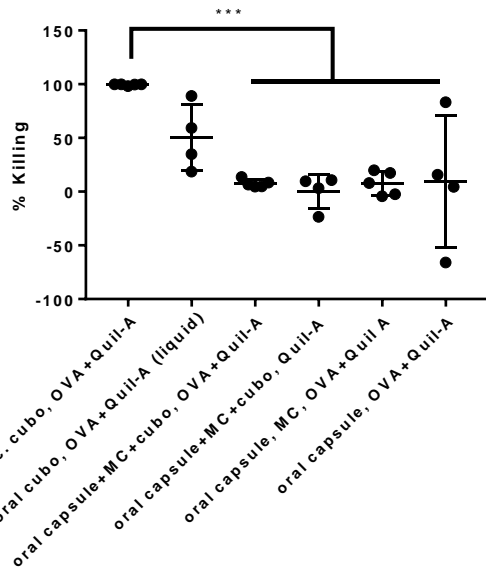


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 512 CD4<sup>+</sup>  
 513 cells  
 514 (b and  
 515 d) in

516 lymph nodes (a and b) and spleens (c and d). Mice were given vaccines three times orally or  
 517 two times s.c. as indicated, with 14 days between each vaccination. Data shown are results  
 518 from individual mice together with the mean and SD from one of two independent  
 519 experiments (n = 4-5 mice). \*p < 0.05, cubo = cubosomes, MC = microcontainers.

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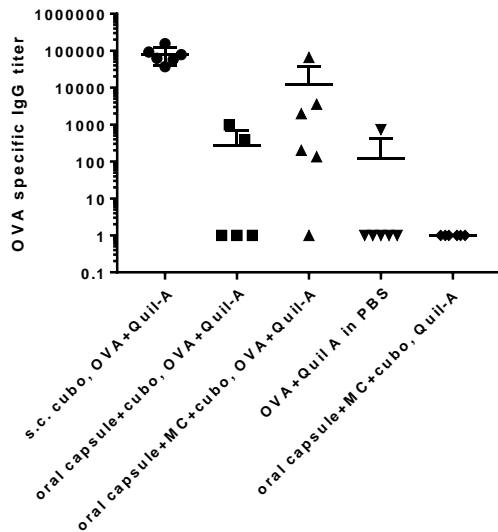
522 **Figure 9.** *In vivo* killing of adoptively transferred OVA-peptide labelled T cells in spleens.  
 523 Mice were given vaccines three times orally or two times s.c. as indicated with 14 days  
 524 between each vaccination. Data shown are results from individual mice together with mean  
 525 and SD from one of two independent experiments (n = 4-5 mice/experiment). \*\*\*p < 0.001,  
 526 cubo = cubosomes, MC = microcontainers.

527

### 528 3.8. Oral cubosomes in microcontainers have some effect as booster vaccine

529 Doherty et al. reported that after an s.c. injected prime, an oral boost could stimulate a similar  
 530 level of protective immunity as a s.c. boost, although the oral vaccine was unable to prime an  
 531 immune response [49]. We therefore investigated if microcontainers with cubosomes with  
 532 OVA and Quil-A could stimulate an immune response when used as oral boost after an s.c.  
 533 injected prime of cubosomes.

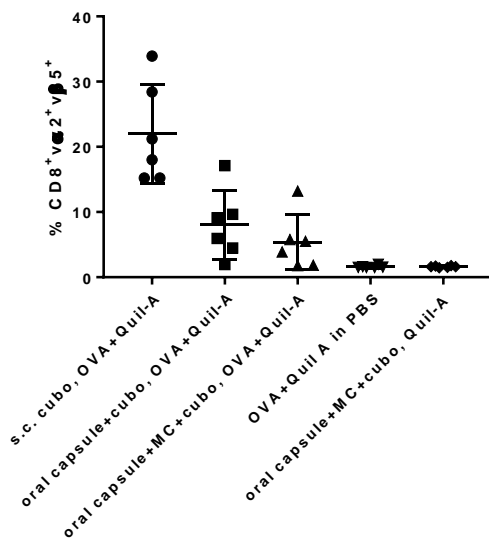
534 Mice were given s.c. primes followed by two oral boosts (or s.c. boosts for the positive  
 535 control). In this experiment, the oral dose was reduced ten times to be the same as the s.c.  
 536 dose (10 µg OVA and 0.67 µg Quil-A). OVA-specific serum IgG titers stimulated by s.c.  
 537 cubosomes was stronger than all oral groups (p < 0.001, Figure 10). Nonetheless, oral  
 538 boosters of cubosomes in microcontainers gave a slightly better antibody response than oral  
 539 boosters of cubosomes without microcontainers, indicating that the microcontainers help  
 540 deliver cubosomes orally as a booster vaccine (not significant, Figure 10). However, this  
 541 effect was not seen in the cellular response where CD8<sup>+</sup> expansion after oral treatment with  
 542 cubosomes in microcontainers was similar to the CD8<sup>+</sup> expansion after oral treatment with  
 543 cubosomes without microcontainers (Figure 11). The results could indicate that a parenteral  
 544 primer is necessary in later studies.



545

546 **Figure 10.** OVA-specific serum IgG antibody titers. Mice were given a priming dose of  
 547 vaccine by s.c. injection and then two s.c. or oral boosters as indicated. Vaccines were given  
 548 at 14 day intervals. In groups that received oral boosters with microcontainers, the s.c. prime  
 549 was injected without microcontainers. Data shown are from individual mice from a  
 550 representative experiment of three independent experiments together with mean and SD (n =  
 551 5-6 mice/experiment). cubo = cubosomes, MC = microcontainers.

552



553

554 **Figure 11.** *In vivo* expansion of OVA-specific CD8<sup>+</sup> cells in spleens. Mice were given a  
 555 priming dose of vaccine by s.c. injection and then two s.c. or oral boosters as indicated.  
 556 Vaccines were given at 14 day intervals. In groups that received oral boosters with  
 557 microcontainers, the s.c. prime was injected without microcontainers. Data shown are from  
 558 individual mice from a representative experiment of three independent experiments as well as  
 559 their average and SD (n = 5-6 mice/experiment). cubo = cubosomes, MC = microcontainers.

560

#### 561 **4. Conclusion**

562 Microcontainers hold great promise for protection of their cargo through the GI tract until  
563 release in the mucus of the small intestine. The transit time of microcontainers through the  
564 small intestine of mice is approximately 30-60 min. After release of the vaccine in the  
565 intestine, these lids caused a change of the particle morphology from cubosomes to  
566 hexosomes, which were stable for at least 21 min in intestinal fluid. However,  
567 microcontainers were not successful in allowing oral delivery of a vaccine consisting of  
568 cubosomes with OVA and Quil-A, but were able to slightly improve the humoral response to  
569 oral boosters using the same vaccine at low doses. This work indicates that an oral delivery  
570 system such as microcontainers should be focused on increasing the potency of vaccines that  
571 have some immunogenicity after oral administration. This study further indicates that oral  
572 vaccination is more easily achieved for booster vaccines after an injected primer.

573

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587 beamline at the synchrotron light source ELETTRA (Trieste, Italy).

588

#### 589 **Conflicts of interest**

590 The authors have no conflicts of interest to declare.

591

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755 **Supplementary information**

756

757 **Table S1:** Vaccinations in study 1, where microcontainers were tested as an oral vaccine  
758 system.

| Group no. | Vaccine formulation?                       | Dosage form               | Administrated on days |
|-----------|--|---------------------------|-----------------------|
| 1         | Microcontainers + cubosomes + OVA + Quil-A | Oral capsule              | 0, 14 and 28          |
| 2         | Microcontainers + OVA + Quil-A             | Oral capsule              | 0, 14 and 28          |
| 3         | Cubosomes + OVA + Quil-A                   | In 100 $\mu$ L PBS orally | 0, 14 and 28          |
| 4         | OVA + Quil-A                               | Oral capsule              | 0, 14 and 28          |
| 5         | Microcontainers + cubosomes + Quil-A       | Oral capsule              | 0, 14 and 28          |
| 6         | Cubosomes with OVA and Quil-A              | In 200 $\mu$ L PBS s.c.   | 14 and 28             |

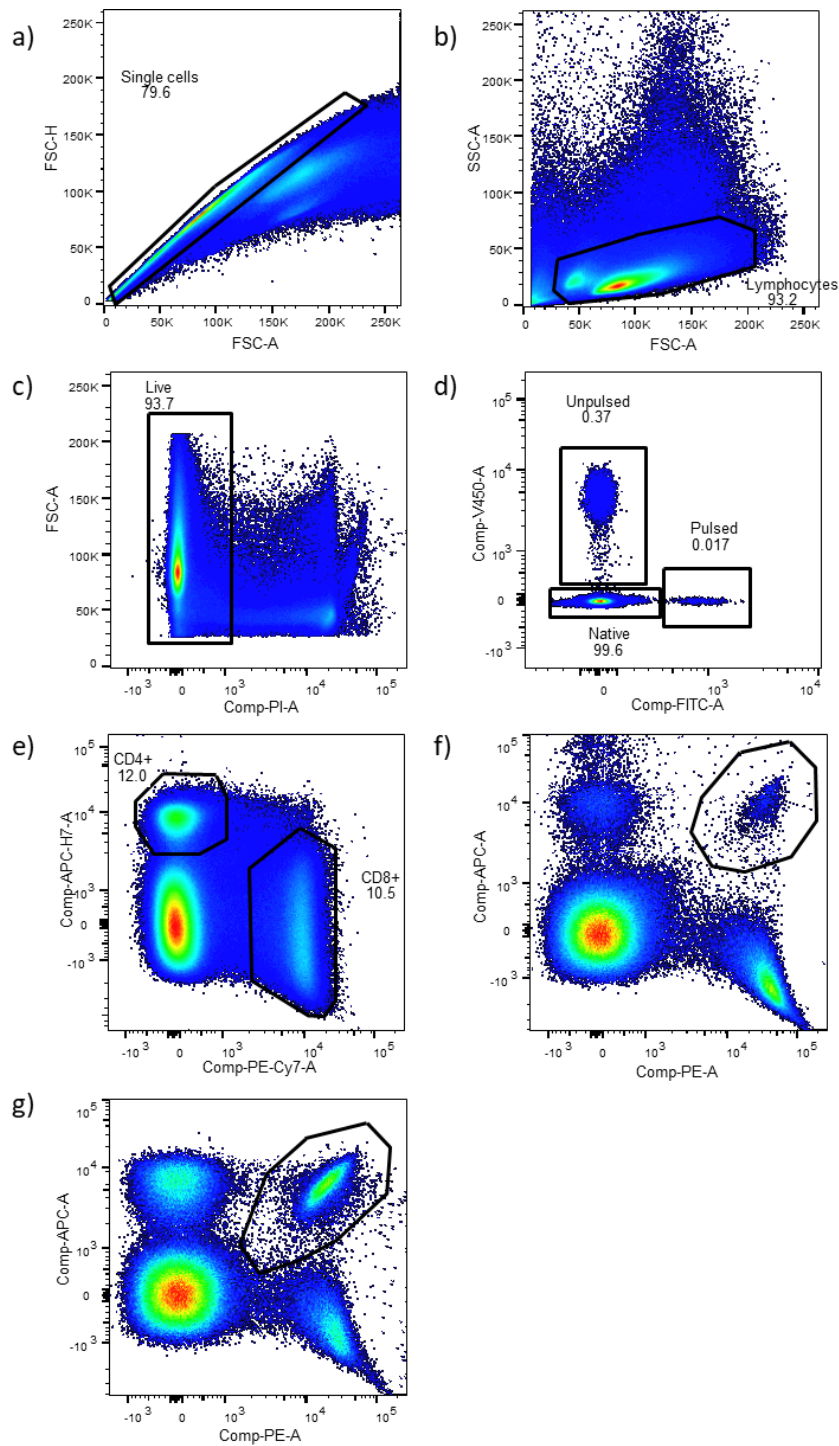
759

760

761 **Table S2:** Vaccinations in study 2 where microcontainers were tested as an oral booster  
762 vaccine system.

| Group no. | Primer (day 0)                                  | Booster (day 14 and 28)                                    |
|-----------|---|--|
| 1         | Cubosome + OVA + Quil-A in 200 $\mu$ L PBS s.c. | Microcontainers + cubosomes + OVA + Quil-A in oral capsule |
| 2         | Cubosome + OVA + Quil-A in 200 $\mu$ L PBS s.c. | Cubosomes + OVA + Quil-A orally in 100 $\mu$ L PBS         |
| 3         | OVA + Quil-A in 200 $\mu$ L PBS s.c.            | OVA + Quil-A orally in 100 $\mu$ L PBS                     |
| 4         | Cubosome + Quil-A in 200 $\mu$ L PBS s.c.       | Microcontainers + cubosomes + Quil-A in oral capsule       |
| 5         | Cubosome + OVA + Quil-A in 200 $\mu$ L PBS s.c. | Cubosome + OVA + Quil-A in 200 $\mu$ L PBS s.c.            |

763



764

765 **Figure S1.** Gating setup for FACS analysis of OVA-peptide labelled target cells and  
 766 transgenic OVA-specific CD4<sup>+</sup> and CD8<sup>+</sup> expansion. The example shown is from splenocytes  
 767 from a mouse receiving oral PBS with cubosomes with OVA and Quil-A. Single cells were  
 768 selected (a), followed by selecting lymphocytes (b), and excluding dead cells (c). OVA-  
 769 peptide pulsed and un-pulsed target cells were selected for the *in vivo* cytotoxic T cell killing  
 770 assay and native cells selected for the OVA-specific T cell expansion assay (d). Gates were  
 771 then set to select native cells expressing CD4<sup>+</sup> and CD8<sup>+</sup> receptors (e), and then transgenic  
 772  $v\alpha 2^+$  and  $v\beta 5^+$  receptors on CD4<sup>+</sup> cells (f), and CD8<sup>+</sup> cells (g).

773

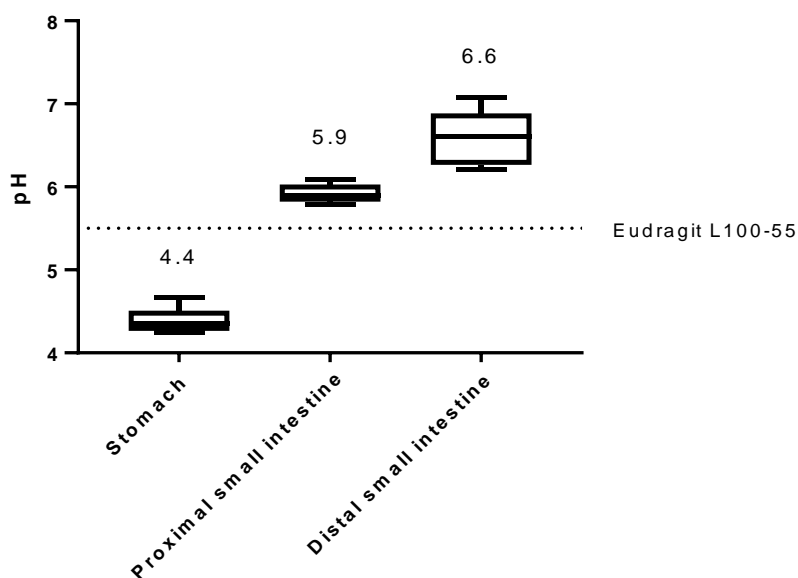
## 774 pH of the GI-tract of mice

### 775 Methods

776 Mice were sacrificed and their stomach and small intestine collected. The small intestine was  
777 divided into two segments (proximal and distal) of equal length. Segment fluid from four  
778 mice were pooled to allow complete submersion of a pH-microelectrode (Metrohm, Herisau,  
779 Switzerland) connected to a SensION+ pH31 pH meter (HACH®). Immediately after  
780 collecting the gastric and intestinal fluids, pH measurements were performed at 37°C on five  
781 independent samples.

### 782 Results

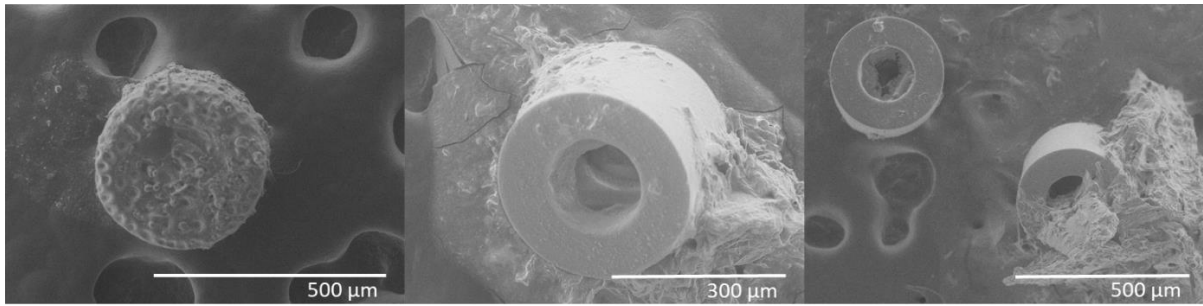
783 The pH of the fluid from the stomach and the proximal- and distal half of the small intestine  
784 was measured immediately after sacrificing the mice. The average pH in the stomach was 4.4  
785 and the highest individual pH-value recorded in the stomach was 4.7 (Figure S2). The  
786 average pH of the small intestine was 5.9 (proximal segment) and 6.6 (distal segment). The  
787 lowest individual pH-value recorded in the intestine was 5.8 and the highest 7.1. These  
788 results showed that the pH-sensitive polymer must be stable below pH 4.7 and must dissolve  
789 at pH-values above 5.8. EL100-55 was therefore chosen as it dissolves at pH-values above  
790 5.5.



791

792 **Figure S2.** pH of fluids from the GI tract from mice measured at 37°C immediately after  
793 sacrifice. Whiskers on the boxplots indicate maximum and minimum (n = 5). Averages are  
794 written above each group, and pH 5.5, above which EL100-55 begins to dissolve, is marked  
795 on the figure.

796



797

798 **Figure S3.** Representative SEM images of microcontainers after 30 min submersion into  
799 fluid from the stomach (a), proximal segment of the small intestine (b), and distal segment of  
800 the small intestine (c), *in vitro* at 37°C and rotation at 120 rpm.

801