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

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Abstract

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

Keywords

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

1. Introduction

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

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

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

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

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

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

2. Materials and Methods

2.1. Materials

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

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

2.2. Mice

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

2.3. Spray drying cubosomes

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

2.4. Fabrication, loading and sealing of microcontainers

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

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

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

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

2.5. Microcontainer characterization and qualitative release study

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

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

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

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

2.6. Small angle x-ray scattering of hydrated particles and particles released from microcontainers

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

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

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

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

2.7. Kinetics of microcontainer transit in the mouse GI tract

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

2.8. In vivo immunological investigation of microcontainers loaded with cubosomes as oral vaccines

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

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

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

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

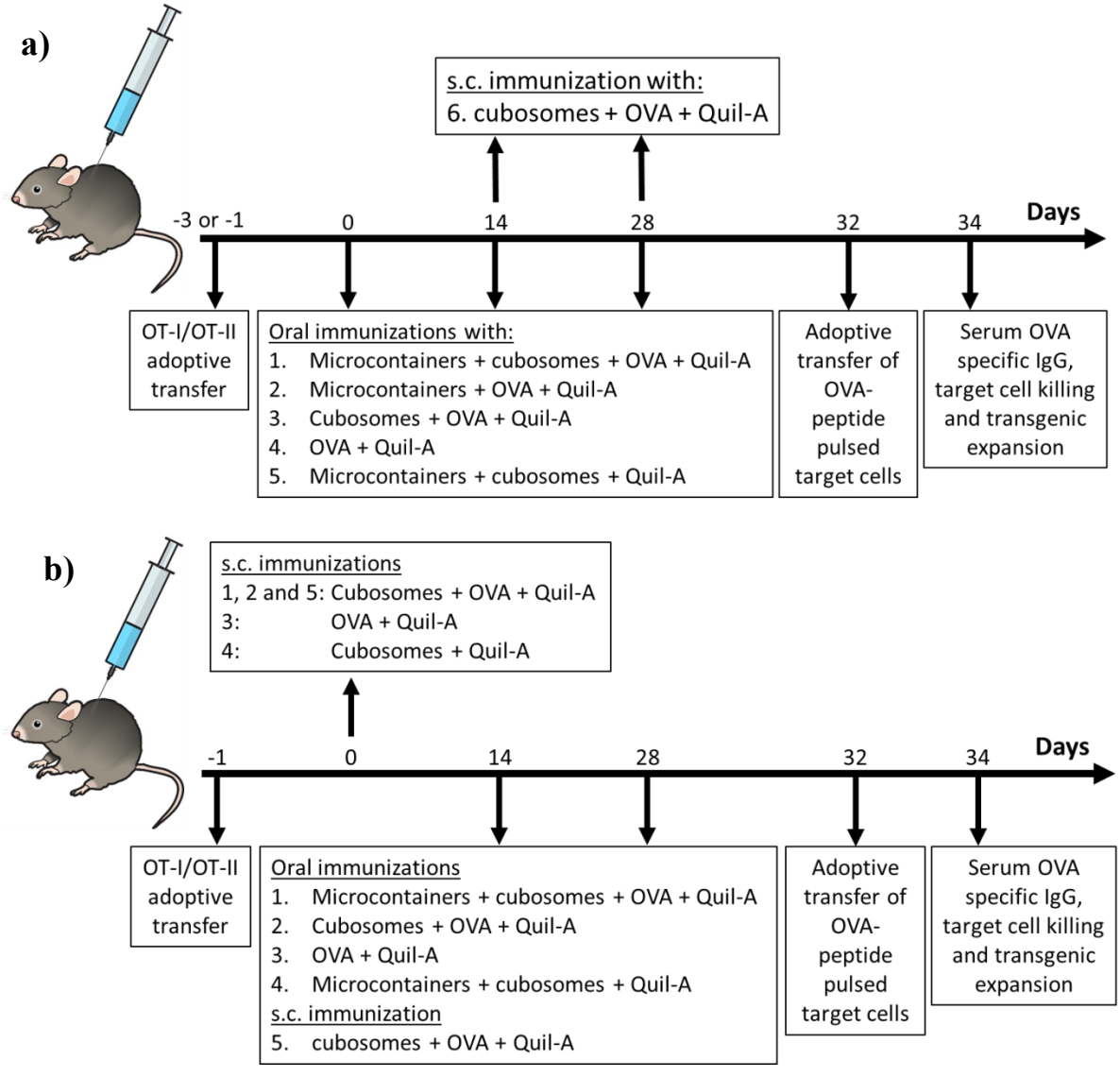


Figure 1. Schematics of the *in vivo* studies investigating (a) oral prime and boost or (b) s.c. prime followed by oral boosts. Positive control groups received s.c. prime and boost in both studies. Numbering of groups correspond with those of Tables S1 and S2.

2.9. Flow cytometry

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

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

Sera were separated from whole blood and OVA-specific serum IgG was measured by ELISA as previously described [26]. Briefly, wells were coated with OVA and then blocked with 2 % w/v BSA in PBS (9.5 mM, pH 7.3). Sera were diluted 1:100, serially diluted across well plates (high-binding 96 well plate, Corning inc. Corning, NY, USA) and incubated for 2 h. 225 ng/mL HRP Goat anti-mouse IgG was used as detection antibody and color was developed using a substrate reagent pack (R&D SYSTEMS®, MN, USA). Color development was stopped with 2 M H_2SO_4 and absorbance at 450 nm was read using a Polarstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany)

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

2.11. Statistics

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

3. Results and discussion

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

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

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

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

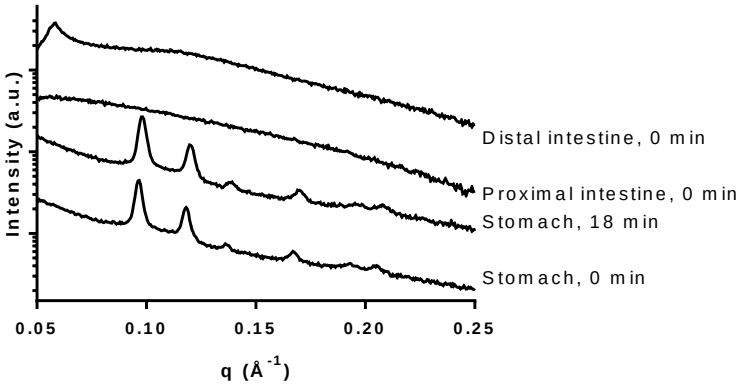


Figure 2. q vs. intensity patterns obtained from SAXS measurements of cubosomes with OVA and Quil-A mixed *in vitro* with fluids from segments of the GI tract of mice at 37°C. Measurements were performed at 0 and 18 min after mixing cubosomes into stomach fluid and 0 min after mixing cubosomes into fluid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

3.2. Fabrication, loading and coating of microcontainers

Microcontainers were fabricated with an outer diameter of 326.3 ± 1.0 µm and height of 255.4 ± 6.0 µm, and with an inner diameter of 231.5 ± 0.9 µm and height of 216.8 ± 6.4 µm, giving a reservoir volume of 9.1 ± 0.3 nL (n = 4, mean ± SD). Microcontainers were loaded with 6.6 ± 4.6 µg/microcontainer of powder and then sealed with EL100-55 lids. The average thickness of the lid coating was measured by contact profilometry to be 29.7 ± 3.5 µm with a surface roughness (SD of the thickness of the individual chip) ≤ 1.2 µm.

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

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

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

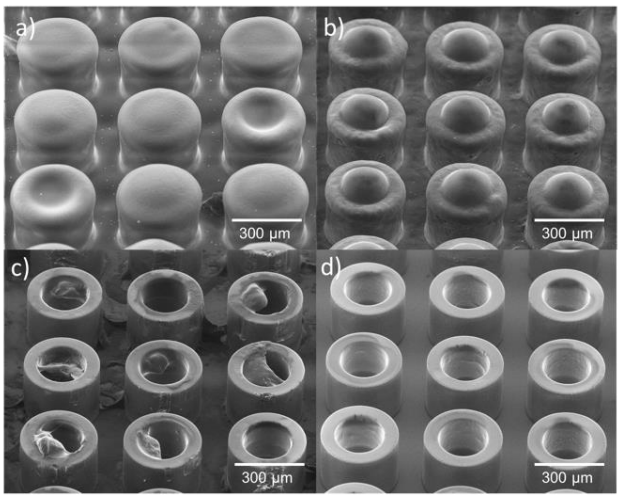


Figure 3. SEM images showing dry microcontainers on a chip after loading with cubosomes and coating with EL100-55 (a). Microcontainers were soaked for 60 min in 2 mM maleic acid at pH 4.7 simulating the pH of the mouse stomach (b) and then in 10 mM maleic acid at pH 6.6 simulating pH of the mouse intestine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

3.4. Effect of microcontainers on particle morphology

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

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

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

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

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

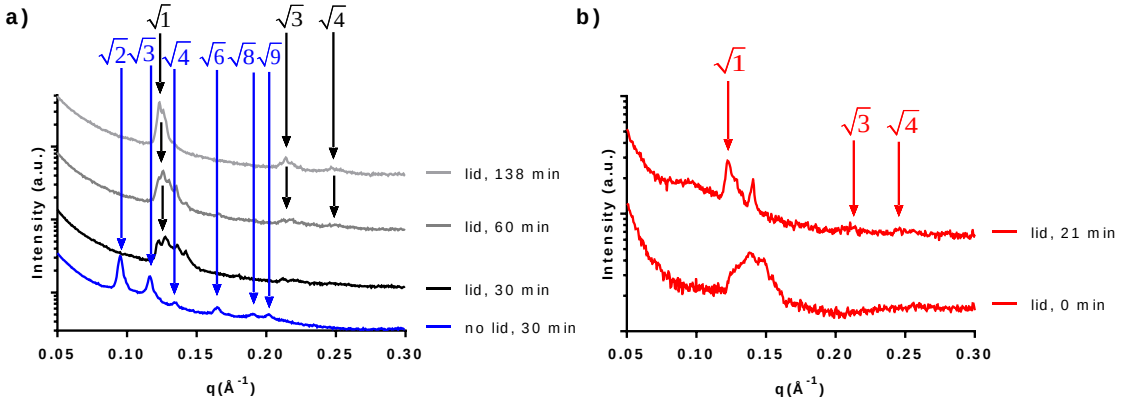


Figure 4. q vs. intensity patterns obtained from SAXS measurements of particles with OVA and Quil-A released from microcontainers at (a) 37ºC in PBS and (b) in mouse intestinal fluid. SAXS patterns shown in (a) are from particles released from microcontainers without lid after 30 min and with lid after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

3.5. Kinetics of microcontainer transit in the mouse GI tract

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

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

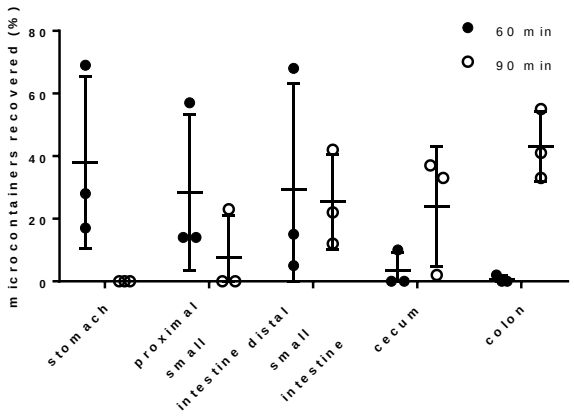
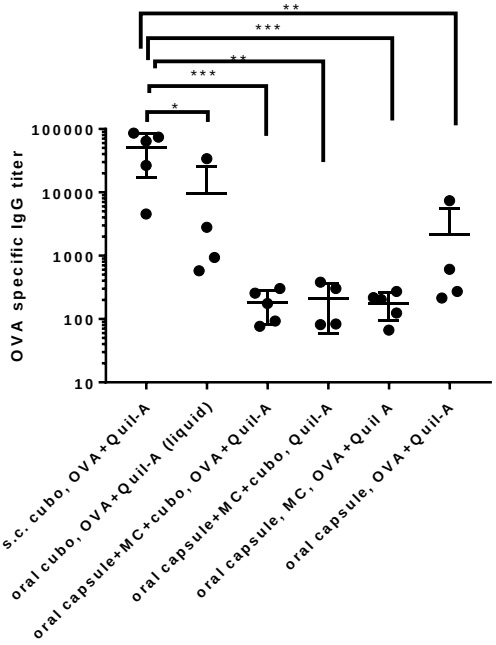


Figure 5. Relative numbers of recovered microcontainers found in the stomach, proximal- and distal half of the small intestine, cecum and colon in mice killed 60 or 90 min after oral administration. Error bars represent mean ± SD (n = 3).

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

Microcontainers appear to have promise as an oral delivery system and were therefore evaluated *in vivo*. Microcontainers were administered in capsules because their pH-sensitive lids would dissolve in buffer at neutral pH. OVA-specific serum IgG titers were measured by ELISA to evaluate the humoral immune response to the vaccines. A strong anti-OVA IgG response was seen after s.c. administration as previously reported [26]. Oral cubosomes resulted in slightly increased IgG response in one experiment (Figure 6), but not in the replicate of the experiment (not shown). OVA and Quil-A delivered in capsules or in 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

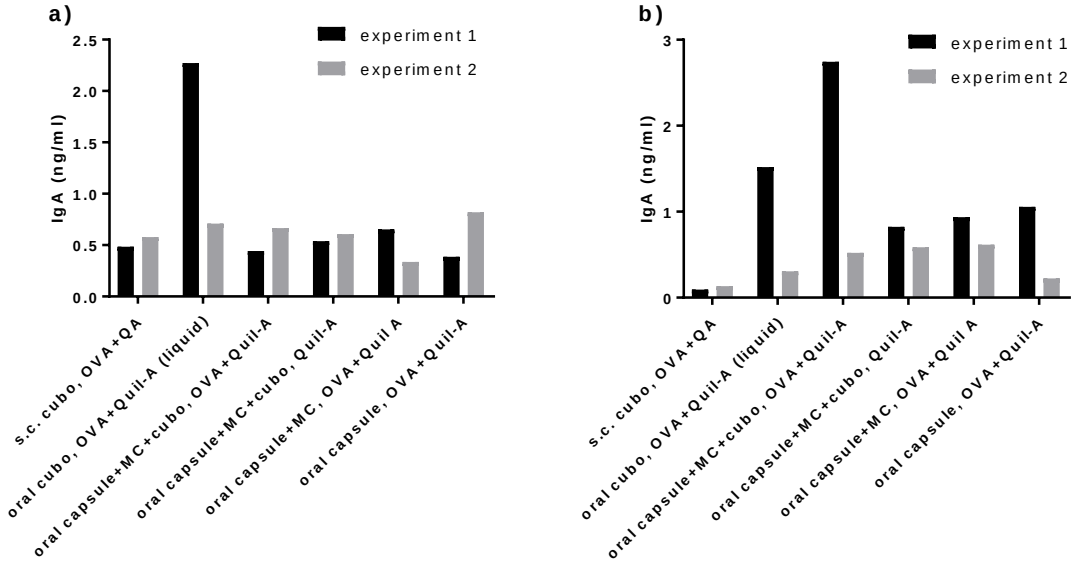


Figure 7. OVA-specific IgA from (a) fecal samples collected at the time of sacrifice and (b) serum samples. Mice were given vaccines three times orally or two times s.c. as indicated with 14 days between each vaccination. Data are from pooled mice with 4-5 mice/group. cubo = cubosomes, MC = microcontainers.

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

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

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

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

form, it is not suitable for oral delivery, even if using an oral delivery system. However, both cubosomes and microcontainers have the potential to be further developed and microcontainers can be coupled to different vaccine formulations warranting further studies.

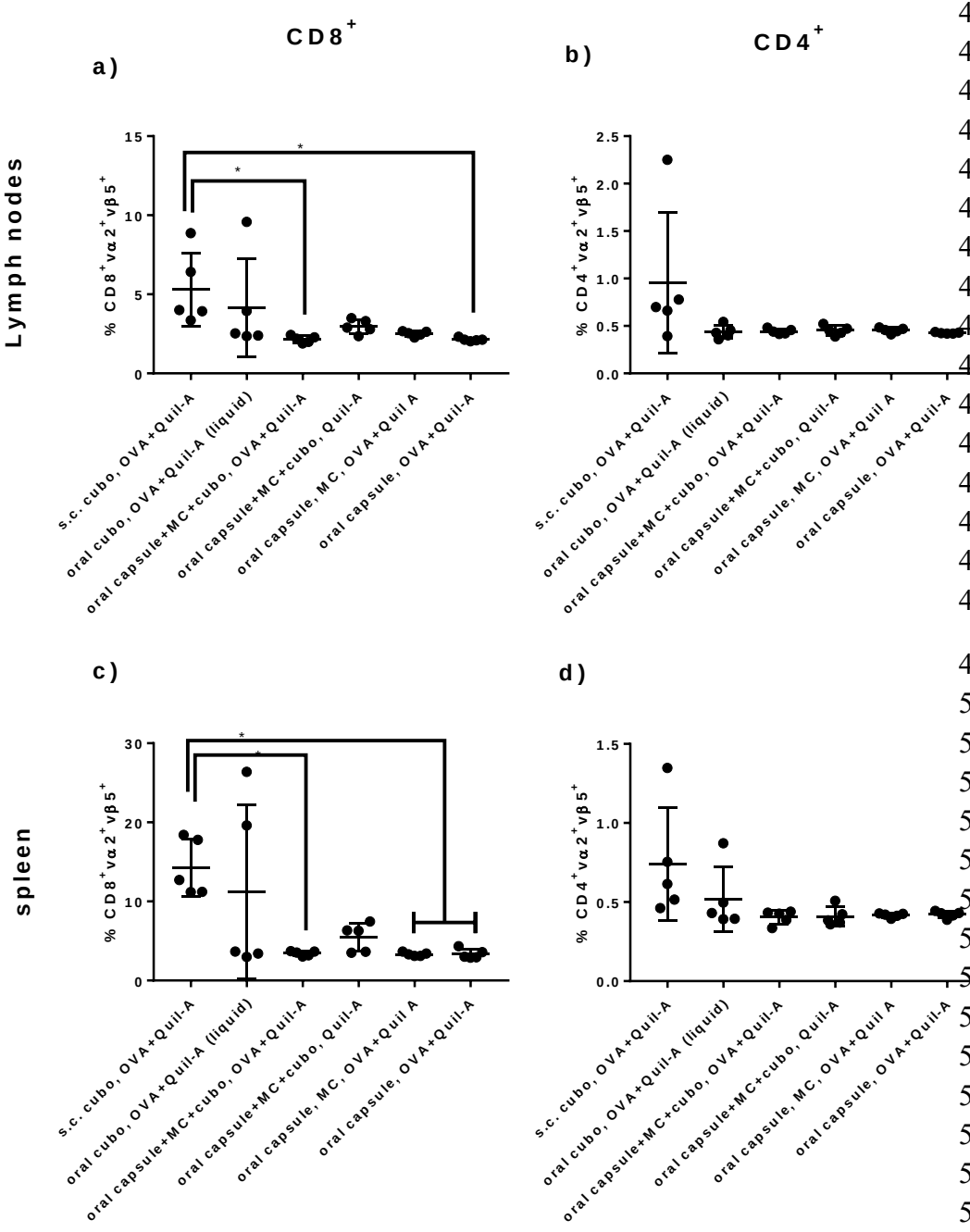


Figure 8. *In vivo* expansion of OVA-specific $CD8^+$ cells (a and c) and $CD4^+$ cells (b and d) in lymph nodes (a and b) and spleens (c and d). Mice were given vaccines three times orally or two times s.c. as indicated, with 14 days between each vaccination. Data shown are results from individual mice together with the mean and SD from one of two independent experiments (n = 4-5 mice). *$p < 0.05$, cubo = cubosomes, MC = microcontainers.

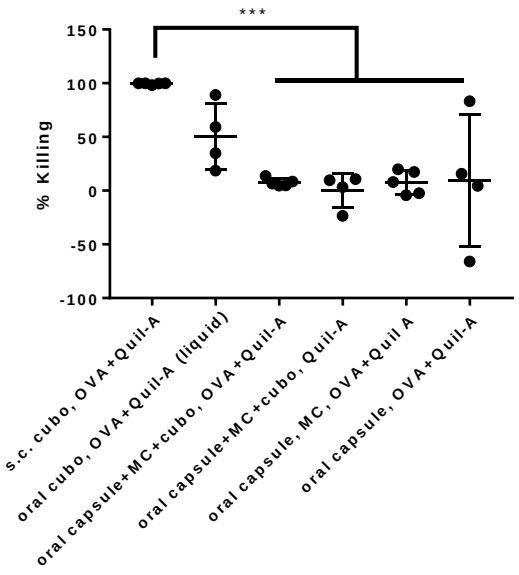
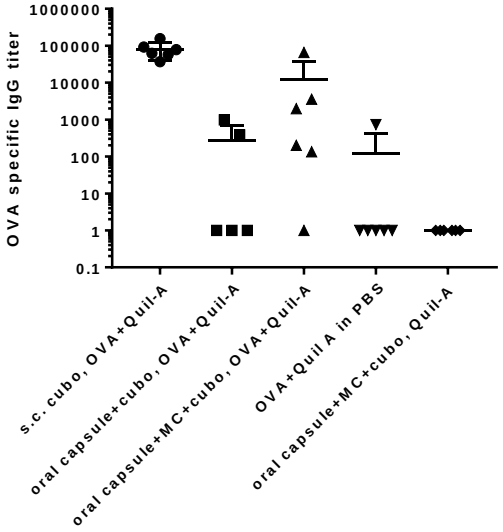


Figure 9. *In vivo* killing of adoptively transferred OVA-peptide labelled T cells in spleens. Mice were given vaccines three times orally or two times s.c. as indicated with 14 days between each vaccination. Data shown are results from individual mice together with mean and SD from one of two independent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

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

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

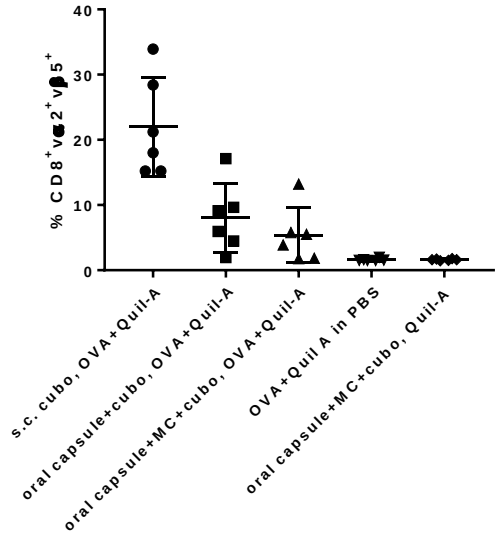
Mice were given s.c. primes followed by two oral boosts (or s.c. boosts for the positive control). In this experiment, the oral dose was reduced ten times to be the same as the s.c. dose (10 µg OVA and 0.67 µg Quil-A). OVA-specific serum IgG titers stimulated by s.c. cubosomes was stronger than all oral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

4. Conclusion

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

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Conflicts of interest

The authors have no conflicts of interest to declare.

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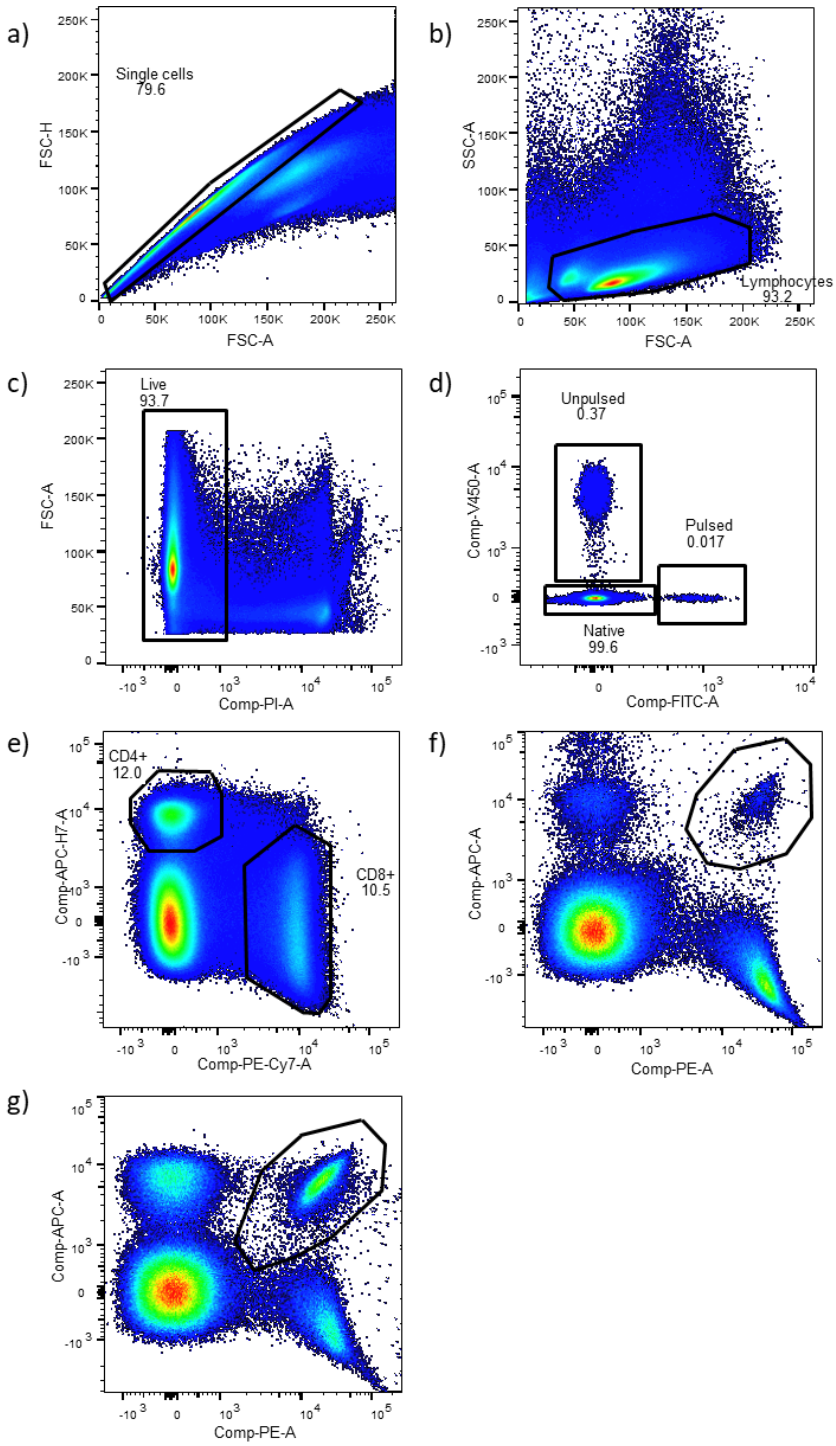
Supplementary information

Table S1: Vaccinations in study 1, where microcontainers were tested as an oral vaccine 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 µL 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 µL PBS s.c.	14 and 28

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

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



764

765 **Figure S1.** Gating setup for FACS analysis of OVA-peptide labelled target cells and
766 transgenic OVA-specific $CD4^+$ and $CD8^+$ 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 $CD4^+$ and $CD8^+$ receptors (e), and then transgenic
772 $v_\alpha 2^+$ and $v_\beta 5^+$ receptors on $CD4^+$ cells (f), and $CD8^+$ cells (g).

pH of the GI-tract of mice

Methods

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

Results

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

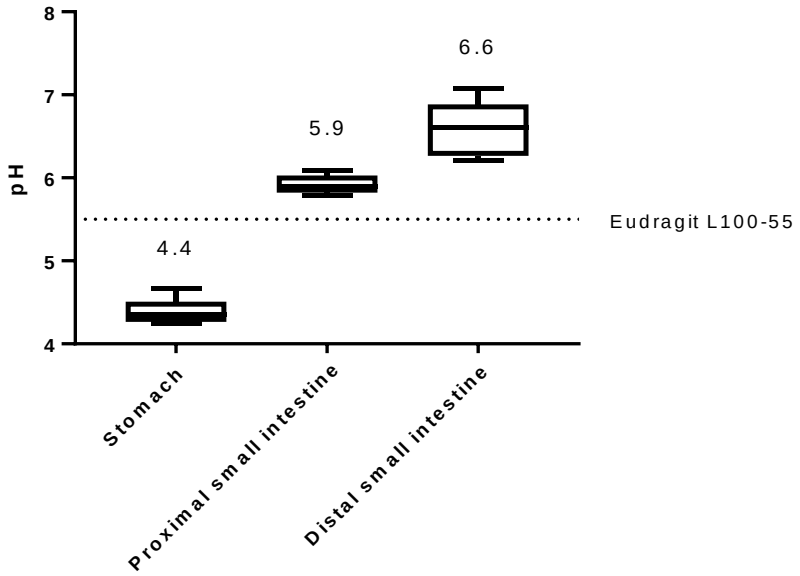


Figure S2. pH of fluids from the GI tract from mice measured at 37ºC immediately after sacrifice. Whiskers on the boxplots indicate maximum and minimum (n = 5). Averages are written above each group, and pH 5.5, above which EL100-55 begins to dissolve, is marked on the figure.

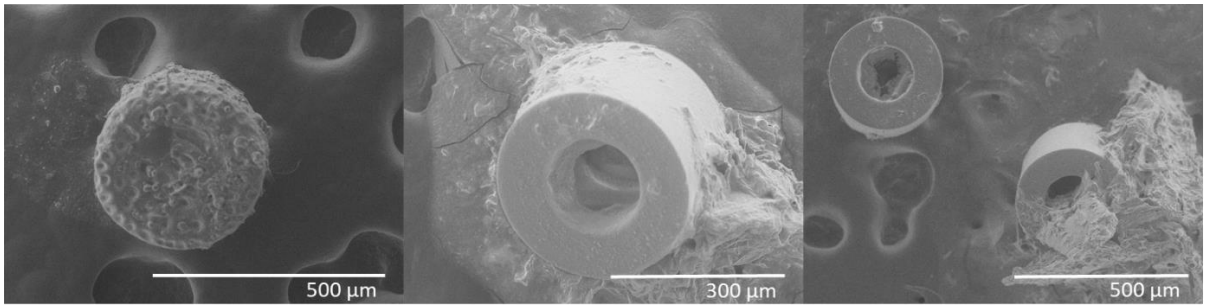


Figure S3. Representative SEM images of microcontainers after 30 min submersion into fluid from the stomach (a), proximal segment of the small intestine (b), and distal segment of the small intestine (c), *in vitro* at 37ºC and rotation at 120 rpm.