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Ph.D. Thesis

Philip Hassing Ronøe Carlsen

# Microcontainers for oral delivery of vaccines

STATENS SERUM INSTITUT

Danmarks Grundforskningsfond
Danish National Research Foundation

IDUN

VILLUM FONDEN

Ph.D. thesis

**Philip Hassing Ronøe Carlsen**

# Microcontainers for oral delivery of vaccines

September 2022

DTU

STATENS SERUM INSTITUT

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# Preface

The present thesis was submitted to meet the requirements to obtain a PhD degree at the Technical University of Denmark (DTU). The research was carried out in the section “Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics” (IDUN), Department of Health Technology, and the Department of Infectious Disease Immunology at Statens Serum Institute from 1st of May 2019 to 30th of April 2022. Additionally, some experiments were conducted at the National Institute of Aquatic Resources (DTU AQUA) and the Centre for Medical Parasitology (CMP), Department of Immunology and Microbiology, University of Copenhagen. The project was a part of the IDUN project funded by the Danish National Research Foundation (grant no. DNRF122) and Villum Fonden (grant no. 9301). The work was performed under the supervision of Professor Anja Boisen, Associate Professor Line Hagner Nielsen, Department of Health Technology, Technical University of Denmark, and Senior researcher Dennis Christensen, Vaccine Adjuvant Research, Department of Infectious Disease Immunology, Statens Serum Institute.

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Finally, a big thanks to my friends and family for always being encouraging and supportive. Thanks to mom, dad, Thomas, Anna, Oline, and Otto for always being there for me. To my girlfriend Catrine, I could not have accomplished this without you. Thanks for always supporting and listening to me, both in the good and the bad times.

I am genuinely grateful to have been given this opportunity, and I have learned more than I could have imagined. Thank you all so much.

## List of publications and other contributions

The findings of this PhD thesis led to two manuscripts, which are either submitted or in preparation for submission. Additional contributions included one popular science article and three conference contributions

**Manuscripts**

Paper I — Oral vaccination using microdevices to deliver α-GalCer adjuvanted vaccine afford mucosal immunity
Philip H. R. Carlsen, Rolf B. Kjeldsen, Gabriel K. Pedersen, Dennis Christensen, Line Hagner Nielsen, Anja Boisen. *In review in Journal of Controlled Release, received from editor with minor revisions on 30-08-2022*

Paper II — Oral delivery of the AP205-SpyCatcher capsid virus-like-particle using microdevices
Philip H. R. Carlsen*, Kara-Lee Awes*, Line Hagner Nielsen, Anja Boisen, Adam Sander Berthelsen. *Manuscript in preparation. *These authors contributed equally to the work.*

**Other contributions**

Poster I — Oral induction of mucosal immune responses against CTH522, using various adjuvants in combination with microcontainers for protection and delivery through the GI tract of mice. *BioBarriers, 2021, Virtual conference*
Philip H. R. Carlsen, Rolf Bech Kjeldsen, Gabriel Pedersen, Dennis Christensen, Line Hagner Nielsen, and Anja Boisen

Poster II — Oral induction of mucosal immune responses, using various adjuvants in combination with microcontainers for protection and delivery through the GI tract. *World meeting of Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology (PBP), March 2022, Rotterdam, Netherlands*
Philip H. R. Carlsen, Rolf Bech Kjeldsen, Gabriel K. Pedersen, Dennis Christensen, Line Hagner Nielsen and Anja Boisen.

Oral talk — Microcontainers for oral delivery of vaccines. *European Network on Vaccine Adjuvants (ENOVA), September 2021, Genova, Italy*
Philip H. R. Carlsen, Rolf Bech Kjeldsen, Gabriel K. Pedersen, Dennis Christensen, Line Hagner Nielsen and Anja Boisen.

Popular science article — Mikrocontainere - Fremtidens vacciner kan være på størrelse med sandkorn
Philip H. R. Carlsen, Rolf Bech Kjeldsen, Juliane Fjelrad Christford
1. December 2021, Kvant p. 1-5, https://www.kvant.dk/issue.php?n=4&y=2021

## Abbreviations

| | |
|---|---|
| 2'-F-c-di-GMP | 2'-fluoro-cyclic-de-GMP |
| α-GalCer | α-Galactosylceramide |
| ALR | Absent in melanoma (AIM)-like receptor |
| AS | Adjuvant system |
| AA | Amino acid |
| APC | Antigen presenting cells |
| BaSO4 | Barium sulfate |
| cVLP | Capsid virus-like-particle |
| CAF | Cationic adjuvant formulation |
| C. trachomatis | Chlamydia trachomatis |
| CPAF | Chlamydial protease-like activity factor |
| CTA | Cholera toxin A |
| CTB | Cholera toxin B |
| C. rodentium | *Citrobacter rodentium* |
| Tc | Collapse temperature |
| CLSM | Confocal laser scanning microscopy |
| CQA | Critical quality attributes |
| CLR | C-type lectin receptor |
| cAMP | cyclic AMP |
| c-di-GMP | cyclic-di-GMP |
| CpG | Cytosine phosphoguanine |
| DC | Dendritic cell |
| dmLT | Double mutant heat-labile toxin |
| DLS | Dynamic light scattering |
| ETEC | Entereotoxigenic E. Coli |
| E. coli | Escherichia coli |
| EL100 | Eudragit L100 |
| EL100-55 | Eudragit L100-55 |
| FAE | Follicle-associated epithelium |
| G. lamblia | *Gardia lamblia* |
| GI | Gastrointestinal |
| GC | Germinal center |
| Tg | Glass transition temperature |
| GALT | Gut-associated-lymphoid-tissue |
| H. pylori | Helicobacter pylori |
| HIV | Human immunodeficiency virus |
| HPV | Human papillomavirus |
| Ig | Immunoglobulin |
| IRF | Interferon regulating factor |
| IFN-γ | Interferon-γ |
| IL | Interleukin |
| i.m. | Intramuscular |
| i.n. | Intranasal |

| | |
|---|---|
| i.p. | Intraperitoneal |
| LT | Labile toxin |
| LP | Lamina propria |
| LPS | Lipopolysaccharide |
| MOMP | Major outer membrane protein |
| MMR(V) | Measles, mumps, rubella and varicella |
| MC | Microcontainer |
| M cell | Microfold cell |
| MP | Microparticles |
| MMG | Monomycolyl glycerol |
| MPL | Monophosphoryl lipid A |
| MALT | Mucosal-associated-lymphoid-tissue |
| mmCT | Multiple-mutated cholera toxin |
| DDA | N,N-dimethyl-N,N-dioctadecylammonium |
| NP | Nanoparticles |
| NKT | Natural killer T cells |
| NLR | *Nod-like receptor* |
| NLR | Nucleotide-binding domain, leucine-rich repeat-containing protein receptor |
| ODN | Oligodeoxynucleotides |
| OMP | Outer membrane proteins |
| PAMPs | Pathogen associated molecular patterns |
| PPs | Peyer's patches |
| P. pastoris | Pichia pastoris |
| PLLA | poly (L-lactic acid) |
| PLGA | Poly(lactic-co-glycolic) acid |
| PMMA | Poly(methyl methacrylate) |
| pIgA | Polymeric IgA |
| pIgR | Polymeric immunoglobulin receptor |
| RGNNV | Red grouper nervous necrosis virus |
| RLR | Retinoic acid-inducible gene-I (RIG-I)-like receptor |
| S. cerevisiae | Saccharomyces cerevisiae |
| S. typhimurium | *Salmonella typhimurium* |
| SEM | Scanning electron microscope |
| sIgA | Secretory IgA |
| SPEDs | Self-configurable Proximity Enable Devices |
| STD | Sexually transmitted diseases |
| Si | Silicon |
| SmPill | Single-Multiple Pill |
| SpyC | SpyCatcher |
| SpyT | SpyTag |
| STING | Stimulator of interferon genes |
| s.c. | Subcutaneous |
| TBK | TANK binding kinase |
| Th1 | T-helper 1 cells |

| | |
|---|---|
| Th17 | T-helper 17 cells |
| Ti\|Au | Titanium\|Gold |
| TLR | Toll-like receptor |
| TEER | Transepithelial/transendothelial electrical resistance |
| TGF | Transforming growth factor |
| TEM | Transmission electron microscope |
| Treg | T-regulatory cells |
| TDM | Trehalose-6,6′-dimycolate |
| TNF | Tumor necrosis factor |
| VD4 | Variable domain 4 |
| VNN | Viral nervous necrosis |
| VLP | Virus-like-particle |
| TDB | α,α-trehalose 6,6’-dibehenate |

# Abstract

Vaccination via the oral route is highly sought after, due to improved logistics and patient compliance. The intestine is a natural target for oral vaccines, as this region harbors an accumulation of immune cells. Current oral vaccines are based on live attenuated and whole-cell killed pathogens, whereas next-generation vaccines are most often subunit based vaccines presenting the antigens as proteins or peptides. However, this type of vaccine-technology is often not immunogenic and prone to the challenges of intestinal delivery. This has caused advancement in the fields of vaccine adjuvants, which can induce activation of the mucosal immune system (immunestimulators) and/or mediate targeted release of the vaccine (delivery systems). Microfabricated devices, microcontainers (MCs), have been used for oral delivery of drugs and observed to protect the content through the stomach and capable of targeted and retained release in the intestine. Previously, a vaccine containing the model antigen ovalbumin, formulated with cubosomes and the adjuvant Quil-A were orally delivered in MCs to mice. However, an efficient mucosal immunological response was not established. In this PhD thesis, it is hypothised that by employing vaccine formulations, designed for mucosal immune stimulation, with the MCs, a robust response could be stimulated.

The MCs were combined with the vaccine candidate CTH522 antigen derived from the mucosal pathogen *Chlamydia trachomatis* (*C. trachomatis*). In addition, the adjuvants cholera toxin subunit B (CTB), cyclic-di-GMP (c-di-GMP), and α-Galactosylceramide (α-GalCer), were chosen for the vaccine formulation. After loading the vaccine formulations into the MCs, they were equipped with a polymeric lid, for the purpose of targeting and tailoring the release from the microdevices. Three coatings were employed here, namely poly(lactic-co-glycolic) acid (PLGA), chitosan or the pH degradable polymer Eudragit L100-55 (EL100-55). Screening studies in mice revealed α-GalCer and EL100-55 as the most promising adjuvant and coating, respectively, for oral administration of CTH522. Mice receiving a sub-cutaneous prime with CTH552 and the liposomal adjuvant CAF01®, followed by oral boosters with α-GalCer and CTH522 in MCs coated with EL100-55, demonstrated a trend to increase systemic Th17 cells in addition to local Th1, Th17, and IgA responses. Furthermore, oral administration solely with MCs did stimulate significantly higher local Th1, Th17, and IgA responses compared to naïve mice. Additionally, the intestinal transit time of the MCs was investigated in mice by X-ray and CT-scan and was comparable to the standard transit time of food in the mice GI tract. It is likely that this time window is simply too narrow for the vaccine formulation to interact with the underlying cells for proper establishment of an immune response, suggesting the devices need to be redesigned to increase the retention time.

The MCs were further applied to orally deliver the AP205 capsid virus-like particle (cVLP) platform. This system is capable of functioning as a scaffold for the presentation of unrelated antigens, in order to adapt the underlying immunogenicity of the cVLP. The AP205 cVLPs were lyophilized, which has not been done with this platform before. Reconstituted cVLPs were checked with SDS-PAGE and transmission electron microscopy (TEM) and revealed no apparent signs of aggregation or degradation. Subsequently, the lyophilized particles were loaded into MCs and orally dosed to rats. However, no stimulation of a mucosal immune response was observed. A reason for this could be that the cVLPs are not able to stimulate an oral mucosal immune response by themselves and should most likely be formulated with a mucosal adjuvant. Furthermore, it is also possible that, as in the previous study, the MCs are not retaining the vaccine formulation long enough in the intestine for a response to be established.

Finally, MCs were explored in European sea bass as a potential tool for oral vaccination in fish, which would be significantly less laborious and time-consuming compared to injectable vaccination in a fish farm setting. This was the first time the MCs were administered to fish, and therefore needed to be verified as safe and functional. First, a method for oral administration of MCs to the sea bass was established. Following administration, the fish were monitored for any visual discomfort, which was not observed. Dissection and visual investigation of the GI tract, revealed no signs of inflammation, further suggesting the MCs to be safe for employment in sea bass. Subsequently, were MCs loaded with a VLP developed from the red grouper nervous necrosis virus (RGNNV) and coated with Eudragit L100. *In vitro* and *in vivo* investigation of the functionality of the MCs were then conducted, revealing them only to release the content when reaching the intestine. Sea bass were then orally immunized with the RGNNV VLP loaded into MC and subsequently a challenged with the RGNNV virus. However, many practical complications occurred leaving the results of the study inconclusive and, thus, a requirement for repetition.

Conclusively, this work shows the potential of the MCs as a tool for oral delivery of vaccines. However, it does also report a need to optimize the device technology, along with highlighting the impact of the chosen vaccine components and animal models. Designing the devices to increase retention in the intestine would most likely be beneficial and would properly be more feasible to conduct in larger animal models. Furthermore, formulation with effective mucosal adjuvants seems essential, especially in the case of subunit-based vaccines and should be incorporated. It would be interesting to identify other novel adjuvants to induce mucosal responses in the intestine which could have the potential to optimize the technology. Furthermore, other microfabricated devices designed to increase retention in the intestine could prove more promising for the oral delivery of vaccines.

# Resumé

Vaccination via den orale rute er eftertragtet på grund af forbedret logistik og patient-compliance. Tarmen er et naturligt mål for orale vacciner, da denne region huser en ophobning af immunceller. Nuværende orale vacciner er baseret på levende svækkede og helcelle-dræbte patogener, hvorimod moderne vacciner oftest er baseret på 'stykker' (subunits) af patogerne, der præsenterer antigenerne som proteiner eller peptider. Imidlertid er denne type vaccineteknologi ofte ikke immunogen og svært ved at overkomme udfordringerne ved levering til tarmen. Dette har forårsaget fremskridt inden for vaccine-adjuvanser, som kan inducere aktivering af det mukosale immunsystem (immunestimulatorer) og/eller mediere målrettet frigivelse af vaccinen (leveringssystemer). Mikrofabrikerede enheder, mikrocontainere (MCs), er blevet brugt til oral levering af lægemidler og er observeret til at beskytte indholdet gennem maven og i stand til målrettet og fastholdt frigivelse i tarmen. I et tidligere studie blev en vaccine som indeholdt model-antigenet ovalbumin, formuleret med cubosomer og adjuvansen Quil-A, leveret oralt i MCs til mus. Et effektivt mukosalt-immunologisk respons blev imidlertid ikke etableret. I denne Ph.D.-afhandling antages det, at ved at anvende vaccineformuleringer, designet til mukosal-immunstimulering, kan et robust respons stimuleres ved levering med MCs.

MC'erne blev kombineret med vaccinekandidaten CTH522-antigenet afledt af patogenet *Chlamydia trachomatis* (*C. trachomatis*). Derudover blev adjuvanserne Cholera Toxin B (CTB), cyclic-di-GMP (c-di-GMP) og a-Galactosylceramid (a-GalCer) valgt til vaccineformuleringen. Efter fyldning af vaccineformuleringerne i MC'erne blev de udstyret med et polymerlåg med det formål at målrette og skræddersy frigivelsen fra mikro-kapslerne. Tre belægninger blev anvendt her, nemlig poly(mælke-co-glykol)syre (PLGA), chitosan eller den pH-nedbrydelige polymer Eudragit L100-55 (EL100-55). Screeningsundersøgelser i mus afslørede α-GalCer og EL100-55 som henholdsvis den mest lovende adjuvans og coating til oral administration af CTH522. Mus, der modtog en subkutan priming med CTH552 og det liposomale adjuvans CAF01®, efterfulgt af orale boostere med α-GalCer og CTH522 i MC'er belagt med EL100-55, viste en tendens til at øge systemiske Th17-celler ud over lokale Th1, Th17 og IgA-respons. Desuden stimulerede oral administration udelukkende med MC'er signifikant højere lokale Th1-, Th17- og IgA-responser sammenlignet med naive mus. Derudover blev den transittiden for MC'erne gennem tarmen undersøgt i mus ved røntgen og CT-scanning og var sammenlignelig med standard transittid for mad i musens tarmsystem. Det er sandsynligt, at dette tidsvindue er for snævert til, at vaccineformuleringen kan interagere med de underliggende celler for korrekt etablering af et immunrespons, hvilket tyder på, at enhederne skal re-designes for at øge retentionstiden.

MC'erne blev yderligere anvendt til oral levering af AP205 capsid virus-like particel (cVLP) platform. Dette system er i stand til at fungere som en platform for præsentationen af ikke-

relaterede antigener for at overføre den underliggende immunogenicitet af cVLP'en. AP205 cVLP'erne blev fryse tørret, hvilket ikke er blevet gjort med denne platform før. Rekonstituerede cVLP'er blev kontrolleret med SDS-PAGE og transmissionselektronmikroskopi (TEM) og afslørede ingen tydelige tegn på aggregering eller nedbrydning. Efterfølgende blev de frysetørret partikler fyldt i MC'er og oralt doseret til rotter. Der blev imidlertid ikke observeret nogen stimulering af et mukosalt-immunrespons. En årsag til dette kunne være, at cVLP'erne ikke er i stand til at stimulere et oralt mukosalt-immunrespons af sig selv og højst sandsynligt bør formuleres med en mukosal adjuvans. Endvidere er det også muligt, at MC'erne, som i den tidligere undersøgelse, ikke bevarer vaccineformuleringen længe nok i tarmen til, at der kan etableres et respons.

Endelig blev MC'er undersøgt i europæisk havaborre som et potentielt værktøj til oral vaccination i fisk, hvilket ville være betydeligt mindre besværligt og tidskrævende sammenlignet med injicerbar vaccination i en dambrug. Dette var første gang, at MC'erne blev administreret til fisk, og de skulle derfor verificeres som sikre og funktionelle. Først blev en metode til oral administration af MC'er til havbarsen etableret. Efter administration blev fiskene overvåget for ethvert visuelt ubehag, hvilket ikke blev observeret. Dissektion og visuel undersøgelse af mave-tarmkanalen afslørede ingen tegn på betændelse, hvilket yderligere tyder på, at MC'erne er sikre til brug i havaborre. Efterfølgende blev MC'er fyldt med en VLP udviklet fra red grouper nervous necrosis virus (RGNNV) og belagt med Eudragit L100. Der blev derefter udført in vitro og in vivo undersøgelser af funktionaliteten af MC'erne, hvilket viste at de kun frigav indholdet, når de nåede tarmen. Havaborre blev derefter oralt immuniseret med RGNNV VLP i MCs og efterfølgende inficeret med RGNNV virus. Der opstod imidlertid mange praktiske komplikationer, hvilket efterlod resultaterne af undersøgelsen inkonklusive og burde gentages.

Afslutningsvis viser dette arbejde potentialet af MC'erne som et værktøj til oral levering af vacciner. Den rapporterer dog også et behov for at optimere teknologien sammen med at optimere virkningen af de valgte vaccinekomponenter og dyremodeller. At designe anordningerne til at øge retentionen i tarmen ville højst sandsynligt være gavnligt og ville være mere gennemførligt at udføre i større dyremodeller. Ydermere synes formulering med effektive mukosale adjuvanser essentiel, især i tilfælde af subunit-baserede vacciner og bør inkorporeres. Det ville være interessant at identificere andre nye adjuvanser til at inducere slimhinderesponser i tarmen, som kunne have potentialet til at optimere teknologien. Yderligere kan andre mikrofabrikerede enheder designet til at øge retentionen i tarmen vise sig at være mere lovende til oral levering af vacciner.

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# 1. Introduction

Since their initial discovery by E. Jenner, vaccines have greatly influenced the health and longevity of humans and animals, together with improved hygiene and sanitation.[1] Decades ago, the effectiveness of vaccines in preventing infectious diseases became established, resulting in the majority of the human population receiving them routinely. Measles, a virus that is highly contagious, is an illustration of this. Prior to the advent of a vaccination for the measles in Denmark in 1987, which was subsequently added into the MMR (or MMRV) vaccine, practically all children were exposed to the disease. This has ultimately resulted in the measles being under control in Denmark, with only few isolated outbreaks of foreign origin since 2017.[2] A more recent instance is the COVID-19 pandemic, which focused global attention on vaccination as the most effective method of disease prevention. Since the end of 2020, 12.1 billion doses of COVID-19 vaccines have been administered worldwide (at the time of writing).[3] Numerous modeling studies have been produced and published to estimate the impact of vaccines, a topic that is still highly debated. According to a recent mathematical modeling study, the rapid development and distribution of the COVID-19 vaccinations prevented the deaths of 19.8 million individuals in 185 countries between December 8, 2020, and December 8, 2021.[4]

With 25 vaccinations already approved globally and hundreds more under development, the COVID-19 pandemic boosted vaccine invention and development. The technologies used for these vaccine candidates range from an innovative use of the traditional inactivated and attenuated vaccines to the more modern mRNA vaccines, several of which use advanced adjuvants.[5] However, all the approved vaccines are injection vaccines, which are generally invasive, laborious, dependent on trained personnel, time-consuming, and impractical in a mass vaccination setting.

Oral vaccination can potentially enhance logistical efficiency and patient compliance. However, the digestive system and biological barriers strongly limit the potential for vaccines to be administered by this route, especially subunit type vaccines, which are less immunogenic and more susceptible to degradation than, for example, whole-cell killed or live attenuated vaccines. The development of oral vaccine delivery systems has been made possible by the emergence of adjuvant and vaccine technologies, that harness the mucosal immune system, along with state-of-the-art micro- and nanotechnology. Combining these approaches may help overcome the challenges of oral vaccination and could be a vital asset in the development of next-generation oral vaccines.

## 1.1 Hypothesis and aims

In earlier research, oral administration of a vaccine composed of the model antigen ovalbumin, formulated with cubosomes and the adjuvant Quil-A to increase immunogenicity, was accomplished using drug delivery systems known as microcontainers (MCs). However, this formulation did not succeed in establishing strong mucosal immunity.

Therefore, it was hypothesized that a compositional change in the vaccine could enable an effective mucosal immune system stimulation while still using the MCs to target the intestine for release and absorption.

To support this hypothesis, the main goal of this study was to evaluate the potential of MCs as an oral delivery method by combining the microdevices with potent mucosal adjuvants and antigens. Following oral administration of the selected vaccine components to several animal models in polymer-coated MCs, experimental evaluations of the MCs' potential to enhance the mucosal immune response were conducted. The project was divided into three parts with the following research objectives:

I) To screen mucosal adjuvants for formulation with the *Chlamydia trachomatis (C. trachomatis)* vaccine candidate CTH522, to be orally delivered in MCs to mice

II) To test MCs' ability to orally deliver the capsid virus-like particle (cVLP) AP205, a generic platform for presenting antigens, in rats

III) To realize MCs as an oral administration tool for European sea bass and test their ability to deliver the red grouper nervous necrosis virus (RGNNV) VLP

## 1.2 Outline of the thesis

The main results obtained from this thesis are presented through three research projects (**Projects I–III**), as illustrated in the overview (Fig. 1). Two of these projects have been written into manuscripts (**Projects I** and **II**).

The following background section introduces the relevant physiology and mucosal immunology of the gastrointestinal (GI) tract. It briefly explains the theory behind vaccines, focusing on mucosal vaccines and the types used in this study. This section will be followed by a description of adjuvants and information on the technologies used in **Project I**. Moreover, the challenges for oral vaccination will be highlighted, followed by a description of the technology utilized for oral delivery of vaccines in this thesis. In this section, dry powder lyophilization will also be covered in this section as a method of vaccine production.

In the “Outcomes and Discussion" section, the major conclusions of Projects I and II will be briefly summarized, followed by a discussion of the experimental considerations and points for future approaches for each project. The two manuscripts based on the findings from Projects I and II are included in the appendix section. Results and discussion points already included in these manuscripts will not be addressed in the Outcomes and Discussion section. **Project III**’s justification, methods, and findings will be presented and discussed as it was not included as an manuscript.

Additional considerations and results will be presented in the Outcomes and Discussion section. This includes a review of the potential of MCs to deliver vaccines orally, which is examined from a global perspective. The use of animal models while examining microdevices as a delivery system is discussed in the section that follows, based on the technical and methodological approaches and complications from the projects. Moreover, the development and characterization of an *in vitro* M-like-cell model to evaluate the release of vaccine formulations from the MCs will be described, along with the results from a small pilot study, investigating the visualization and kinetics of MCs and a new delivery technology named “Foils” in rabbits.

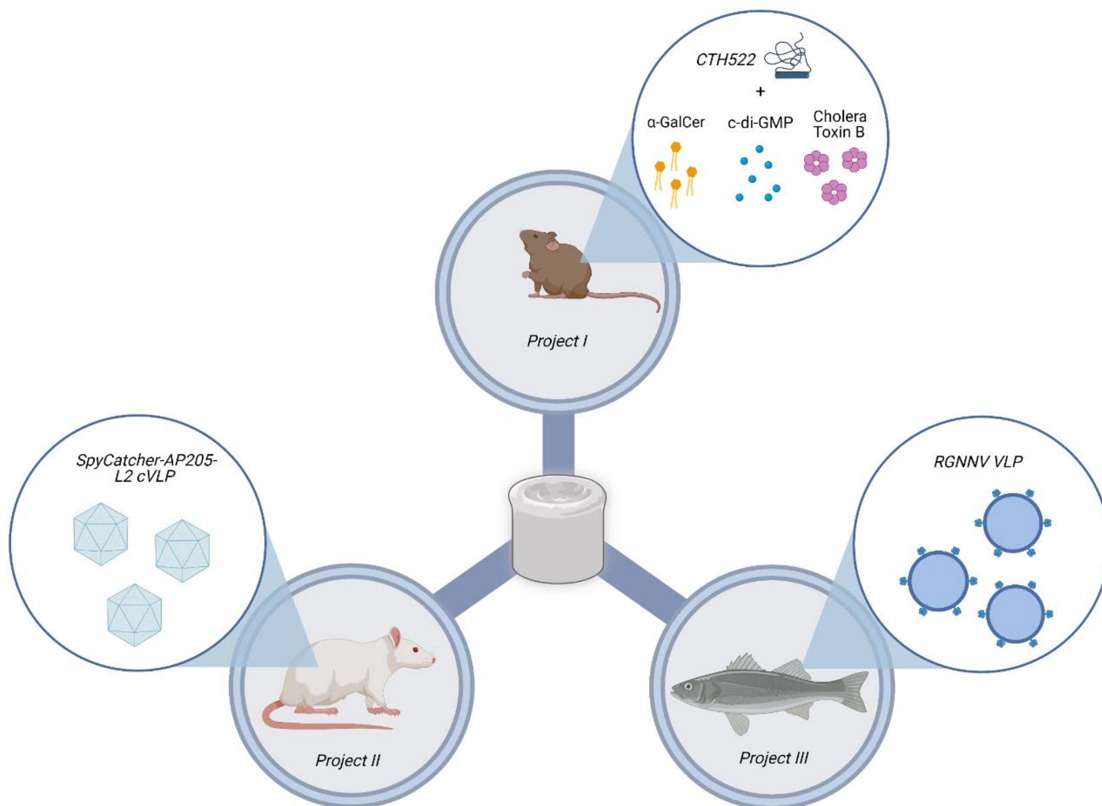

**Fig. 1.** An overview of the main projects presented in this thesis, revolving around the use of microcontainers (MCs) for oral vaccine delivery. In **Project I**, the *C. Trachomatis* antigen, CTH522, was screened with the adjuvants α-GalCer, c-di-GMP, and cholera toxin B, and delivered orally in MCs to mice. In **Project II**, rats were orally immunized with the capsid VLP (cVLP) SpyCatcher-AP205-L2 loaded into MCs. In **Project III**, MCs were trialed in the European sea bass, and the red grouper nervous necrosis (RGNNV) virus-like particle (VLP) was orally administered in MCs. *Created with Biorender.com*

# 2. Background

## 2.1 Physiology and mucosal immunology

The development and study of mucosal vaccines is rapidly expanding as mucosa-infecting pathogens/mucosal infectious pathogens continue to pose a global threat.[6] The mucosal tissue in the body acts as a frontier, serving as both a wall and a door for pathogens. The main goal of the mucosal vaccines is to strengthen the wall by developing an interplay between the physical barrier and the underlying immunological mechanisms.[6,7] The immune-related sites of the mucosal tissue are defined as mucosal-associated-lymphoid tissue (MALT), which can be subdivided into anatomical regions. One of these regions is the gut-associated lymphoid-tissue (GALT), which includes the immunological activities of the GI tract, the primary region of interest with regard to oral vaccination.[8]

### 2.1.1 The gastrointestinal tact

The GI tract is part of the digestive system that functions as a semipermeable barrier to absorb nutrients and excrete waste products for maintaining body homeostasis. The stomach, small intestine, large intestine, rectum, and mouth make up the human GI tract (Fig. 2.).[9] Peristalsis, which starts as a result of brain signals after swallowing, is the physical driving force of digestion. Peristaltic movements—wave-like movement of the muscles that push contents forward—are found in each of the GI tract's internal organs and are an essential component of the system.[9,10] After consuming food, the first round of digestion takes place in the stomach. There are proteolytic enzymes that may break down large and complex protein structures into molecules in this region, which also contains acids with a pH range of 1 to 6, depending on whether the stomach is fed or fasting. The molecules will then enter the small intestine, the primary region of absorption, after being expelled from the stomach. The environment in the small intestine is radically different, with fluids in the more neutral (pH 5–7) range and other symbiotic bacterial species known as the microbiota.[9,11]

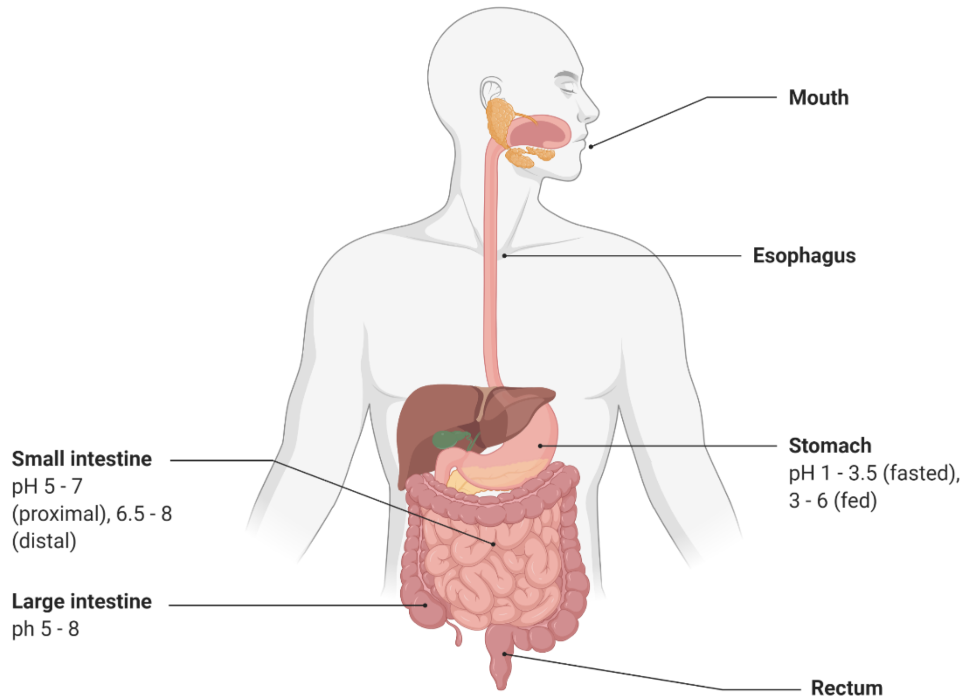


**Fig. 2.** The human digestive system, with the organs of the gastrointestinal tract highlighted, along with the pH values of the stomach and small and large intestines. *Created with Biorender.com*

In an adult person, the small intestine's surface area is estimated to be ~30 $m^2$, which aids in the absorption of molecules and nutrients.[12] The surface folds into plicae containing crypts and villi structures, and the majority of the cells are absorptive epithelial cells that project their distinctive microvilli toward the intestinal lumen.[12] Enterocytes, which are epithelial cells, can absorb molecules either through intracellular transportation or paracellular absorption (Fig. 3). Several pathogens can use these transportation pathways to infect the host through the mucosal tissues. The effective vaccination of these sites could prevent this invasion. Another type of cells found on the intestinal surface is goblet cells that secrete a covering protective layer known as mucus.[12,13]

#### *2.1.1.1 Mucus and the mucosal tissue of the intestine*

Mucus consists mainly of water and mucins, a glycoprotein type that forms a complex hydrogel-like structure.[14] The GI tract is covered with mucus, which varies in function and thickness depending on the location. For instance, the mucus in the stomach serves to shield the underlying epithelial tissue from the acidic environment. In contrast, intestinal mucus acts as a filter to aid the absorption of "correct" molecules.[14] This filtering trait of mucus results from its dual function as an interactive barrier—which can bind molecules via electrostatic interactions—a steric barrier, which filters molecules and particles down to the nm size owing to the density of the mucin fibers. Lastly, mucus is also a dynamic barrier, constantly being secreted and shed, carrying away any

waste or compounds embedded in it.[14,15] Many attempts have been undertaken in the pharmaceutical and vaccination fields to make use of the physical and chemical characteristics of mucus in order to efficiently deliver medications and vaccines at mucosal sites in the body.[16]

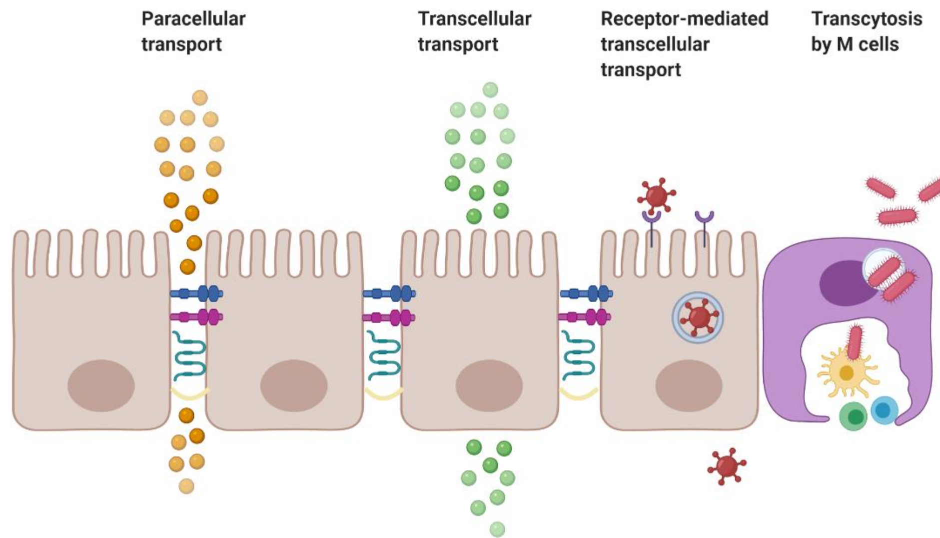


**Fig. 3.** Simplified illustration of the different transportation pathways of the enterocytes in the gut. Paracellular transport, a mechanism where molecules are transported in between the cells through the junctions. Transcellular transport, where selected molecules are transported into the enterocytes and secreted on the basolateral side for absorption. Receptor-mediated transcellular transport, a mechanism that can be utilized by some pathogens to enter the host. The same pathway is also used for the secretion of secretory IgA from the lamina propria into the lumen. Furthermore, microfold (M) cells are also depicted, displaying their unique ability to transport larger particles and organisms by transcytosis. M cells are a vital part of the mucosal immune system and a tool to absorb and present antigens, for instance, from vaccines. *Created with Biorender.com*

The mucosal tissues in the intestines also serve as a natural habitat of the microbiota, which mainly consists of foreign bacteria benefiting from the environment and aiding in digestion.[11,17] However, the mucosal tissue is also susceptible to many foreign infectious pathogens. It is therefore paramount that the GALT is be able to distinguish between pathogenic and nonpathogenic organisms or molecules in addition to providing protective immune responses when required. A tool used for this is the maintenance of immune homeostasis, which includes processes capable of protecting the body while simultaneously inducing tolerogenic reactions to food, commensals, and self-antigens.[13,18,19] Inappropriate responses against such molecules can lead to inflammatory disorders such as celiac disease and inflammatory bowel disease. These immunological processes are governed by a branch of the immune system termed the mucosal immune system.

### 2.1.2 The mucosal immune system of the gastrointestinal tract

The GALT is one of the largest immunologic tissues in the body and has a distinctive architecture that separates inductor and effector sites to limit and control immune responses.[18,20] Briefly, the antigen is sampled at the inductor sites and presented by antigen-presenting cells (APCs) for the maturation and migration of immune cells to the effector sites, where their immune specific role is conducted.[19] APCs and other immune cells of the innate immune system function by recognizing and binding molecules on the surfaces of pathogens, apoptotic host cells, and damaged cells via pattern recognition receptors (PRRs). Upon activation, these receptors trigger immune-regulated responses against infections, antitumor responses, or tolerogenic responses.[21]

Various families of PRRs have been identified, with Toll-like receptors (TLRs) being one of the most well-known and earliest discovered receptors. Since then, numerous other PRR families have been identified, with C-type lectin receptors (CLR), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding domain, leucine-rich repeat-containing protein receptors (NLRs), along with absent in melanoma (AIM)-like receptors (ALRs), being some of the main families.[22] PRRs recognize pathogen-associated molecular patterns (PAMPs), which are specific and conserved molecular structures in pathogenic organisms. These include proteins, lipids, and nucleic acids such as foreign DNA, lipopolysaccharides (LPS), and membrane proteins.[21,22] Innate immune cells make advantage of the unique molecular characteristics of PAMPs to discriminate between self- and foreign molecules by means of PRRs. Once a ligand is recognized by a receptor, a series of downstream signaling pathways will be initiated, which in turn will activate different branches of the immune system, depending on the receptor and the ligand.[21,22] Information and response from the innate immune cells are essential for stimulating an adaptive immune response. An example is TLR4's recognition of the bacterial component of gram-negative bacteria LPS. Myeloid cells such as granulocytes and macrophages express TLR4, and ligand recognition can signal activation of the transcription factor nuclear factor-κB (NFκB). This results in the production of inflammatory cytokines, which will enable and influence the adaptive immune response.[23] APCs are present in large quantities at the GALT's inductive sites because they are a crucial component in maintaining and regulating the immunological mechanisms.

#### *2.1.2.1 Peyer's patches and antigen sampling*

The inductive sites include Peyer's patches (PPs), a type of organized lymphoid clusters or follicles.[24] PPs are typically found in the distal ileum of the small intestine in humans and harbor an abundance of immune cells. Here antigens are sampled and presented through various means. One of these is by a population of APCs, which includes both macrophages and dendritic

cells (DCs) and expresses the chemokine receptor CX3CR1$^+$. These immune cells have a unique morphology trait that enables them to extend protrusions into the lumen to catch pathogens (Fig. 4). A study by *Mazzini et al.* investigated the CX3CR1$^+$ cell population and found macrophages to be the main driver of antigen sampling.[25] Once the antigen had been sampled, the macrophages would transport it to CD103$^+$ DCs using a gap junction–dependent mechanism, to be presented to T cells in the draining lymph nodes.[25] Other possible methods for antigen sampling include the capture of virally infected epithelial cells, the passage of small soluble materials through goblet cells, or the sample of translocated IgA immune complexes.[26] Furthermore, the PPs are covered by a membrane of cells known as the follicle-associated epithelium (FAE), which has a unique epithelial cell type called microfold (M) cells that can transport antigens into the *lamina propria* (LP) (Fig. 4.).[24] Similar to APCs, M cells recognize luminal antigens by surface receptors and have the special ability to actively engulf and transport these from the apical surface of the lumen to the basolateral LP by transcytosis. Compared to normal enterocytes, M cells are able to transport big particles and can uptake particles between the sizes of 50 nm and 10 µm.[24,27–29] Additionally, M cells contain specific transcellular pores through which dendrites can be extended to the sample antigen.[18,24,26]

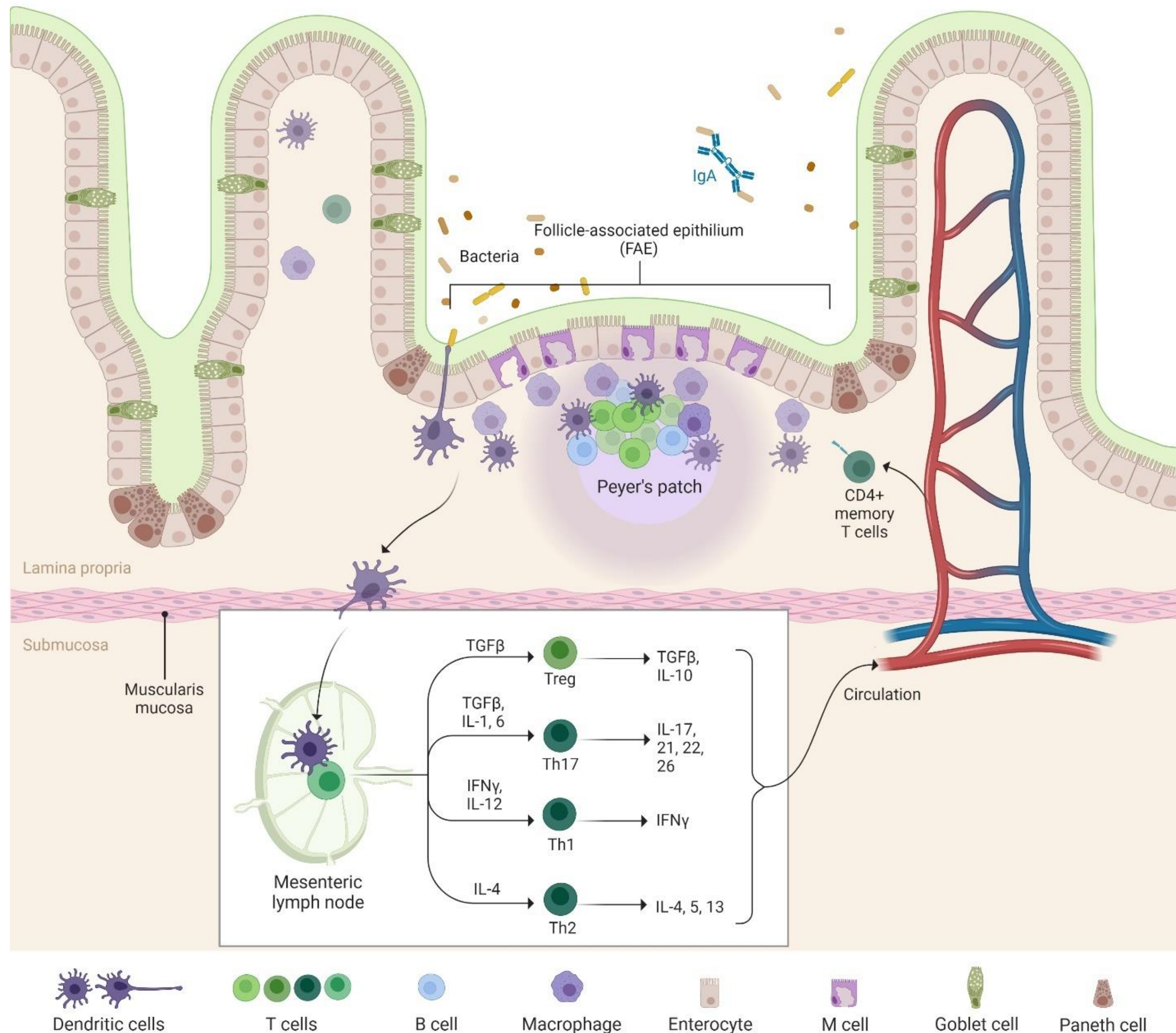


**Fig. 4.** Immunological processes that govern the B-, Th1-, T17-, and $T_{reg}$-cell responses in the gut lymphoid-associated epithelium (GALT) in the small intestine. Antigens can be taken up by macrophages or dendritic cells by transepithelial protrusions or transported by M cells to be presented to naïve T and B cells which constitute the Peyer's patches. Depending on the antigen, a variety of T-cell populations can be induced and sent into circulation to suppress, maintain, or enhance the immune response. This is mainly regulated by the secretion of cytokines, which also impact several functions in maintaining the epithelium. Activated B cells will proliferate and differentiate to produce antibodies, such as IgA, which can be secreted into the lumen, becoming sIgA, to bind and prevent infectious pathogens. *Created with Biorender.com*

### *2.1.2.2 T-cell responses in the intestine*

The PPs contain T-cell zones wherein antigens endocytosed by DCs are presented to naïve T cells, promoting DC maturation and T-cell activation (Fig. 4). T cells primed in the intestinal mucosal tissue rapidly migrate to mesenteric lymph nodes, where they differentiate into effector or memory T cells.[30] Interferon-γ (IFN-γ) is one of the dominant cytokines secreted upon mucosal

infection of the gut.[30,31] IFN-γ has been associated with Th1 responses, essential for developing an effective host defense against intracellular viral and bacterial pathogens.[32] Studies on the function of intestinal and colonic IFN-γ have reported that it is associated with processes such as proliferation and apoptosis of epithelial membrane cells along with promoting secretion of IL-17 and CXCL10 for expansion of Th1 and Th17 cells. Furthermore, it has been proposed that IFN-γ plays an essential role in regulating the microbiota and level of antimicrobial peptides.[33] Concerning intestinal infection, an earlier study investigating *Salmonella typhimurium* (*S. typhimurium*) infection in mice revealed that IFN-$\gamma^{-/-}$ animals exhibited an impaired immune response, whereas wild-type mice displayed an increase in $CD4^+$ and $CD8^+$ T cells.[34] However, there is little evidence available on the precise function of IFN-γ in intestinal infection. Studies on chlamydia infections in other mucosal tissues, such as the genital mucosa, have revealed that Th1 responses—which are strongly regulated by IFN-γ—play a prominent role in clearing genital infection.[35–37] However, other studies have contradicted these assumptions by stating that tumor necrosis factor (TNF-α) is the main cytokine in chlamydia infection or that IFN-γ signaling is significant, but Th1 cells are not necessary for clearance of the disease.[38,39] Nevertheless, these examples suggest that IFN-γ plays a vital role in the establishment of protective responses against mucosa-infecting pathogens.

Th17 cells are another type of T cell present in the GALT. Since the discovery of the cytokine interleukin (IL) 17, extensive research has been conducted to determine the precise role of cells secreting this cytokine, such as Th17 cells.[40,41] Studies on infections have demonstrated that antigen-stimulated $CD4^+$ cells enhance the production of Th17 cells, which are thereafter detected in high quantities in the LP.[42,43] Their presence is observed in all segments of the intestine strategically, indicating their importance in protecting the mucosal tissue.[43,44] Th17 cells have been associated with the promotion of tight junction formation, mucus production, antimicrobial peptide production, IgA production, and epithelial regeneration.[42–46] Th17 has been found to be necessary to eliminate the infection in some pathogenic intestinal bacteria, such as *Citrobacter rodentium* (*C. rodentium*).[47] This appears to be the case in other mucosal tissues as well, such as the stomach. Th17 accumulation and elevated levels of IL-17 in the stomach tissue were observed in mice in *Helicobacter pylori* (*H. pylori)* challenge trials. One of the first cytokines to be identified after infection was IL-17, which was discovered to play a critical role in activating and recruiting neutrophils for barrier function maintenance.[48]

Another crucial T-cell population of the GALT is the regulatory T cells ($T_{regs}$), which are particularly significant because they are vital for maintaining immunological homeostasis.[46] Their specific

marker, FoxP3, characterizes the primary subset of $T_{regs}$ localized in the intestine. $FoxP3^+$ $T_{regs}$ regulate immune reactions to infections and sustain tolerance to commensal flora, environmental, and self-antigens by limiting inflammatory responses.[46,49] An example is the immune regulation against the microbiota, which consist of a vast load of foreign bacteria. Research has indicated that a $FoxP3^+$ subset that expresses the RAR-related orphan receptor γt (RORγt) is a primary driver of mediating tolerance to the microbiota.[50,51] The microbiota, or certain metabolites related to it, such as secondary bile acids and short chain fatty acids, are necessary for RORγt T-cell activation. To sustain immune tolerance after activation, the cells release high levels of the anti-inflammatory molecules IL-10, CTLA-4, and ICOS.[50] $T_{regs}$ have been discovered to have significant roles in immune responses to intestinal pathogens, which can vary depending on the type of invading pathogen. For example, $RORγt^+$ cells were upregulated in *Giardia lamblia* (*G. lamblia*) and *Helicobacter hepaticus* infections to prevent the growth of antigen-specific Th17 cells, which hampered the protective response.[52,53] In contrast, was IL-2, an inhibitor of Th17 cell growth, absorbed by $T_{regs}$ in the event of infection with *C. rodentium* or *Candida albicans*, thereby supporting protective Th17 responses.[54,55] Although $T_{regs}$ are crucial for immune and GI tract functions, the tolerance they mediate makes oral vaccination difficult.[56,57] This is explained in more detail in section 2.2.3.

#### *2.1.2.3 Immunoglobulin A generation and function*

The activation of T cells, often referred to as the cellular response, can stimulate the priming of B cells, which in turn mediate the humoral response by secreting antibodies, when the antigen is delivered to naive lymphocytes. Antigen-primed B cells will cluster in germinal centers (GCs) or migrate to GCs in peripheral lymph nodes, where they differentiate and undergo affinity maturation to become plasma cells.[56] In the LP, the primary type of antibody is immunoglobulin (Ig) A, which is secreted by $IgA^+$ plasma cells.[56] In order to regulate and mediate the differentiation of plasma cells to secrete polymeric IgA (pIgA), activated $CD4^+$ T helper cells secrete cytokines IL-2, IL-5, and IL-10 (Fig. 5). pIgA is transported across the epithelial barrier by recognizing the polymeric immunoglobulin receptor (pIgR), expressed on the basolateral surface of the epithelial cells. Following proteolytic cleavage of pIgR, pIgAs are secreted and released into the luminal region. Secretory immunoglobulin A (sIgA) refers to the pIgA molecule attached to the cleaved, extracellular region of pIgR. sIgA is typically secreted as a dimer, which enhances the binding capacity and stability of the antibody.[56,58,59] Polymeric IgA, either in the lumen or LP, has three main functions: (1) To bind to antigens or pathogens in the lumen, preventing them from infecting the epithelial cells. (2) To bind to antigens already present in LP and target them for transport out into the lumen using the pIgR. (3) To bind to the pIgR of an infected cell, target the antigen within

the cell and transport it back into the lumen.[58,59] Additionally, monomeric IgA can be produced and transported into the blood vessels underlying the PPs.[60] Although IgA is the main isotype in the mucosal tissue, IgG is also present and may support the systemic and local adaptive immune defenses in the gut.[60,61]

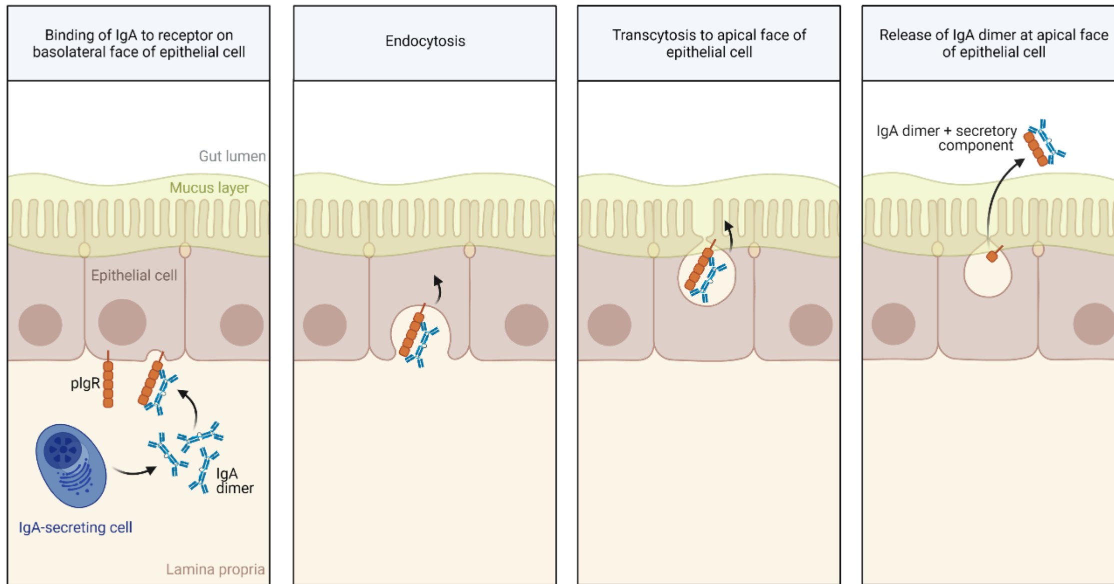


**Fig. 5.** Secretion of IgA into the gut lumen by binding to the pIgR molecule on enterocytes enables endocytosis, followed by transcytosis. IgA is secreted into the lumen bound to the secretory component of pIgR and is now characterized as sIgA. *Created with Biorender.com*

#### *2.1.2.4 The animal models and scalability*

These immunological functions are defined in terms of their occurrence in the human gut. Prior to human clinical trials, however, research is undertaken on novel vaccine candidates using animal models, which is a crucial phase in the process. Initial immunization studies of a vaccine candidate typically involve rodent models, such as mice and rats, with the inclusion of larger animals, such as ferrets, pigs, and nonhuman primates, at a later stage in the development. Mice and rats are used in **Projects I** and **II**, respectively. Both of these models have been extensively used in vaccine research, with mice being the most commonly used. This has led to the development of various strains of mice and rats by genetic modification, which can provide vital information on the immunological function of a vaccine component or make the animal susceptible to certain diseases for challenge studies. For example, transgenic mice have been developed to express the poliovirus receptor, CD155, rendering them susceptible to polio and, by extension, polio vaccine research.[62] Due to the similarities between the mouse GALT and the human GALT63, much of what is known about the immunological responses of the GALT has been acquired from

mice.[63] Similar types of immune cells are present in the intestines of mice, although their numbers vary. For example, mice generally have a higher M-cell frequency than humans. Several variations in the differentiation of certain $CD4^+$ T-cell subsets have also been reported. For instance, murine Th17 differentiation is induced by IL-6 and TGFβ in mice, whereas this type of signaling is insufficient for Th17 differentiation in humans. However, Th17 can be induced in humans using IL-1β in conjunction with IL-23 or IL-6. In terms of humoral responses and origin of IgA, the subset of B cells present in the germinal centers of the GALT reveals the most significant variations. However, there are many similarities between the class-switching and function of the $IgA^+$ plasma cells in mice and humans. Gibbons *et al.*[63] provides a thorough analysis of these differences. Rats appear to share numerous immunological traits with humans, as evidenced by their ability to manufacture IgA, promote Th1 and Th17 cell proliferation, and the presence of M cells.[64,65] However, there is currently no information available on the fundamental distinctions between rats and mice or between rats and humans. Nevertheless, the rodents serve as well-established and feasible animal models for the research of oral vaccines.

In **Project III**, the fish species, European sea bass (*Dicentrarchus labrax*), is the target animal for the developed vaccine investigated. The mucosal tissues of fish and mammals have many similar characteristics. The mucosal tissues of fish also serve as a site of absorption and defense against infections. In terms of mucosal immunology, the MALT of fish can also be subdivided according to the anatomical location, and fish thus harbor a GALT site.[66] The main difference is that PPs are not generated in fish, but the GALT does harbor many of the same cell populations observed in higher vertebrates, such as DCs, T cells (Th1, Th17, $T_{regs}$), B cells, gut-associated macrophages, and specialized antigen-sampling (M-like) cells, which, similar to mammals, make the intestine a noteworthy target for oral vaccination.[67] The adaptive immune response of fish, which only has three Ig subtypes (IgM, IgD, and IgT) and does not experience B-cell class switching, is another significant difference in evoking protective immunity.[66,68] IgM is the primary antibody of the circulatory system, whereas IgT is assumed to dominate on the mucosal surfaces. Both have been identified in sea bass.[69,70] IgM is commonly assessed as a reaction to immunization and/or exposure to the pathogen.[71,72] **Project III** focuses on viral nervous necrosis (VNN), a condition that can be brought on by the betanodavirus RGNNV. Studies have indicated that most viral infections in fish require a systemic response to ensure complete protection against the pathogen.[73] When infected with a viral pathogen, the host will respond by activating both innate and adaptive immune mechanisms. It was observed that sea bass infected with betanodavirus were capable of establishing an adaptive immune defense, deeming it possible to develop potential vaccines against the disease. It was documented that IFN responses were

upregulated along with activation of IFN-stimulated genes, such as myxovirus resistance proteins, which are antiviral proteins capable of inhibiting viruses by various mechanisms depending on the host and pathogen.[74] In infection studies with RGNNV in sea bass, circulatory IgM and $CD4^+$ T cells in the serum were detected, suggesting these to be vital in establishing protection against this virus.[74,75] Although weaker compared to intraperitoneal (i.p.) administration, studies have demonstrated that oral immunization of fish can indeed stimulate a systemic response, among others, as seen in the papers by Wi *et al.* and Gonzalez-Silvera *et al.*[76,77,73]

The underlying physiological mechanics and intestinal immune processes of the GI tract are vast and complex. Understanding these mechanisms is key in establishing protection against infectious pathogens in this region. Gut immunity constitutes both the secretion of antibodies and the proliferation of tissue-resident T cells. Together they represent a potent tool for disease prevention, which in principle could be harnessed by vaccines.

## 2.2 Vaccines

Vaccines continue to be one of the most efficient ways to prevent infectious diseases. Because of their enormous effectiveness, thousands of research studies and clinical trials have been conducted to modify, develop, and optimize vaccines. This has led to the development of different vaccine technologies and designs over time, a repertory that is still expanding. Some of the first generation of vaccines, which were based on whole bacteria or viruses, remain the most effective vaccines to date, particularly oral vaccines.[1,78] The techniques for attenuating or inactivating pathogens are not always practical, despite their effectiveness. Modern vaccine strategies are now centered on using certain components, or "subunits," from pathogens, such as specific peptides and proteins. Due to the lack of PAMPs, which inactivated and attenuated whole-cell vaccine types benefit from, subunit vaccination technology is often less immunogenic despite being highly adaptable and generally deemed to have an excellent safety profile.[79]

### 2.2.1 Subunit vaccines

The term “subunit” in vaccinology broadly refers to any component of a pathogen used as a vaccine candidate. This definition naturally includes a wide range of technologies and vaccine opportunities, many of which have already been developed.[79] Recombinant protein antigens have been used in the current work and will therefore be the main topic of discussion. It’s important to note that other technological platforms, including DNA and mRNA-based vaccines, also fall under the category of subunit-type vaccines because they only carry the genetic code of a small subset of antigens, such as the coronavirus spike protein.

The development of recombinant vaccine types, which contain massive potential and are already used extensively in research, was made possible by recombinant genetic engineering and DNA technology. These are produced using various expression systems, such as bacteria like *Escherichia coli (E. coli*) or yeast strains like *Saccharomyces cerevisiae (S. cerevisiae)* and *Pichia pastoris (P. pastoris*), which allow for the production of large-scale quantities of recombinant proteins.[80–82] Recombinant vaccine design has become much more reasonable due to knowledge of pathogen biology, which proteins are conserved and implicated in pathogenesis, and which specific immune responses need to be generated for successful protection. To enter the host through recognizing receptors in the biological barriers of the host (i.e., mucosal tissues), many pathogens, for instance, are equipped with surface or membrane proteins.[83] Disease prevention has frequently been using these membrane proteins as vaccine targets. The *C. trachomatis* vaccine candidate CTH522, the antigen used in **Project I,** is based on a membrane-bound surface protein.

#### 2.2.1.1 The *Chlamydia trachomatis* antigen—CTH522

One of the most common sexually transmitted diseases (STDs) is *C. trachomatis*, a gram-negative bacteria that causes mucosal infection.[84] Currently, *C. trachomatis* is classified into 19 serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2, L2a, and L3) that are defined according to specific epitopes on the major outer membrane protein (MOMP).[85] Natural immunity can be gained after infection, but it is serovar-specific and short-lived, leaving people vulnerable to immediate reinfection.[86] Following infection, the bacteria undergo a distinctive life cycle that encourages immune system evasion.[87] Although the immune evasion of *C. trachomatis* is still not fully understood, significant fieldwork has helped to identify some of the evading mechanisms. In its life cycle, *C. trachomatis* invades host cells as an elemental body (EB) and differentiates into a reticular body (RB) once in the host cells.[87] The main differences of the two bodies are that the EB facilitates infection whereas RBs are metabolically and reproductively active, facilitating replication. The RBs secrete inclusion bodies that act to inhibit cellular defense mechanisms, here among alteration of the PRRs TLR2, NLR1 and stimulator of interferon genes (STING), which are specifically involved in recognition of *C. trachomatis.*[87] This prevents inflammatory and immunological responses by inhibiting the synthesis of protective cytokines. Chlamydial protease-like activity factor (CPAF), a substance released by *C. trachomatis* after infection, has been demonstrated to inhibit the synthesis of pro-inflammatory cytokines and NFκB pathway subunits, hence preventing the expression of innate immunity genes.[88–90] These immune evasion traits make removing the bacteria from the host nearly impossible.

Antibiotic therapy can control *C. trachomatis*, but the prevalence suggests that a potent vaccination is necessary for complete disease control. *C. trachomatis* has been extensively researched as a potential vaccine candidate after studies with MOMP of *C. trachomatis* showed that it is highly immunogenic in both humans and animals.[91–94] A novel antigen construct based on the MOMP sequence was developed at the Statens Serum Institute. However, full-size MOMP is reportedly not to be a feasible antigen, as *in vivo* immunization studies yielded conflicting data about the establishment of protection.[92] Therefore, the constructs were based on the MOMP sequence's variable domains, which were found to be abundant in neutralizing target epitopes. In particular, variable domain 4 (VD4) was of interest because it has a conserved species-specific epitope that can trigger neutralizing responses to various serovars. Olsen *et al.* conducted a comparative research study in which different VD4-based constructs were tested by immunization in mice, indicating CTH522 to be the most promising construct. This construct consisted of a recombinant MOMP (rMOMP) immunogen sequence derived from *C. tracho*matis serovar D, fused with the variable domain 4 (VD4) regions from four different *C. trachomatis* serovars (D, E, F, and G).[91] The resulting construct benefits from the VD4 neutralizing epitopes and several T-cell epitopes in the rMOMP sequence. In a recent clinical phase I investigation, it was discovered that CTH522 induces the production ofneutralizing systemic and genital IgG and IgA antibodies in humans.[93]

In addition to having the ability to subvert the immune system, *C. trachomatis* has been seen to reinfect women who have received treatment for genital infections.[95] This was linked to *C. Trachomatis* residing in the GI tract, which renders a natural habitat for the bacteria.[96] The intracellular life cycle of *C. trachomatis* contributes significantly to the innate immune system downregulation and reduced competitiveness with other bacteria in this region.[95–97] Autoinoculation from the GI tract can result in continued reinfection unless cleared or prevented, which is challenging to accomplish with antibiotics. It may be possible to overcome this issue by using oral vaccination with a prominent antigen, such as CTH522, to create immune protection against *C. trachomatis* in the GI tract.

Membrane proteins such as CTH522 generally have low immunogenicity as they lack PAMPs. However, recombinant technologies have made it possible to use the effect of PAMPs and the pathogenic morphology while maintaining safety. A method to do this is by isolating the capsid protein responsible for the structure of the pathogens and using these as vaccine candidates. The virus-like particles that comprise this subunit technology are used in **Projects II** and **III**.

#### 2.2.1.2 Virus-like particles

The structural resemblance of a live virus can make VLPs efficient in activating the innate immune system, which can facilitate strong adaptive immune responses. The size of VLPs allows draining into the lymph nodes and permits absorption by APCs. APCs can recognize the highly repetitive surface of the VLPs, which provides a display of closely spaced epitopes mimicking the PAMPs of live viruses. Compared to soluble antigens, the VLPs' size and particulate structure make them easier for the DCs to absorb. This "virus-like" epitope display is most frequently detected by PRRs on DCs.[98] Effector B and T cells then multiply as a result of the PAMP recognition and uptake of VLPs, which triggers DC maturation. Furthermore, the multimeric epitopes on the surface of VLPs, which can facilitate cross-linking to the B-cell receptors, can directly excite B cells. VLPs can stimulate both humoral and cellular responses because this link has the potential to be potent enough to activate the B cells to produce antibodies.[99–101] VLPs are ideal for usage as vaccines and vaccination platforms due to their characteristics and the decrease in safety concerns. Many VLP-based technologies are in pre-clinical and clinical development, and current licensed VLP vaccines include the two vaccines against human papillomavirus (HPV), Cervarix and Gardasil.[102]

VLPs have the potential to be used in oral vaccination because of their ability to trigger mucosal immune responses.[103–106] One example is the study by *Serradell et al.* where VLPs were equipped with surface proteins from *G. lamblia*, which has the ability to grow in the upper part of the small intestine.[103] The exact mechanism of how *G. lamblia* survives is unknown, although the bacteria are covered with variant-specific surface proteins (VSPs), which are a part of the bacteria's immune suppression and avoidance strategy. In the study, a retrovirus-derived VLP was equipped with VSPs in addition to hemagglutinin (HA) from Influenza as a model antigen. Oral immunization of mice against Influenza and HA-expressing tumors resulted in a protective immune response, which was not observed with VLPs lacking VSPs. In another recent study, Zhai et al. studied the use of MS2 bacteriophage VLP to defend against human papillomavirus (HPV).[107] L2, the minor capsid protein of HPV, is highly conserved among HPV variations and is engaged in important events in the virus life cycle, including facilitating encapsulation, promoting binding to epithelial cells and trafficking of the viral DNA.[108] Therefore, L2 has been proposed as a prominent antigen candidate for the development of a broadly protective HPV vaccine. The vaccine formulation in the study comprised two different MS2 VLPs, one equipped with conserved L2 epitopes from HPV 31 and 16 and another displaying a consensus L2 epitope derived from an alignment of different HPV variants. Mice orally immunized with a mix of the two MS2 VLPs were found to be protected against both oral and genital infection from 11 HPV types.[107]

## The AP205 VLP and the SpyTag/Catcher system

VLPs are platforms for the presentation of heterologous antigens in the two research listed above. This approach can adopt the immunogenicity of the underlying VLP to the antigen by this repetitive virus-like presentation. Numerous platforms and methods for conjugating antigens onto capsid scaffolds, such as genetic fusion and chemical conjugation, each with advantages and disadvantages, have been established.[109–113] The AP205 capsid VLP (cVLP), developed from the *Acinetobacter* phage's capsid protein and used in **Project II**, is a potential platform for the general display of antigens. The AP205 self-assembles into cVLPs when expressed in *E. Coli,* and quality controls and trial-and-error have shown it to be highly stable and modifiable.[114,115] The AP205 cVLP utilizes a split-protein conjugation system called the SpyTag/SpyCatcher system (Fig. 6). This system is comprised of a peptide (SpyTag) and a protein (SpyCatcher) derived from the fibronectin-binding protein FbaB of *Streptococcus pyogenes*. These counterparts will recognize and interact to reform when in solution by spontaneously forming an isopeptide bond.[116–118] Recently, this technique has been used as a platform for the COVID-19 spike protein.[119] In **Project II**, the AP205 cVLP, expressing the SpyCatcher protein and the HPV 16 antigen peptide L2 as model antigens, is used to investigate the platform's potential for inducing mucosal immunity when taken orally.

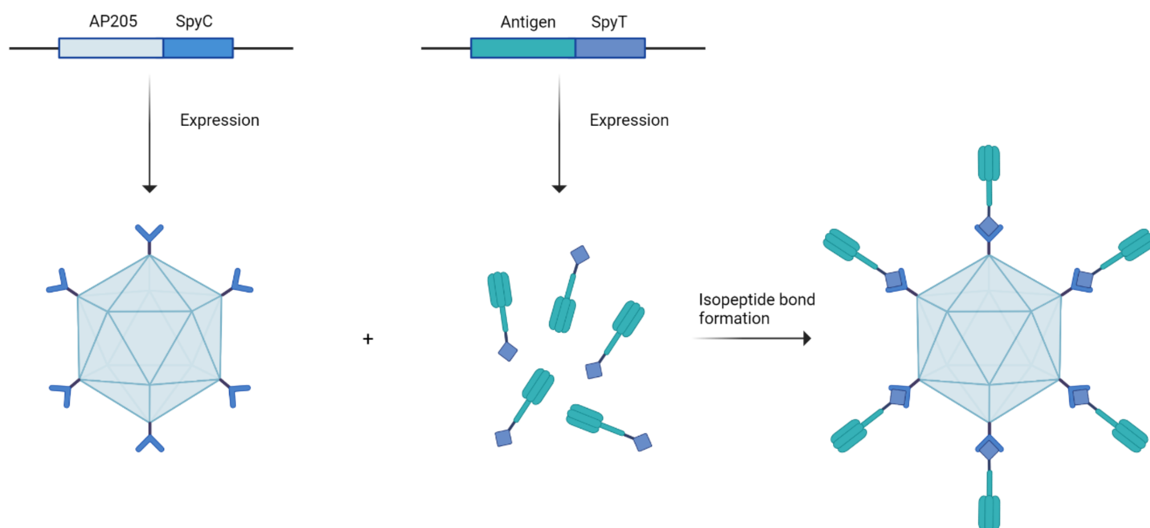


**Fig. 6.** Schematic of the SpyTag (SpyT) and SpyCatcher (SpyC) conjugation system on the AP205 cVLP. SpyC is genetically fused to the AP205 cVLP and recombinantly expressed. Similarly, it is an antigen of interest fused to SpyT and expressed. When mixed in solution, the SpyC/SpyT counterparts will localize and reform the isopeptide bond, resulting in the display of the antigen on the cVLP. *Created with Biorender.com, inspired by*[118].

## Red grouper nervous necrosis virus-like-particle

In **Project III,** the species in question is the European sea bass, which accounts for nearly 50% of the aquaculture production in the Mediterranean aquaculture production.[120] This species is

susceptible to viral nervous necrosis caused by RGNNV and can result in devastating production losses. Varying degrees of losses have been reported, with up to 100% mortality in hatcheries and pregrowing sites and 0.1%–60% in juvenile and adult sea bass in ongrowing sites, simulating a decrease in mortality with increasing size/age.[121,122] In the pursuit of preventive measures, the RGNNV capsid protein has been modified and recombinantly produced into VLPs as a potential vaccine. Several VNN VLPs have been produced and tested in susceptible fish species.[76,123,124] The RGNNV used in **Project III** was supplied by the w42 GmbH facility in Dortmund, Germany, and the exact sequence and structure is a patented secret. However, studies have defined the RGNNV capsid and identified numerous possible target epitope sites. Using polyclonal rabbit anti-sera, Panzarin *et al.* discovered that amino acid (AA) positions 217–256 of RGNNV contain neutralizing epitopes.[125] According to another study, AA positions 1–32, 91–162, and 181–212 of the capsid are important B-cell epitopes.[126] This was further confirmed in an immunization study, where a recombinant fragment containing AA 91–220 induced a protective immune response.[127]

The construct used in **Project III** has been extensively studied by Barsøe *et al.* determining it to induce long-lasting protection against the disease by activating both innate and adaptive immune responses.[128,129] Currently, most of the developed vaccines are injectable, which in a fish farm setting is laborious and stressful for the animals due to rigorous handling.[130] In addition, the mortality rates indicate that immunization of smaller fish is more feasible for preventing VNN. Nonetheless, injecting vaccines into small fish is impractical and, in some cases, impossible. This demonstrates the necessity for an alternative immunization approach for small fish, such as the oral route.

Although recombinant subunit antigens such as VLPs possess intrinsic immune-stimulating properties, most subunit-type vaccines require immune-boosting molecules. These substances are known as adjuvants and have slowly become an indispensable component of most modern vaccination formulations.

### 2.2.2 Adjuvants

Since the introduction of the first licensed adjuvants, numerous immune-stimulatory compounds, including aluminum salts, emulsions, PAMP molecules, and others, have been developed (Table 1). Depending on the type, these adjuvants have very diverse modes of action and effects, allowing them to modify and target specific immune responses against the antigen in question. Aluminum salts, such as aluminum potassium sulfate, are an example of one of the oldest commercial adjuvants. Glenny and colleagues observed that aluminum potassium sulfate, also

known as alum, stimulates a more powerful Th2 and antibody response than soluble toxoid.[131] Since then, aluminum salts such as aluminum hydroxide and aluminum phosphate have been added to human vaccines. Due to the efficacy and safety of aluminum salts, they have been used in vaccines against several diseases such as hepatitis A/B, HPV, tetanus, and diphtheria.[132,133] Many other types of adjuvant have been developed, such as the Adjuvant System (AS) of GlaxoSmithKline, which are meant to induce immune responses specific to target diseases. The need and modifiability of adjuvants have given rise to a significant amount of research and development in this field. In the mucosal field, however, there are currently a lack of demonstrated beneficial adjuvants. Many effective adjuvants for parental administration are not ideal for mucosal delivery, which is partly attributable to the extremely peculiar architecture of the mucosal tissues and the underlying immune mechanisms, as well as the challenges of the mucosal administration routes.[6,57] This section will present potential mucosal adjuvant types used for vaccine formulation in **Project I**, with detailed theory on the specific candidates used.

**Table 1:** Various types of adjuvants approved by the FDA, along with their immune-modulating component(s), type of delivery system, and some examples of FDA-approved vaccines they are included in.

| Adjuvant | Immunomodulator | Delivery system | Vaccines |
|---|---|---|---|
| Aluminum compounds | - | Aluminum gel/precipitate | DTaP vaccines, pneumococcal conjugate vaccines, hepatitis B vaccines |
| AS01 | MPL and QS21 | Liposomes | Shingrix (Shingles) |
| AS03 | Squalene and a-tocopherol (vit. E) | Emulsion | H5N1 Influenza |
| AS04 | MPL (TLR4 agonist) | Aluminum gel | Cervarix (HPV) |
| CpG | CpG | - | Heplisav-B (Hepatitis B) |
| LNPs | - | Lipid nanoparticle | COVID-19 (Pzifer/BioNTech, Moderna) |
| MF59 | Squalene | Emulsion | Fluad (Influenza) |
| Matrix-M | QS saponin | Lipid nanoparticle | COVID-19 (Novavax), R21/Matrix-M (Malaria) |
| Virosomes | Surface markers on virosomes (i.e., HA) | Virosomes | Nasalflu (Infuenza A+B), Invivac (Infuenza A+B), Epaxal (Influenza A + Hepatitis B) |

### *2.2.2.1 Lipid-based adjuvants*

When it comes to lipid-based adjuvants, there are currently a plethora of choices and options. Some lipids function as ligands to PRRs, directly stimulating immune system branches, whereas others are combined to create liposomes or lipid nanoparticles, which serve as carriers for antigens and adjuvants and may also possess adjuvanticity. In recent years, lipid-based adjuvants have received much attention due to their effectiveness in boosting mucosal immune responses.

#### Cationic Adjuvant Formulation 01

The Cationic Adjuvant Formulations (CAF®) are, as their name suggests, a class of cationic liposomal adjuvants.[134] Liposomes are lipid-based particles with the potential to attach proteins and molecules to the surface or absorb them, serving as a delivery system for pharmaceuticals and antigens.[134–136] Owing to their amphiphilic properties, liposomes self-assemble in aqueous solutions, with hydrophilic ends facing outward and hydrophobic ends facing each other. This enables the incorporation of hydrophobic peptides and proteins, such as the majority of antigens, in between the hydrophobic tails, whereas hydrophilic compounds, such as some small molecule medications like doxorubicin, can be integrated into the core (Fig. 7).[136,137138]

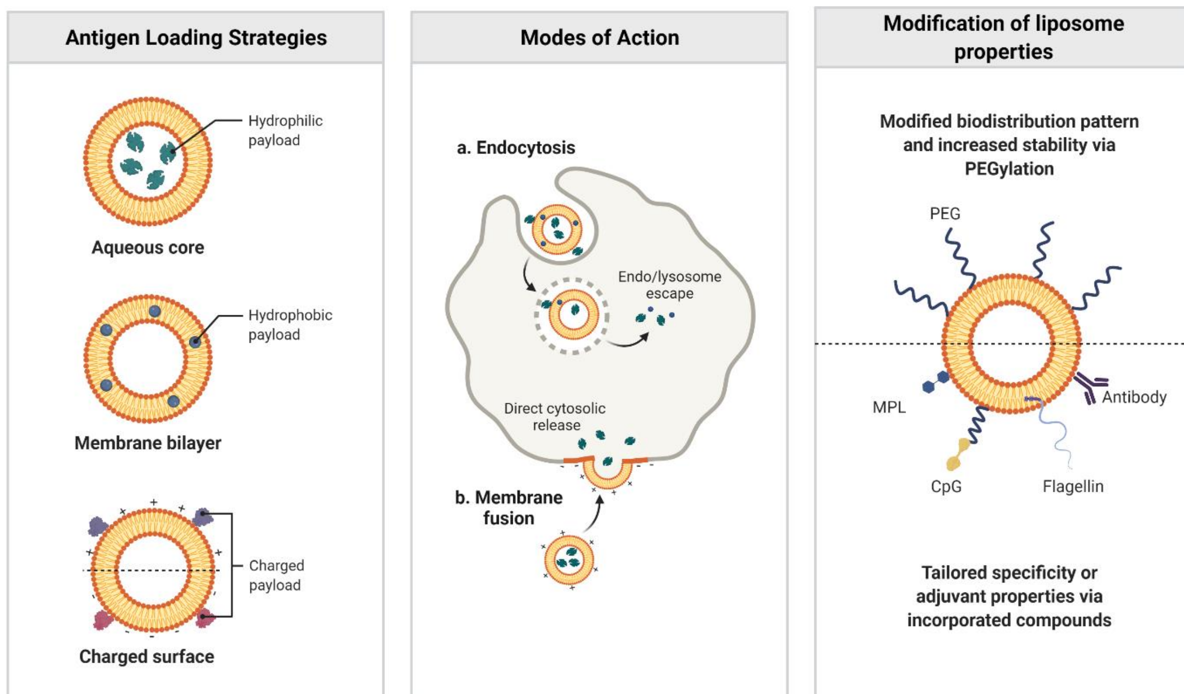


**Fig. 7.** Properties of liposomes in regard to loading strategies, where antigen can be absorbed into the core or between the bilayers, as well as attached to the surface by charge. Simplified primary modes of action of the liposomes are also depicted, where the particles can either undergo endocytosis or fuse with the membrane, depending on the type of liposome, to deliver the payload. Liposomes can be highly modifiable and allow for tailoring by, for example, PEGylation, which can affect half-life, mucoadhesion, and the biodistribution pattern.[136] Other molecules and proteins can also be

attached, such as specific antibodies, monophosphoryl lipid A (MPL), cytosine phosphoguanine (CpG), or flagellin to modify specificity and adjuvant properties. *Created with Biorender.com*

Furthermore, the surface charge of the particles can also be utilized to attach antigens; for example, in a study by Ma et al., high charge density increased the uptake of ovalbumin by liposomes.[139] From a vaccinology perspective, the fundamental principle of a liposomal adjuvant is to deliver the antigen of interest at an optimum concentration for improving antigen presentation and activating APCs.[136,137] The particulate shape of the liposomes facilitates interaction with the immune cells, which can result in an enhanced response. In addition, some liposomes, such as CAF01, are capable of retaining the antigen at the injection site, which enhances uptake by migrating DCs. In addition, the properties of liposomes are highly adaptable, allowing for the size, charge, lamellarity, and attached molecules to be altered to meet the requirements of a vaccine. Liposomes, such as the CAF adjuvants, have demonstrated potential for inducing mucosal immune responses, following the alteration of their properties for the activation of mucosal immune cells.[140–144]

The backbone of CAF liposomes is the quaternary ammonium surfactant N,N-dimethyl-N,N-dioctadecylammonium (DDA), which has been extensively studied for its adjuvant properties and is primarily identified as an inducer of Th1 and Th17 responses.[145] It was initially developed as an adjuvant for a tuberculosis subunit vaccine, focusing on inducing a robust Th1 response. To this end, DDA was formulated with the glycolipid α,α-trehalose 6,6′-dibehenate (TDB) to promote this type of immune response.[146] Additionally, it was shown that the DDA:TDB formulation enhanced the stability of the liposomes by facilitating hydrogen bonding with the surrounding water.[147] This formulation, termed CAF01, is used in **Project I** due to its mucosal immune-stimulating properties.

The mode of action of CAF01 is linked to its core components, DDA and TDB. As previously stated, DDA is an inducer of Th1 response, but it has also been reported to moderately promote Th2 stimulation.[148,149] TDB is a synthetic analogue to the mycobacterial glycolipid trehalose-6,6′-dimycolate (TDM). In addition to stabilizing DDA-based cationic liposomes, TDB functions as an immunostimulant, augmenting the Th1 responses generated by DDA.[146] TDB is an activator of the macrophage-inducible C-type lectin (Mincle) receptor, whose binding results in the phosphorylation of the immunoreceptor tyrosine activation motif of the FcRγ chain, followed by SYK activation and Card9–Bcl10–Malt1 signaling of NF-κB (Fig. 8).[150] In addition, several studies have demonstrated that CAF01 is a potent inducer of IL-17 and, by extension, Th17 responses.[151–153] Werninghaus *et al.* demonstrated that this is a result of the Syk-FcRγ-Card9–Bcl10–Malt1 pathway being activated.[150] Further research by Desel *et al.* revealed that TDB

stimulates antigen-specific Th1 and Th17 responses by stimulating Mincle to generate IL-1 to activate MyD88-dependent NF-κB stimulation.[154] A recent study by Wørzner *et al.* investigated how the degree of antigen absorbed to CAF01 influenced the immune response and found that binding to CAF01 is crucial for the Th1 and Th17 response, but not for the antibody production.[155]

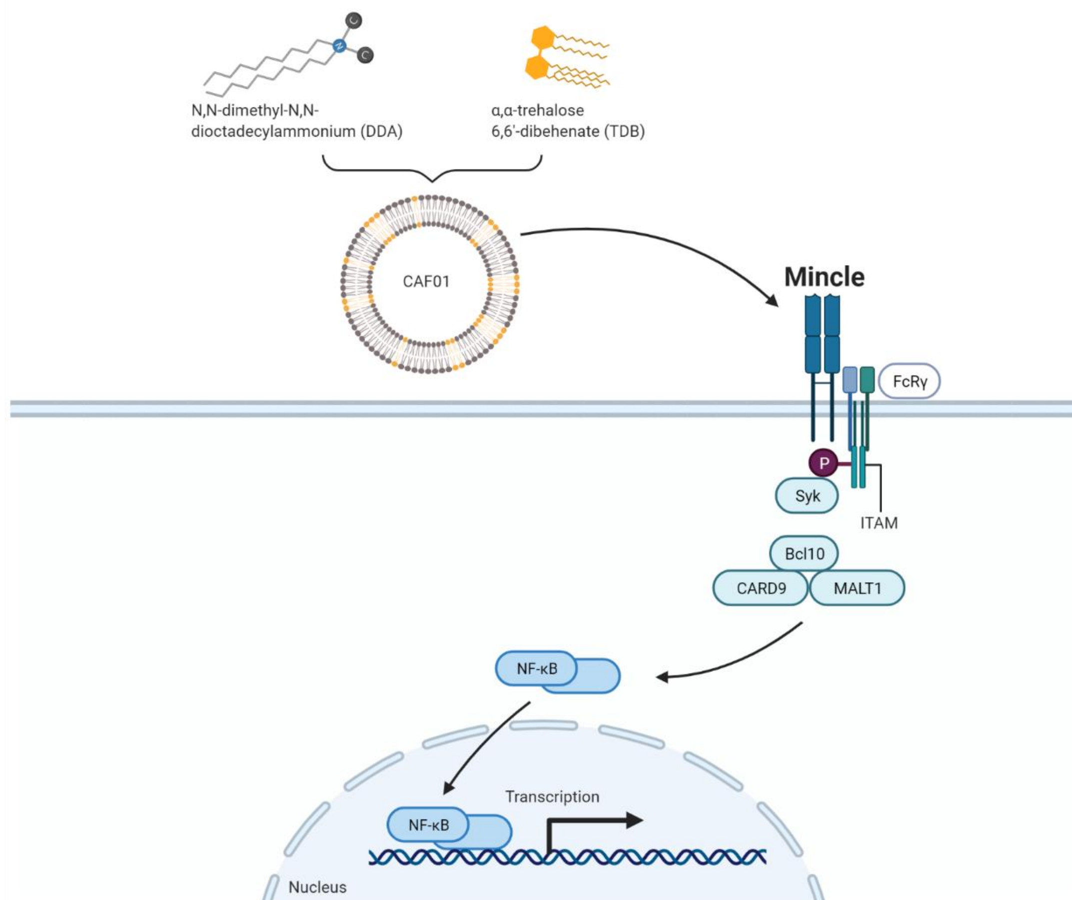


**Fig. 8.** Formulation of CAF01 with N,N-dimethyl-N,N-dioctadecylammonium (DDA) and glycolipid α,α-trehalose 6,6′-dibehenate (TDB). CAF01 recognizes the Mincle receptor, which leads to phosphorylation of the immunoreceptor tyrosine activation motif (ITAM), resulting in signaling through SYK and Card9–Bcl10–Malt1 to activate the NF-κB pathway (simplified illustration). *Created with Biorender.com*

In addition, CAF01 has shown potential as a mucosal adjuvant by robust stimulation of Th1, Th17, and IgA in the nasal and genital tissues of animals.[156,157] In the CTH522 clinic phase I study, CTH522 was adjuvanted with CAF01 and compared to aluminum hydroxide (AH). In this study, the CAF01-adjuvanted CTH522 surpassed the AH-adjuvanted CTH522 in inducing 5- to 6-fold higher IgG titers. In addition, IgG and IgA were reported to be stimulated in the nasal and genital mucosal tissues, which is consistent with earlier *in vivo* animal research. In contrast, IgA antibody production was not detected in the AH formulation.[93]

### α-Galactosylceramide

The glycolipid α-Galactosylceramide is another potential lipid-based adjuvant for inducing the mucosal immune system. α-GalCer, a synthetic lipid derived from a marine sponge, has been observed to be a potent inducer of natural killer T (NKT) cells.[158] When taken up by APCs, α-GalCer is presented by the CD1d molecule, which activates invariant NKT (iNKT) cells, resulting in the production of cytokines such as IFN-γ and IL-4, which leads to stimulation of innate and adaptive immune cells (Fig. 9).[159] Initially, these traits were used to induce immunity against viral infections and tumors. α-GalCer has also been observed to have potential as a mucosal adjuvant. Courtney et al. and Lindqvist et al. conducted some of the earliest research on this topic.[160,161] Both studies investigated the adjuvant in combination with antigens against the STDs—human immunodeficiency virus (HIV) and genital herpes (HSV-2)—respectively. Courtney et al. reported that intranasal (i.n.) and oral dosing induced HIV-specific cytotoxic T cells and increased mucosal and systemic levels of IFN-γ.[160] Lindqvist *et al.* likewise immunized i.n., where they reported protection against HSV-2 in the vaginal canal, revealing the unique ability of the mucosal immune system to acquire protection at distant mucosal sites.[161] They then investigated the mechanism of action of α-GalCer by verifying the significance of the CD1d molecule by discovering that $CD1d^{-/-}$ mice did not develop immunity. Recent studies have demonstrated the mucosal adjuvant abilities of α-GalCer, to induce local proliferation of Th1 and Th17 cells along with IgA antibodies in combination with various antigens, especially following oral administration. Longet *et al.* demonstrated protective Th1 responses against *H. pylori*, following oral administration of a whole-cell *H. pylori* antigen adjuvanted with α-GalCer.[162] In this study, it was also shown that the antigen-specific Th1 response induced by α-GalCer was dependent on CD1d, IL-1R, and IL-17R signaling, indicating that activation of the mucosal iNKT populations by α-GalCer can provide effective adjuvanticity when administered orally. Davitt *et al.* utilized an oral delivery technology along with α-GalCer adjuvanted enterotoxigenic *E. Coli* (ETEC) to induce local antigen-specific IgA in the intestine as well as systemic IgG responses.[163] In another study, the same technology was used for oral delivery of the cholera vaccine Dukoral adjuvanted with α-GalCer, which outperformed Dukoral formulated with the bacterial component cholera toxin B (CTB) in terms of the induced mucosal immune responses.[164]

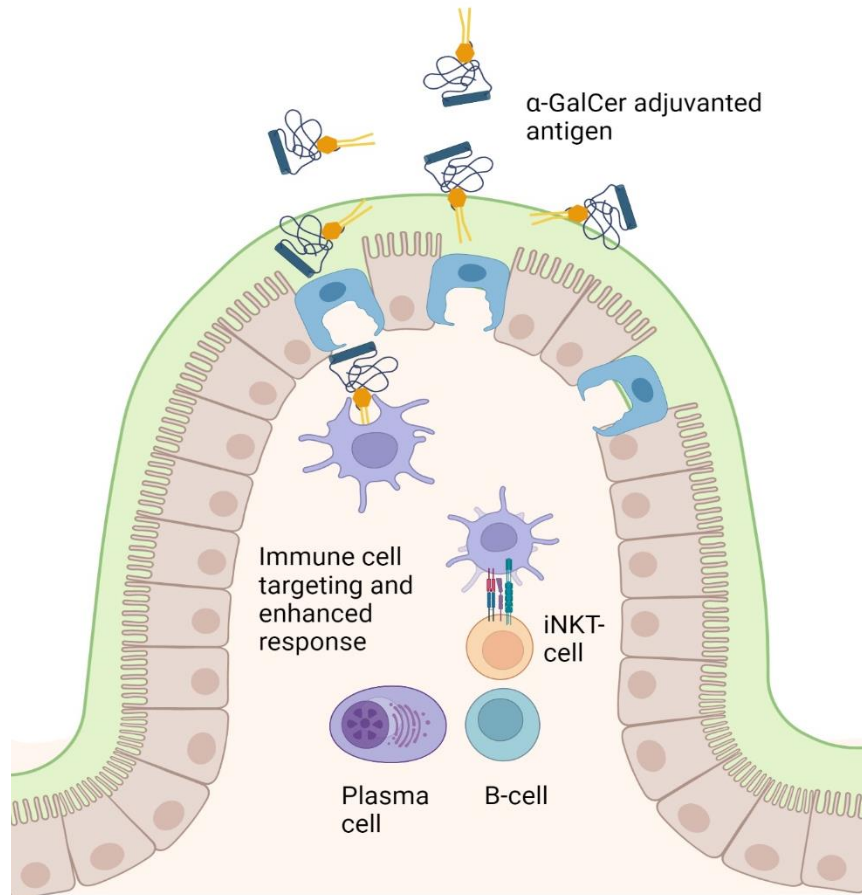


**Fig. 9.** Supposed mode of action of an α-GalCer-adjuvanted antigen. The adjuvanted antigen will be transported by M cells across the epithelial cell membrane in the intestine, to be phagocytosed by dendritic cells (DCs). Presentation by DCs on the CD1 molecule will activate invariant natural killer T cells (iNKT cells), resulting in an enhanced immune response. *Created with Biorender.com, inspired by*[6].

### 2.2.2.2 Bacterial components

Bacterial components, such as toxins, peptides, proteins, and membrane compounds, have been used as adjuvants for a long time. These components were the first to be used as immune modulators, laying the groundwork for the adjuvant concept.[165] Being equipped with PAMPs, bacterial components often possess high immunogenicity. PAMP molecules can activate the most efficient immune response against the pathogen in question, given that different types of bacteria target the immune system in different ways.[166] This trait makes it possible to utilize PAMP molecules to manipulate and direct specific types of immune responses (e.g., Th1, Th17, and IgA responses). As a surplus of bacteria invades through the mucosal tissues, the mucosal immune system has evolved to recognize and respond to specific bacterial components, making these components potential mucosal adjuvants.

### Cholera Toxin B

The bacteria *Vibrio cholera* secretes an $AB_5$-complex toxin composed of two units, one A unit and five B units. Extensive research has confirmed that the B subunit is nontoxic and a strong regulator of the immune cells in the gut.[167] CTB recognizes the receptor GM1, which is present on the surface of a variety of cells, including the epithelial cells in the mucosal barrier of the gut, enabling these cells to serve as an entry point for CTB. In addition, GM1 is also expressed by macrophages, DCs, and B cells, facilitating CTB uptake by these immune cells. In vaccine formulations, CTB is most often recombinantly fused to the antigen in question, which, due to its pentameric form, optimally allows a 5:1 molecular ratio.[167,168] In addition to enhancing APC uptake of the antigen via GM1 affinity, this enables CTB to function as an antigen carrier. However, recombinant fusion can be time-consuming and has limitations, particularly when it comes to larger antigens with more complex structures. As an alternative, CTB has demonstrated promise when chemically conjugated to antigens or when co-administered. CTB has repeatedly proved its capacity to stimulate the mucosal immune system via various delivery routes and in conjunction with several antigens.[169–171] However, CTB classification as an adjuvant has been questioned by the presence of residual cholera toxin or LPS in CTB preparations. It has thus been a challenge to separate adjuvanticity from toxicity, and it seems that highly purified CTB is not an effective promoter of the mucosal immune responses when administered orally or intranasally.[6,172] Compared to studies employing the entire $AB_5$ complex, the immune-enhancing benefits of only the B unit are poor.[172] On the contrary, it appears that conjugating antigens to CTB is an effective method of developing tolerance, which may be advantageous for treating autoimmune diseases.[173] In a clinical trial by Stål *et al.,* CTB were used in the treatment of Crohn's disease, an inflammatory bowel disease, to downregulate the Th1 response that facilitates chronic inflammation.[174] CTB were proven to be safe for use in humans, with 40% of the patients responding to the treatment. In another study by Sun *et al.,* naive B cells were incubated with OVA and CTB for the development of suppressive B cells as a treatment for autoimmune and allergic diseases. The study showed that by transferring the OVA/CTB-treated B cells to mice, proliferation of $FoxP3^+$ $T_{reg}$ cells could be induced upon immunization with OVA.[175]

### Cyclic-di-GMP

Other adjuvant bacterial components are the bacterial second messengers cyclic dinucleotides. These have been linked to central bacterial processes, such as virulence, stress survival, motility, antibiotic production, and biofilm formation.[176] The most investigated are c-di-AMP and c-di-GMP, which play significant roles in gram-positive and gram-negative bacteria, respectively.[177] Furthermore, these compounds have been identified as signaling molecules in mammalian cells,

where they operate as innate immune agonists. More specifically, they stimulate the cytosolic DNA sensor STING.[178] Studies of viral infection have demonstrated that STING activation stimulates TANK binding kinase 1 (TBK1), resulting in the phosphorylation of interferon regulatory factor 3. (IRF3). IRF3 and NFB collaborate to trigger type 1 interferon gene expression. Type 1 interferons mainly include IFN-α and IFN-β, which trigger downstream pathways to stimulate the activation of multiple interferon-stimulated genes, increased expression of MHC I complexes on several cell types, and activation of NK cells. Blaauboer *et al.* investigated the mechanisms for mucosal immune activation by c-di-GMP *in vivo* and discovered that type I IFN signaling is not necessary for the mucosal adjuvant activity of c-di-GMP, but that STING-mediated TNF-α facilitates this activity.[179] Moreover, they demonstrated that c-di-GMP promotes antigen absorption in both APCs and non-APCs, which was dependent on the expression of STING.[180] This has been further verified in a study by Madhun *et al*. where c-di-GMP was used as an adjuvant in an H5 influenza vaccine and administered i.n. to mice.[181] Similar to the study of Blaauboer et al., the authors found Th1-cell proliferation, stimulation of a robust mucosal and systemic antibody response, and an increase in antigen absorption. In another study, Svindland et al. combined chitosan, another compound with mucosal adjuvant properties, with c-di-GMP and found increased cytokine and IgG responses against influenza H5N1 when delivered i.n.[182] C-di-GMP has shown potential in triggering strong mucosal immune responses, but predominantly in respiratory tract mucosal tissue when administered i.n.[6,183] Oral administration of c-di-GMP may induce mucosal immune responses in the intestinal tissues, as it shares many similarities with respiratory tissues. However, being a small molecule, c-di-GMP may be susceptible to breakdown in the stomach in the absence of a mechanism that protects against the gastric environment.

The aforementioned adjuvant technologies have demonstrated promise in successfully eliciting mucosal immune responses and, in some instances, been successful in providing protection. This is with the exception of CTB, where research indicates that it does not appear to improve immunity when it is purified and is instead more effective as a vehicle for the activation of immunological tolerance. CTB is currently the only subunit antigen formulated in a commercial oral vaccine, specifically the cholera vaccine Dukoral. CTB has some boosting effects in the presence of the whole bacteria and is included to induce cholera toxin–specific antibodies. Nevertheless, this demonstrates the necessity for and absence of effective adjuvants that can be used in oral vaccination and induce the numerous mucosal immune system branches. Since effective novel

adjuvants appear to be required to overcome the challenges of oral vaccination, this need has sparked interest in the topic in recent years.

### 2.2.3 The challenges of oral vaccination

Sabin, Vivotif, and Dukoral are examples of currently approved oral vaccinations that use either inactivated or live attenuated microorganisms. The effectiveness of these vaccines can be attributed to their innate immunogenicity and the fact that they are generated from viruses that actively infect portions of the GI tract in their wild-type form.[6,78] However, the processes of inactivation and attenuation are not feasible for all pathogens and can be time-consuming. As previously indicated, modern vaccination techniques have evolved toward the use of subunit vaccines that are more amenable to modification and new adjuvants. However, subunit antigens are often fragile and susceptible to the challenges of oral vaccination (Fig. 10). Numerous factors, including the physiology of the GI system and immunological tolerance at the cellular level, contribute to the complexity of the oral route.[8] The first challenges are found in the stomach, where acids and proteolytic enzymes destroy and break vaccine components. In certain instances, the stomach can serve as the target organ, such as in the case of vaccines against *H. pylori,* which infects the gastric mucosal tissue.[184] However, the majority of oral vaccines will likely be designed to continue into the intestine to take advantage of the high concentration of immune cells in this region. In the intestine, the vaccine will come into contact with intestinal fluids and degrading enzymes and bacteria. Furthermore, to establish an immune response, the vaccine components need to cross the mucus and epithelial membrane to reach and interact with the underlying immune cells. Antigen retention at a certain site can frequently improve interaction and identification by immune cells. However, peristaltic movements and intestinal flow will propel the vaccine formulation further along the GI tract, narrowing the absorption window making detection more difficult. Moreover, immune tolerance, a trait controlled by the $T_{reg}$ cells, is also one of the significant challenges of oral vaccination.

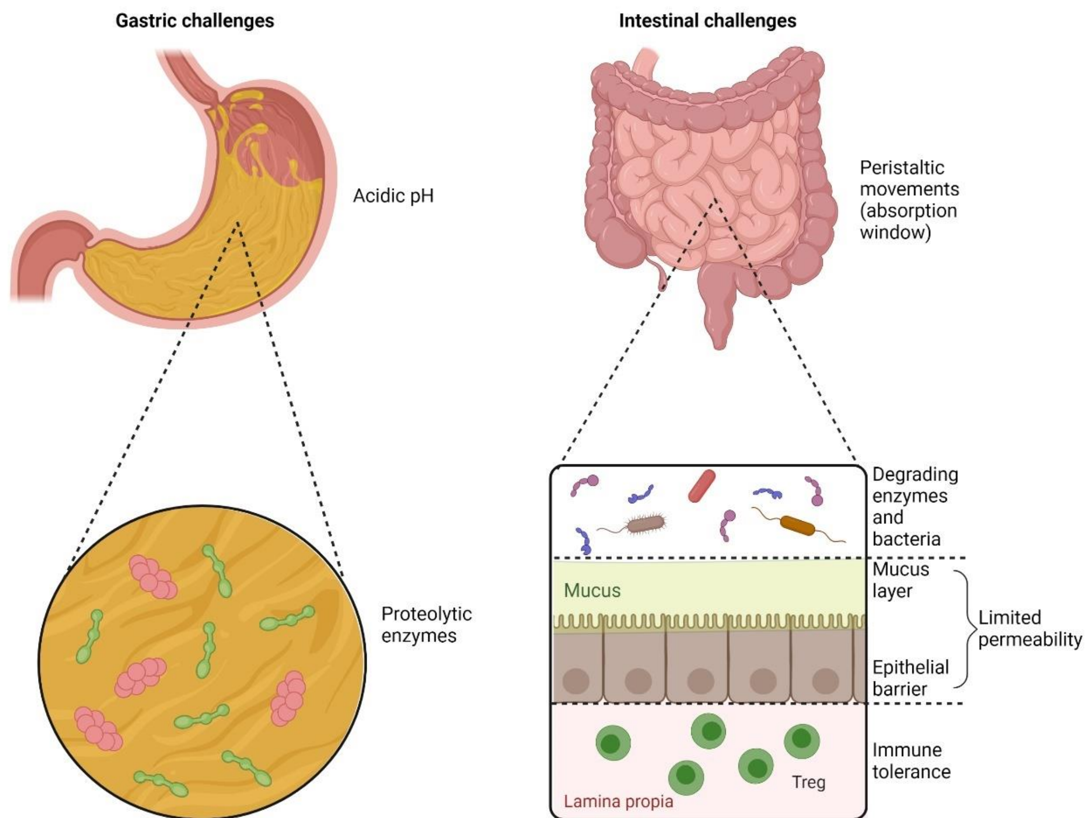


**Fig. 10.** The major challenges of oral vaccination summarized for the gastric and intestinal regions. In the gastric environment, acidic pH and proteolytic enzymes will degrade proteins such as antigens. In the small intestine, which often will be the target site of release, degrading enzymes are also present along with degrading bacteria from the microbiota. To increase the chance of inducing a response, the antigens should be in close proximity to the epithelial barrier, either to be taking up by macrophages and DCs or be transported across the epithelial membrane by M cells. Here the mucus layer and epithelial membrane represent barriers, which limit the permeability. Once in the lamina propria, the vaccine formulation needs to stimulate a robust immune response, but tolerance will most likely be induced by the regulatory T cells ($T_{reg}$). Thus, the vaccine formulation must contain an effective adjuvant or immunogenic antigen to overcome the immunological tolerance. At all times, peristaltic movements will push the vaccine further down the intestine and keep it mobile, which will limit the time window in which the formulation can interact with the underlying cells. *Created with Biorender.com*

#### 2.2.3.1 Oral immune tolerance

When soluble proteins are administered orally, the mucosal membranes become unresponsive, known as the tolerance mechanism. Not only in the GI tract but also in the majority of mucosal membranes, such as the respiratory system, tolerance is the default response route. As mentioned, is $T_{reg}$ cells the dominant facilitator of mucosal immune tolerance.[46,49] The primary subset of these cells in the intestine expresses the FoxP3 transcription factor and secretes transforming growth factor (TGF)-β along with IL-10, which inhibits T-cell priming and effector

functions.[185] For example, $T_{reg}$ can inhibit the proliferation of Th1 and Th17 cells, decreasing the production of IFN-γ and IL-17 to prevent inflammation and tissue damage. This is a vital feature of the mucosal immune system, as impaired tolerogenic responses can lead to inflammatory bowel diseases. However, this mechanism also inhibits vaccine responses, especially for subunit antigens, typically immunogenic to a low degree. Inactivated and attenuated vaccines feature the ability to express several PAMPs, which is the most prevalent method for overcoming tolerance. Overcoming tolerance for a subunit vaccine formulation thus depends on the adjuvants contained in the formulation and, in some situations, the type of the antigen, such as when VLPs are used. Recent studies have shown that the draining lymph nodes in the proximal intestine stimulate $T_{regs}$, whereas distal lymph nodes facilitate effector T-helper cell responses.[49] This information indicates that it may be "easier" to overcome tolerance at designated sites in the distal part of the intestine, which then could be a more promising target of release, as opposed to facilitating release upon initial entry to the intestine.

The reported difficulties of oral administration and immune stimulation indicate that certain factors are necessary for efficient oral vaccination, including (1) protection from the stomach environment, (2) facilitated and retained delivery of antigens in the intestine, and (3) potent stimulation of the mucosal immune system to overcome immune tolerance. Whereas adjuvants and some antigens are tools for immune stimulation, effective delivery systems capable of protection and target delivery might be a solution to these challenges. Polymer- and microtechnology advancements have shown promise in this area and could be a powerful instrument for the oral administration of vaccines.

## 2.3 Polymeric particles and films

Polymers have been found as effective tools for mucosal vaccination due to the advantages they offer in mucosal site interaction. This includes delivery and release to a specific target site, protection of vaccine components from the gastric environment, and the modifiable nature of the polymers. The psychochemical properties of polymers can be modified to optimize factors such as charge, solubility, the ability to form particles, and in this case, the particle size. Furthermore, polymers better interact with immune cells such as M cells and DCs in particle form than in soluble form; hence, they are most commonly utilized as particles.[186] In section 2.2.2 about lipid-based particles, the principle of particles as antigen delivery vehicles has been briefly discussed. Similar to liposomes, encapsulating antigens into polymeric microparticles (MPs) and nanoparticles (NPs) has shown promise in delivering antigens to APCs in the mucosal tissues in a concentrated manner.[187–189] Moreover, the polymers frequently exhibit robust structural stability, which protects

the antigens and enables the incorporation of additional immune modulators. The leading polymers explored in preclinical studies are synthetic polymer poly(lactic-co-glycolic) acid (PLGA) and natural polymer chitosan.[190] In addition, the cellulose acetate phthalate (Eudragit) is often used as a polymeric film to protect encapsulated antigens and target delivery, being insoluble at a low pH of, for example, the stomach, but dissolves at higher pH, which can be found in the intestine. PLGA, chitosan, and Eudragit were used as polymeric coatings in **Project I**, with different variants of Eudragit being utilized in **Projects II** and **III.**

### 2.3.1 PLGA

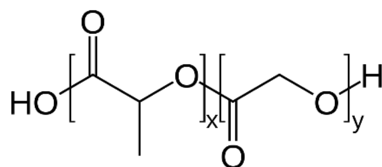


**Fig. 11.** Chemical structure of PLGA

PLGA is a synthetic polymer that is highly modifiable and may be produced with different ratios of lactic acid and glycolic acid, end groups, and molecular weights (Fig. 11). Due to its modifiable nature and excellent safety record, PLGA is utilized in a variety of biomedical applications, including sutures for bone reconstruction, implants, and particles for sustained drug delivery.[191] Upon administration, PLGA begins to hydrolyze back into the original monomers—lactic and glycolactic acids. Multiple parameters, such as pH, morphology, polymer crystallinity, molecular weight, and acid ratio, influence this process. Given that glycolic acid is more hydrophilic than lactic acid, an increase in glycolic acid would result in a faster rate of breakdown in water. In a particulate form, this property can be utilized to change the release profile of entrapped content and to facilitate targeted release with knowledge of the kinetics of the particles.[191] The degradation of PLGA occurs by three hydrolytic pathways: (1) surface degradation, where the cleavage of ester bonds of polymers occurs mainly on the surface, (2) bulk degradation, when degradation medium penetrates polymer matrix and random hydrolysis occurs throughout the polymer bulk, and (3) bulk degradation with autocatalysis, when the bulk degradation results in the formation of a higher concentration of acidic degradation products in the polymer interior compared to the polymer surface, resulting in autocatalysis that accelerates the internal degradation.[192] Following degradation procedures, the payload is subsequently released through diffusion, which is used for the delivery of pharmaceuticals and vaccines.

PLGA NPs have been widely used for the delivery of antigens as they are easy to handle and licensed for use in humans and animals. Compared to soluble antigens, antigen-containing

polymeric particulates have been reported to stimulate strong and long-lasting T-lymphocyte responses.[193] Other characteristics include protection of their payload from proteolytic degradation, sustained antigen availability, peptide dosage reduction, minimized immunization times, reduced toxicity, and accelerated degradation when taken up by APCs due to the acidic pH of the endosomal compartment.[191] Ashhurst *et al.* developed biodegradable PLGA as a carrier for the *M. tuberculosis* lipoprotein MPT83 in conjunction with TDB or MPL.[194] The PLGA-encapsulated antigen induced robust MPT83 antibody and Th17 responses when administered i.n. to mice.

### 2.3.2 Chitosan

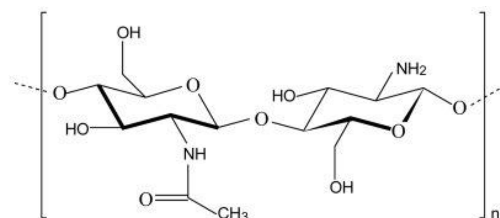


**Fig. 12.** Chemical structure of chitosan

Chitosan is a natural polymer derived from the deacetylation of chitin, a component of crustacean and insect shells (Fig. 12). Chitosan has amino and carboxyl groups that form hydrogen bonds with the mucus glycoproteins, giving mucoadhesive properties to the polymer.[186] Chitosan is degraded by a group of chitinases present in mammalian tissues, which hydrolyzes the N-acetyl-β-1,4-glucosaminide linkage, converting chitosan into nontoxic oligosaccharides that can be excreted or incorporated to glycosaminoglycans and glycoproteins.[195] Moreover, chitosan can interact with epithelial cells, DCs, and macrophages, and it is an activator of the cGAS–STING and NLRP3 inflammatory pathways, hence promoting adaptive immunity.[196] The characteristics of the polymer can contribute to improving permeability and stimulating a mucosal immune response, making it a suitable option for vaccination at mucosal sites.[186] Numerous i.n. administrations of chitosan to diverse animal models have elicited IFN-γ and IgG responses in the lungs.[182,188,197] Renu *et al.* recently developed a subunit chitosan NP-based vaccine by using immunogenic outer membrane proteins (OMPs) and the flagellin protein of *Salmonella.*[198] Oral immunization of layer chickens caused the particles to be transported to the Peyer's patches and induced significantly higher OMP-specific mucosal IgA production and antigen-specific lymphocyte proliferation than immunization with soluble antigens. Chitosan, together with c-di-GMP, was used as an adjuvant in the study by Svindland *et al.* to induce a local mucosal immune response in the respiratory tract.[182]

Combinations of PLGA and chitosan have also been manufactured, such as PLGA MPs being coated with chitosan and the conjugation of an M cell homing peptide (CKS9).[199] Oral immunization in mice demonstrated elevated mucosal IgA responses, as well as systemic IgG stimulation.

### 2.3.3 Eudragit

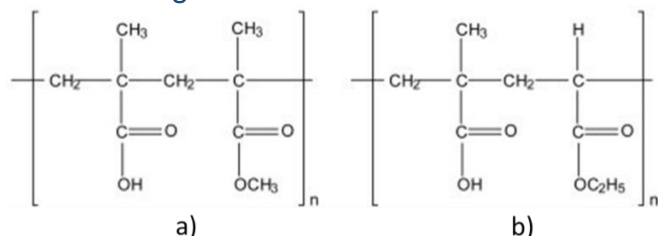


**Fig. 13.** Chemical structure of Eudragit L100 (a) and Eudragit L100-55 (b).

Eudragits are methacrylic acid copolymers, a registered trademark of Rohm Pharmaceuticals (Darmstadt, Germany). Dimethylaminoethyl methacrylates, methacrylic acid, and methacrylic esters are present in varied proportions in various Eudragit polymers. Eudragits are defined as pH-sensitive polymers as they dissolve at various pH values based on the composition of their chemical groups. Similar to the study by Laier *et al.,* **Project I** utilized Eudragit L100-55 (EL100-55) as a coating on the MCs (Fig. 13). EL100-55 supports a controlled drug release at pH levels >5.5, beginning in the duodenum of humans, preserving the drug from the acidic gastric juice. This function also applies to mice, as their gastric pH ranges from 3 to 4, and their intestinal pH <5.2. In **Projects II** and **III,** Eudragit L100 (EL100) was used as a coating on the MCs to target the intestine of rats and sea bass (Fig. 13). EL100 differs from EL100-55 only for the presence of a methyl group instead of an ethyl group, which influences the slightly different dissolution pH threshold for the two polymers, changing the dissolving pH value to be >6, which corresponds to the jejunum of humans. Comparable to mice, rats' gastric pH ranges from 3.2 to 3.9, whereas the pH of their intestines is approximately 6.6. For sea bass, the gastric pH is somewhat higher than the murine models at 5.3–5.7, with an intestinal pH of 6.5–6.8.

Eudragit has often been utilized alone or in conjunction with PLGA and chitosan for drug delivery. According to my knowledge, Eudragits do not possess any adjuvant properties, and Laier *et al.* is the only study to have used them for oral vaccination purposes.

## 2.4 Nonparticular microtechnologies for oral vaccine delivery

The field of targeted oral drug administration has made significant advances in microtechnologies to enhance and improve delivery systems' efficacy. Innovative design and engineering vehicles

other than particles fall short in the arena of oral administration. However, some emerging technologies have demonstrated potential in oral delivery by facilitating protection through the gastric environment and release in the intestine and have additionally been shown to improve the oral delivery of vaccines.

On two separate occasions, Davitt *et al.* demonstrated that oral immunization with α-GalCer-adjuvanted antigens elicits robust mucosal immune responses.[163,164] This was facilitated by the incorporation of Single-Multiple Pill (SmPill), initially a drug delivery system, into the vaccine formulation. Using Eudragit and sorbitol, it was possible to achieve a prolonged, controlled release of antigens in the intestinal medium from SmPills (1–2 mm).

Recent studies have highlighted the potential of nonparticular microdevices to aid in oral vaccination.[200–202] Using these technologies in conjunction with potent mucosal adjuvants could be a powerful tool for developing efficient oral vaccine delivery vehicles. The hypotheses made in this thesis revolve around the use of vehicle microtechnology called MCs.

### 2.4.1 Microcontainers

MCs are microfabricated devices meant for oral delivery of therapeutic agents and medicins.[203–206] MCs are designed with a reservoir-like geometry to ensure the unidirectional release of the content into the intestinal wall, which can be very beneficial in concentrating the content at a specific location (Fig. 14).[207,208] This can be compared to particulate systems, which will enable an omnidirectional release in the lumen. For the technology to profit from the reservoir-based architecture, however, the device must be oriented correctly. This can be challenging due to peristalsis and the constant motility in the GI tract.[209] Retention is a critical feature for both medications and vaccines and should be incorporated into the MCs.

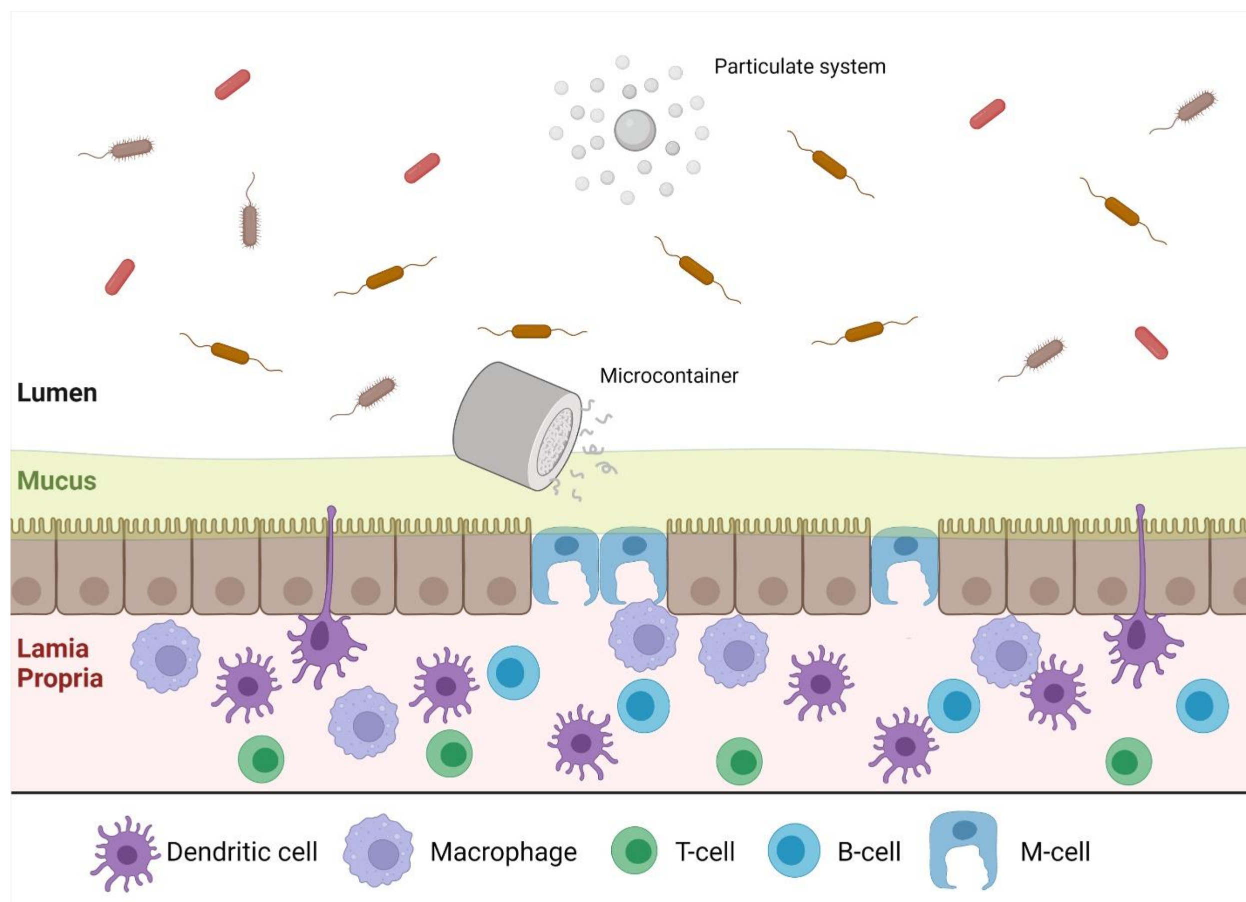


**Fig. 14.** The concept of the microcontainers (MCs) technology compared with a particulate system when reaching the small intestine. After passing through the stomach, the MCs stick to the mucus layer for retention and maintain close proximity to the epithelial membrane. Here, the MC will deliver the payload in a unidirectional and concentrated manner. A particulate system will often display an omnidirectional release, which most likely will require larger doses of the compound in question. *Created with Biorender.com*

The first of this kind of these microfabricated devices were made from silicon (Si) dioxide, poly(methyl methacrylate) (PMMA), and photoresists; however, fabrication in more biocompatible and biodegradable materials has been explored since then.[210,211]

### *2.4.1.1 SU-8 MCs*

At the IDUN Research Center, the most common MCs are currently fabricated from SU-8, a biocompatible photopolymer with adhesive traits. SU-8 is subjected to UV light, which initiates cross-linking of the polymer, as part of a two-step photolithography production procedure.[209,212] The MCs are fabricated on a Si wafer divided into 30 chips containing 625 MCs (Fig. 15). To ease detachment of the MC to be filled in gelatin capsules for animal studies, an antiadhesive layer of titanium and gold (Ti|Au) can be applied. This is a very versatile fabrication process, allowing for various designs of the containers.[213] However, SU-8 is not biodegradable, and it will eventually

be necessary to find a biodegradable replacement for this polymer for the technology to enter clinical trials and be commercialized. To this end, many studies with biodegradable polymers and different fabrication techniques have been conducted. This includes hot punching and hot embossing, used to make MCs of PLGA, poly (L-lactic acid) (PLLA), and polycaprolactone.[205,214,215] However, these methods are still in early stage research and require additional testing before they can undergo fabrication upscaling and be applied in preclinical studies. Several designs of the SU-8 MCs have been produced and studied *in vivo*. At the IDUN Research Center, the most common are containers of a cylindrical design, with an outer height of ~300 µM and an outer diameter of ~250 µm. These have been extensively used for oral delivery of peptides, probiotics, small molecule drugs, and the OVA vaccine formulation by Laier *et al.*[203–208] Cylindrical photolithography fabricated SU-8 MCs were therefore used in **Projects I–III**.

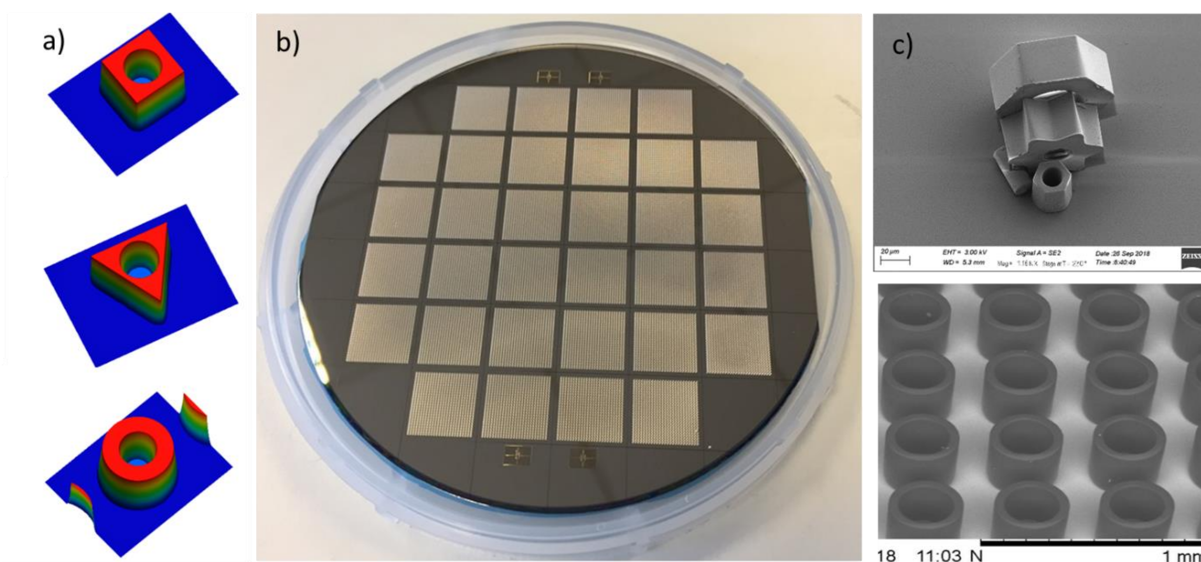


**Fig. 15.** Microcontainers (MCs) can be designed in various shapes and sizes (a) and produced on a silicon wafer by a two-step photolithography process (b). Once fabricated, the MCs are separated into "chips," each containing 625 MCs in the case of the cylindrical MCs. Scanning electron microscope (SEM) is commonly used to visualize the MCs properly and can be used to verify shape and size, among other features (c).

Most devices designed for oral drug delivery follow the same hypothesis that retention and unidirectional release will allow a higher concentration of drug to be absorbed. This concept could very well be translated to vaccines, as it could be assumed that the probability of the vaccine components to reach and be recognized by the immune cells would increase, by being released and retained closer to the epithelium.[207] This hypothesis has previously been investigated by Laier *et al.*, employing MCs together with cubosomes, the adjuvant Quil-A, and the model antigen OVA.[207] In this study, the vaccine formulation was successfully spray dried, loaded into SU-8

MCs, and administered to mice. However, the MCs did not manage to induce a robust immune response. A limitation of the MC technology necessitates the use a drying procedure, as solutions are not compatible for loading into the microcapsules, unless reduced to powder form. In this thesis, lyophilization was used because it is a highly controllable procedure and is believed to be superior to spray drying at retaining proteins.

#### *2.4.1.2 Lyophilization of formulations for MCs*

In the pharmaceutical sector, lyophilization is a well-known procedure for stabilizing thermally unstable substances, which is crucial in a commercial sense. No matter how effective a compound is in the lab, its commercial value becomes heavily limited if it cannot be stabilized for distribution and storage.[216] Vaccines, which are produced on a large scale and found compatible with the process, are lyophilized for the same reasons. Lyophilization is an extensively studied and complex field of research, as novel methods to prolong storage or optimize the lyophilization process are of great value in vaccine production.[216–218] Increased thermal stability is especially advantageous in places where cold chain management is difficult. Lyophilization allows for high control of a wide range of parameters and can be specifically optimized for a particular protein. The process has been found to be optimal at preserving the proteins, which is beneficial when dealing with large and complex proteins or antigens.[216] However, it can be expensive and time-consuming, especially in a large-scale setting.

There are five main stages in lyophilization: formulation, freezing, primary drying, secondary drying, and stop (Fig 16).[219] The vaccine will be formulated with excipients prior to lyophilization, such as trehalose used in **Project I** and sucrose used in **Project II**. These act as stabilizers and lyoprotectants during the lyophilization process and will replace the water molecules when they are removed. In the lyophilization process, the first step is freezing the sample. This is followed by a reduction of pressure, which will cause the water to sublimate, meaning the water will go directly from the solid to the gaseous state to leave the vials. This is the beginning of primary drying, where sublimation is driven by pressure. The majority of the water molecules will be removed at this stage. Temperature will drive sublimation during the secondary drying phase. Increasing the temperature will ensure sufficient removal of tightly bound water molecules. Restoring atmospheric pressure then terminates the operation.

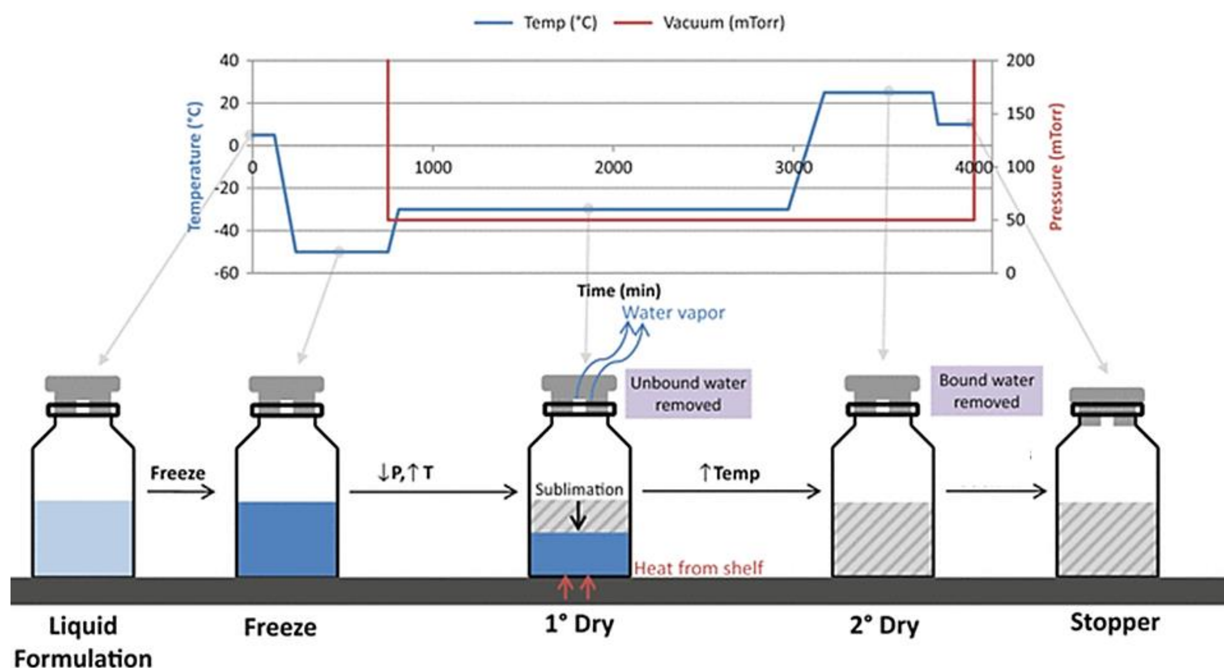


**Fig. 16.** The process of lyophilization, depicting the five main stages: formulation, freezing, primary drying, secondary drying, and stop. The lyophilization will start with freezing the sample. Then primary drying will begin with a decrease in pressure, which allows for the sublimation of the water molecules. During secondary drying, the temperature will be increased to ensure the removal of bound water. The process is stopped by reestablishing atmospheric pressure. *Reprinted and adapted with permission from Springer Nature.*[219]

Several complications can arise during this process and must be addressed and optimized to the specific formulation and lyophilization machine, as many variants exist. Critical quality attributes (CQA) are used to assess the quality of the lyophilized product and typically include appearance, residual moisture, behavior upon reconstitution, retention of biological activity, and long-term stability.[218] Furthermore, knowledge of the critical collapse temperature ($T_c$), the maximum temperature the product can withstand without collapsing or melting during primary drying, and the glass transition temperature ($T_g$), the maximum temperature where the crystallized sample transitions into a glass which can cause collapse or harm to the sample, can benefit the optimization process.[218]

Lyophilization was utilized in all three projects in this thesis. In **Project I,** a lyophilization protocol was developed, which also was used in **Project II** but with different excipients. The *P. pastoris* extracts used in **Project III** were already lyophilized when received. The sample can be loaded into the MCs after being reduced to a powder.

#### *2.4.1.3 Powder loading into MCs*

Loading formulations into the MCs has proven almost a science in itself, with the development and optimization of several loading methods.[220] Loading compounds directly into the MCs has

been achieved using a brush, centrifugation, embossing, or a spatula.[204,209,212,220] These methods are combined with a shadow mask, cut in metal, or molded in polydimethylsiloxane (PDMS) to reduce waste and avoid excess between the containers.[206,221] In this study, vaccines were loaded manually using a shadow mask and a brush, as this method proved easy, efficient, and reproducible.[207,209] More advanced methods are the loading of drugs in polymers by photolithography of drug-loaded hydrogel matrices, hot punching in a spin-coated drug polymer film, and supercritical $CO_2$ impregnation of MCs filled with a polymer.[203,204,222] How effective the loadings methods are for a certain powder depends mainly on its density and texture. However, the mentioned methods are time-consuming and only useful in small-scale studies. In large-scale production, these loading methods would not be feasible. Automation of the loading would greatly benefit the process but would also be a challenge to implement as different types of compounds are used. Liquids have also been successfully loaded into the MCs by using inkjet printing.[223,224] This could be very interesting to explore for vaccines, as these originate in liquid form. However, some drawbacks are that this method is time-consuming, and with the current design of the MCs, it is only possible to load 3 nl of liquid per container. From a vaccination standpoint, this implies that the antigen concentration in the solution must be high or that a large number of MCs must be dosed in order to give sufficient antigen for an immunological response.[219] When loaded, MCs must be sealed with a polymeric lid, which defines the containers' release function.

#### *2.4.1.4 Spray coating polymeric lids on MCs*

To target release in specific regions of the GI tract, the MCs are equipped with polymeric lids similar to some of the technologies described in section 2.3. These lids are applied to protect the content from the gastric environment and facilitate release in specific regions of the GI tract. For instance, polymers such as chitosan have mucoadhesive traits, which could be exploited.[225] Many of these lids have been developed for MCs and are continued to be researched and optimized for targeted release.[225,226] The state-of-the-art method applied for sealing is ultrasonic spray coating[227]. Here, a nozzle moves across the MCs, spraying very fine uniform droplets of the polymer solution created by high-frequency sound vibrations. The method allows for fine-tuning of many parameters, such as spraying pattern, heat, pressure, and vibration power. Different solvents are required depending on the polymer, resulting in solutions with varying densities and viscosities. These variables have a significant impact on the solution's flow during spray coating and should be tuned extensively for each polymer solution. Multiple polymeric coatings were utilized in this thesis for the oral administration of vaccines using microcapsules. In **Project I,** EL100-55, PLGA, and chitosan were assessed and compared for their ability to deliver and improve the mucosal immune response in mice. In **Project II,** Eudragit L100 (EL100) was used

as this polymer dissolves at pH 6–7, corresponding to that of the rat intestine. EL100 was likewise used in **Project II**, as sea bass has similar intestinal pH values as rats.

# 3. Outcomes and discussion

Even when paired with mucosal adjuvants, it has been difficult to produce appropriate immune activation with recombinant antigens. This demonstrates the necessity for additional instruments, such as the MCs, to aid in the immunization process. Laier *et al.* investigated the effect of the MCs to deliver the model antigen ovalbumin incorporated into cubosomes and adjuvanted with Quil-A.[207] Even though it appeared that MCs may target the intestine for release, there was no rapid amplification of the immune response. Some investigations have found mucosal immune induction using Quil-A, but only as a component of lipophilic immune-stimulating complexes (ISCOMs), which are known to stimulate mucosal immune cells.[228] To my knowledge, no other preexisting data are available that defines the vaccine components used by Laier *et al.* as inducers of the mucosal immune system. This may explain why an increased response was not achieved, necessitating the addition of other mucosal-stimulating antigens and adjuvants to the MCs.

## 3.1 Oral delivery of CTH522 and the AP205 cVLP in MCs

This section is based on the results from **Project I,** which are presented in **Paper I**: "Oral Vaccination Using Microdevices to Deliver α-GalCer Adjuvanted Vaccine Afford Mucosal Immunity" (Appendix I) and the results from **Project II** presented in **Paper II**: "Oral Delivery of the AP205-SpyCatcher Capsid Virus-like particle Using Microdevices" (Appendix II). The studies in **Project II** were done in collaboration with Kara-Lee Awes from the University of Copenhagen.

### 3.1.1 Project I discussion

#### 3.1.1.1 Main outcomes

In **Project I,** effective mucosal adjuvants were identified based on published data to test their ability with the CTH522 antigen from *C. Trachomatis* and to be orally delivered in combination with the microcontainers (MCs). Mucosal adjuvants for formulation with CTH522 were screened *in vivo*, designating α-GalCer as the most effective adjuvant for oral administration of CTH522 on account of its induction of local T-cell responses. A lyophilization protocol was developed to facilitate loading into MCs. The optimal buffer for this technique was determined to be 10% (w/v) Trehalose + 10 mM Tris. The lyophilized vaccine was evaluated *in vivo* to determine if the lyophilization process negatively affected the immunogenicity of the antigen. This was not the case, because reconstituted CTH522 + α-GalCer had the same immunogenicity profile as non-

lyophilized CTH522. The effect of the polymeric lids EL100-55, PLGA, and chitosan was also assessed. The quantities of CTH522-specific IgA antibodies produced by MCs with EL100-55 lids were substantially higher than those produced by MCs with PLGA and chitosan lids. CTH522 + α-GalCer was delivered in MCs with EL100-55 lids in the final immunization study and evaluated against designated control groups. A trend of enhanced systemic Th17 along with local Th1, Th17, and IgA levels was observed from administration with MCs following an s.c. prime with CTH522 + CAF01. Prime and boost with MCs alone induced a significantly higher local immune response compared to naive mice. However, the observed responses were relatively weak. This could be due to the transit time of the MCs, which was comparable to the standard transit time of food in mice of 1 h–1.5 h. Therefore, MCs are not maintained in the intestine, which may be required for a robust immunological response.

#### 3.1.1.2 Additional results: Immunization with c-di-GMP + CTH522 in MCs

In regard to c-di-GMP, this adjuvant performed poorly in the oral groups, but did show some promise following the s.c. prime concerning the local Th1 and Th17 responses measured from the PPs. Being a small molecule, c-di-GMP is susceptible to degradation in the stomach; but, if provided with a method capable of protecting the molecule via the stomach for intact transport to the mucosal tissue of the intestine, it may augment the immune response further. Based on this hypothesis and the immune response from the prime-pull administration in the adjuvant screening, c-di-GMP was formulated with CTH522 and delivered orally with MCs. Similar to the screening study, mice were primed with CAF01-adjuvanted CTH522 prior to receiving a booster with c-di-GMP. Mice were then administered CTH522 adjuvanted with c-di-GMP orally, either as MCs or by gavage. Based on the results observed in the screening study, it was determined that it was unnecessary to include a solely oral group. A naive group served as the negative control, and a group receiving nasal boosters served as the positive control, as c-di-GMP has been shown to trigger powerful immune responses via this method.

When fed orally to MCs, the data did not indicate that c-di-GMP could enhance the systemic T-cell response or the local antibody response (Fig. 17). The IFN-γ and IL-17A levels measured in the spleen of the oral boosted groups were comparable to those of the CAF01 (s.c.) group, suggesting the observed response is the result of s.c. prime injection. As expected, nasal treatment greatly increased systemic Th1 and Th17 responses. The levels of antigen-specific IgA levels extracted from feces were similar in all immunization groups, again suggesting this response is on account of the prime immunization.

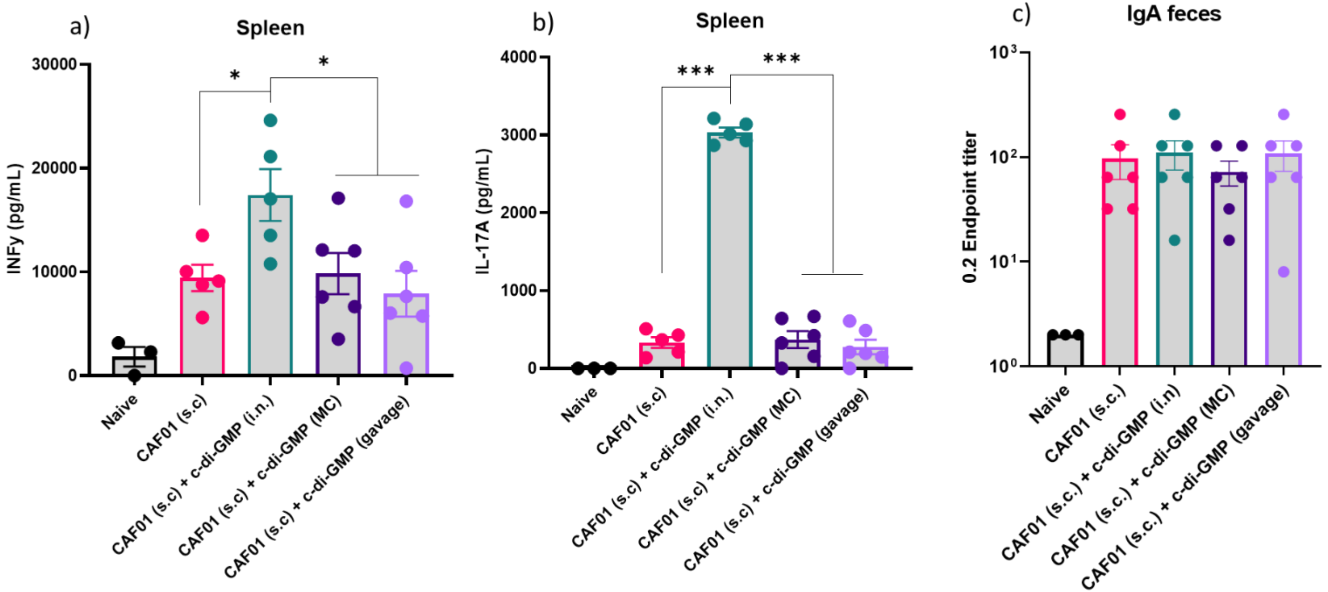


**Fig. 17.** Measured IFN and IL-17A cytokines in the spleen from mice prime s.c. with CAF01 + CTH522 followed by nasal (a) or oral (b) boosters with c-di-GMP + CTH522. Levels from the oral boosted groups were comparable to the group only receiving the s.c. prime, suggesting this administration was driving the response. Antigen-specific IgA measured from the feces of the immunized mice (c). The measured antibody response in all the groups was comparable to the CAF01 (s.c.) group, indicating the response was stimulated on account of the prime immunization.

C-di-GMP-adjuvanted CTH522 did not demonstrate stimulation of a mucosal immune response when delivered in MCs. It is possible that c-di-GMP as an intestine adjuvant is not as effective as when delivered to the respiratory system. Li *et al.* reported a similar phenomenon in their investigation. They observed that oral administration of c-di-GMP failed to elicit an immunological response. The authors chemically modified c-di-GMP, by developing a 2′-fluoro analogue of the molecule (2′-F-c-di-GMP), which was capable of inducing a mucosal immune response when administered orally with an *H. pylori* cell-free sonicate extract as an antigen. It is also likely that the lyophilization process used in **Project I** had an adverse effect on c-di-GMP. C-di-GMP + CTH522 was not quality controlled post lyophilization like the formulation with α-GalCer, and it is unknown whether the process had a damaging impact on c-di-GMP. If this is the case, then it is most likely linked to the reconstitution in the fluids and environment of the intestine, as c-di-GMP is commercially available as a lyophilized powder. To properly determine this, a lyophilized c-di-GMP + CTH522 formulation should be reconstituted in a solution mimicking that of the intestine and subsequently analyzed.

### 3.1.1.3 Considerations on the choice of adjuvants and antigens

Being an isolated membrane protein, the CTH522 antigen is not very immunogenic and needs adjuvants, even when administered s.c. This would most likely also be the case if administered orally. In this study, the adjuvants CTB, c-di-GMP, and α-GalCer were chosen as potential oral adjuvant candidates. From the screening study, α-GalCer was chosen as the most promising adjuvant for oral administration with CTH522. This choice was based on the ability of α-GalCer to induce Th1 responses locally in the PPs by solely oral administration. The formulation also showed promise following the s.c. prime generating slightly higher systemic IFN-γ and IL-17 cytokine levels than the other adjuvants. However, this response was not significantly enhanced compared to only receiving the CTH522 + CAF01 s.c. prime. Nevertheless, α-GalCer outperformed CTB and c-di-GMP, the other adjuvants chosen. Particularly, CTB exhibited low immune augmenting properties, which is not particularly surprising, given that multiple investigations have proven CTB as an inducer of immunological tolerance and not as an immune enhancer when delivered orally. The adjuvant was chosen based on outdated research in which residual LPS was most likely responsible for the enhancing response and was, therefore, not optimum. To improve mucosal responses, it appears that the toxin must be synthesized in the native AB5-complex, yet there are toxicity problems with this formulation. However, new approaches have produced a safe variation of the cholera toxin, which has resulted, among other things, in the production of multiple-mutated cholera toxin (mmCT). Lebens *et al.* constructed a CT derivative that targets the cholera toxin A (CTA) component, which is responsible for both the toxic and adjuvant activities. CTA molecules were altered at numerous places to prevent proteolytic cleavage, which would usually activate ADP ribosyltransferase activity, leading to the generation of cyclic AMP (cAMP), which drives electrolytes and fluids into the intestinal lumen and promotes inflammatory reactions. The mutated sites in the CTA unit were resistant to proteolytic cleavage, and the construct demonstrated a >1000-fold decrease in cAMP activity relative to native CT. The mmCT was then co-administered with multiple antigens including orally and nasally delivered OVA, oral whole-cell cholera, and nasal influenza HA, eliciting robust mucosal cellular and humoral responses, hence sustaining adjuvanticity despite the absence of enterotoxicity. In a recent preclinical investigation, the mmCT adjuvant was combined with an *H. pylori* antigen. In the study, mice inoculated intragastrically with an *H. pylori* antigen adjuvanted with mmCT or CT developed comparable protection with a 50- to 125-fold reduction in *H. pylori* colonization. The vaccine formulations induced robust systemic IgG and intestinal IgA responses, in addition to enhanced IFN-γ and IL-17A responses, displaying mmCT to be just as potent adjuvant as CT but a subvert of the toxic effects.

Another promising toxin adjuvant, which inspired the mutation of CT, is the heat-labile toxin (LT) from ETEC, which has many structural and functional similarities to CT. A portion of the A subunit of LT was shown to be cleaved by trypsin, resulting in the enzyme's toxic effects. Mutation of this region inhibited trypsin cleavage and improved the toxicity profile, although the protein still exhibited residual toxicity. This issue was resolved by the introduction of a second mutation, which produced the double mutant heat-labile toxin (dmLT). In tests using *H. pylori* and *Streptococcus pneumoniae* vaccines, dmLT was found to be a strong oral adjuvant, evoking systemic IgG, local IgA, Th1, and Th17 responses, similar to mmCT. In addition, dmLT has been utilized in a number of clinical trials for safety evaluation, with results suggesting that the toxoid is a safe and effective oral adjuvant. MmCT and dmLT would certainly be superior alternatives to CTB, and their investigation in conjunction with MC technology could be intriguing. Additionally, derivatives of mmCT and dmLT, such as CTA1DD or LThaK, have emerged and likewise shown promise as mucosal adjuvants. These could potentially also be included in further screening studies of mucosal adjuvants to employ in combination with the MCs.

### 3.1.2 Project II discussion

#### 3.1.2.1 Main Outcomes

In **Project II**, the AP205 cVLP platform was used, and the animal model was shifted from mice to rats due to technical difficulties and the rapid MC retention period reported in mice during **Project I**. The cVLP were lyophilized, with the process developed in **Project I** and subsequently analyzed with SDS-PAGE and transmission electron microscope (TEM), as it had never been freeze dried previously. These quality controls did not reveal any aggregation or visible change in conformation. The cVLP appeared capable of undergoing the lyophilization procedure based on these results. Rats were administered MCs loaded with lyophilized SpyC.AP205.L2, coated with the EL100 polymer via oral administration. The specific IgG and IgA responses were measured for the L2 protein and the AP205 backbone, and oral administration with MCs was compared with control groups. Unfortunately, it did not appear that the group administered MCs or any of the other groups receiving oral doses exhibited any detectable response.

#### 3.1.2.2 Considerations on the choice of adjuvants and antigens

In **Project II,** the AP205 platform technology employed in the study is promoted to be capable of establishing robust immune responses without additional immune stimulants other than the virus-like presentation of the antigens.[118] While this might be the case for i.m and i.n administration, the results of this study indicate that adjuvants are required in addition to a delivery mechanism for oral administration. In future studies with this VLP and the MCs, additional groups should be included, and formulated with effective mucosal adjuvants. This could, for example, be α-GalCer,

having generated promising responses with various antigens. In addition, it might be examined if replacing the antigens shown on the AP205 platform, for example, with antigens from oral infections such as *H. pylori* or Poliovirus, will have an effect.

In general, it would adequately be more optimal to test the proof-of-concept of the MCs with commercialized oral vaccines and investigate if they could be improved with the technology. A prominent choice could be the Typhi Vivotif vaccine, as this has already undergone a freeze-drying process before being encapsulated.

### 3.1.3 Lyophilization of the vaccine formulations in Project I and II

As previously explained, powders are currently more viable than liquids for MCs. Due to the vaccine formulation being a liquid solution, a drying technique was required to convert the liquid to a powder. The lyophilization drying procedure is complex and requires tuning of both process parameters and buffers in order to achieve optimal drying and prevent immunogenicity loss in the case of vaccines. First, the buffer conditions were optimized; 10% w/v trehalose, sucrose, lysine, and mannitol were used as excipients, along with 10 mM Tris, 10 mM Tris + 2% Glycerol, and PBS as additives. All experiments, however, resulted in formulation meltback, prompting a reevaluation of the lyophilization procedure (Fig. 18). The previous lyophilization program (not shown) was replaced by the one described in the paper of **Project I**, and the study was repeated. Various excipients at different ratios were lyophilized and evaluated by the consistency and aesthetic appearance in reference to Patel *et al.*[229] To achieve the best-dried state post lyophilization, 10% (w/v) Trehalose + 10 mM Tris was considered the most prominent excipient and additive (Fig. 18).

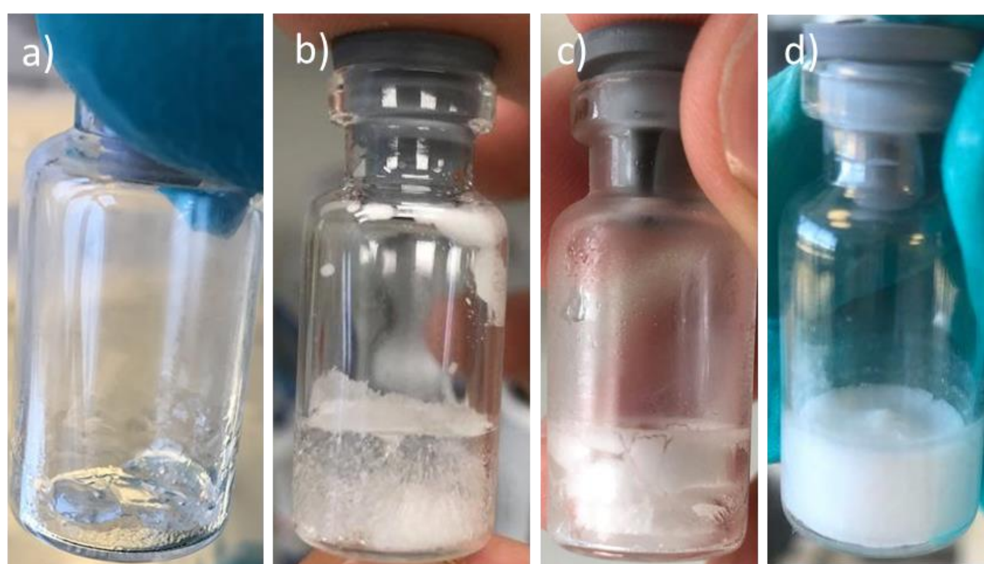

**Fig. 18.** Different dry states obtained by the two lyophilization protocols. Meltback was often reported when using the first lyophilization process along with the collapse of the cake (a, b). Following the optimization of the protocol, more intact compound cakes were observed (c). A well-preserved cake was produced with further optimization of the buffer and excipients, showing no signs of collapse (d). The lyophilized solution contained CTH522 + α-GalCer formulated in 10%(w/v) Trehalose + 10mM Tris.

To investigate whether lyophilization diminished the immunogenicity of the antigen, lyophilized [CTH522 + α-GalCer] was rehydrated and injected subcutaneously to mice as a prime-boost regimen. The study showed that lyophilization had no effect on immunogenicity since there was no difference in the assessed immune response between the lyophilized and non-lyophilized groups. The developed lyophilization process and optimized buffer formulation are thus capable of effectively lyophilizing CTH522 in formulation with α-GalCer, without damaging the antigen and successfully retaining the immunogenicity. The developed protocol was also used to lyophilize the AP205 VLPs in **Project II** and could potentially be used for other such formulations in future studies. After being lyophilized, the formulations for the MCs were kept at −20° alongside the liquid formulations until use. It would have been interesting to explore how the heat stability and storage requirements for lyophilized formulations were imposed. This might be investigated by exposing them to different temperatures for varying amounts of time, followed by immunization trials with reconstituted samples. Moreover, using the established lyophilization technique for commercial oral vaccines may also be of interest, as the existing storage temperature requirements for licensed vaccines range from 2°C to 8°C or −20°C, depending on the vaccine. Functional lyophilization of the current licensed oral vaccines would enable them to be trialed with the MCs. Furthermore, improving the thermal stability of the vaccines would be of great interest for their commercial value.

### 3.1.4 Considerations on immunization regime in Project I and II

The trend observed in **Project I** of the α-GalCer + CTH522 formulation to slightly enhance local and systemic responses from the oral boosters, subsequent to the s.c. prime, displays a prime-pull effect. The prime-pull concept functions by (1) priming systemic immune cell responses by parental vaccination and are followed by (2) recruitment of the primed cells to the target tissue/organ for the establishment of long-term protective immunity.[230] Several recent studies have employed this method and achieved promising results.[231–233] The prime-pull strategy may be the most optimal method for technologies such as the MCs, to induce a robust and protective immune response in the mucosal tissues. Moreover, a recent study on the antibody and germinal center kinetics following immunization with CAF01 reported delayed germinal center formation. It indicated that the first booster should be administered later than four weeks after priming.[234] This

suggests that the employed immunization regime may not be optimal, as boosters were administered three weeks after the CAF01 prime. For **Project II**, it is likely also the case that a prime-pull strategy will be more efficient in eliciting a response. Groups following this regime should be included in future studies with AP205 cVLP and the MCs.

### 3.1.5 MCs kinetics and immunological challenges

Due to the relatively weak measured immune responses in **Project I**, the MCs were hypothesized to be proportionately too large for the mouse gut. If this were the case, the containers would be pushed along the intestine by peristalsis and transiting food and fluids regardless of being mucoadhesive. This was investigated by loading the MCs with $BaSO_4$, as a contrast agent that is visible on CT scan and X-ray. This analysis showed that the transit time from the stomach to the cecum of mice was about 1–1.5 h, corresponding to a mouse's standard transit time of food. Before an adequate immunological response can be stimulated, it may be necessary to lengthen the transit time. Moreover, the average diameter of a mouse's intestine is 2 mm, and the mucus thickness is 20–25 μm.[235] Proportionally, the MCs can be considered quite large compared to the mouse intestine, by taking up 12.5%–15% of the intestinal diameter, depending on the orientation. In addition, due to their size, the MCs cannot be sufficiently embedded into the mucus of mice and will be moved forward by peristalsis, along with fluids and consumed food. These observations suggest that the MCs are not very compatible with the mice model.

Future studies with devices in the 100 μm range will likely require the use of larger animals to accurately evaluate their function. It would also be interesting to investigate if customizing the lid for release in the distal part of the intestine could improve the response. As mentioned in section 2.2.3, this portion of the gut is thought to promote the induction of effector T cells, while the release in the proximal intestines promotes the stimulation of $T_{reg}$.[49] In a similar context, it could be speculated whether release in the correct place is significant to establish a response, rather than focusing on retention. However, some form of retention would still most likely be needed as a means of targeting the "correct location" due to the constant dynamic movement of the intestine.

## 3.2 MCs as a tool for oral vaccination of European sea bass

In this section, the rationale, methods, and results acquired in **Project III** will be presented and discussed, in which the MCs were used to deliver a VNN antigen to European sea bass orally. The studies were performed in collaboration with DTU Aqua and involved the mentioned people below:

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[*]*These authors contributed equally to the work.*

Mass vaccination in fish farms is one of the most effective strategies for preventing disease, but it can be difficult and is connected with the stress of fish handling. The production of European sea bass (*Dicentrarchus labrax*) is severely affected by disease and mortality caused by infection with the betanodavirus RGNNV.[236] Two oil-adjuvanted intraperitoneal injection vaccinations are marketed commercially in several Mediterranean countries. Although these vaccines are effective, they can induce severe side effects such as peritonitis with internal adhesions, melanosis, and reduced growth.[129,130,237] The vaccines are registered for use in fish weighing between 12 and 15 grams, but the disease is more severe in larvae and fry, underscoring the need for a vaccine that can induce protection in smaller fish.[238] For this purpose, the RGNNV VLP has been shown to be effective as a nonadjuvanted injectable vaccine, capable of eliciting protection against RGNNV-caused illness in fish as small as 5 g without the typical side effects of adjuvanted vaccinations. However, injection vaccination in small-sized fish is usually not cost-effective and at the young stages, the fish are more susceptible to complications like physical damage and stress following intensive handling.[130] Oral vaccination could be a key technique for enhancing the logistics of the mass vaccination procedure in fish farms, as it has been shown to produce less stress in the fish, but it has proven less effective for establishing protection.[239] Earlier experiments by *Wi et al.* suggested that RGNNV VLP expressed in *Saccharomyces cerevisiae* extract might be suitable for oral vaccination.[76] In this study, a 57% survival rate was achieved, which was much greater than the included mock group, but should be further optimized before being called effective. Some of the administered antigens are most likely enzymatic and chemically degraded in the stomach, which could be why a higher survival rate was not observed. Further, it is possible that facilitated and targeted delivery to the intestine could enhance the interaction with the immune cells, and in extension the immune response. The MCs were therefore proposed as a delivery tool for RGNNV VLP to overcome the gastric challenges and effectively deliver and retain the antigen in the intestine. However, MCs have never been trialed in a fish model previously and before actual immunization were possible, a dosing method needed to be established along with confirmation that the MCs does not cause harm to the fish, when orally administered.

### 3.2.1 MC administration to sea bass

For oral dosage of MCs to fish, two methods were investigated. First, an attempt was made to "glue" the MCs onto feed pellets, which were then fed to sea bass for voluntary consumption. Fish oil was received from the Danish fish feed company Biomar (Brande, Denmark) along with uncoated feed (BioVet, 0.8 mm pellets). The oil usually coats the fish feed to add fat to the extruded pellet and thereby reach needed dietary requirements. In this case, however, the feed pellets were received dry in order to combine the coating with oil and microcapsules, utilizing the oil as a glue. To seal the oil, 10 g of feed was combined with 500 µL of oil and 2 chips of MCs (a total of 1250 MCs) before being heated at1 37°C for 24 h. Sea bass was isolated in a bucket containing saline water and an airstone. The buckets contained feed containing MCs, which the fish ate voluntarily. Following euthanasia with an overdose of benzocaine solution, sea bass were dissected and examined under a fluorescence microscope 8 hours later (not shown). No MCs were observed in the dissected GI tract in any of the included seabass. The most likely explanation is that the oil was ineffective as an adhesive, causing the MCs to separate from the feed when poured into water. Another explanation could be that the MCs may have already passed through the digestive system upon euthanization, assuming the sea bass consumed the feed. Furthermore, it could be that the sea bass simply did not consume the feed due to the stress of handling and being isolated. Although this method would be most applicable in the field, for research purposes and to investigate the fate of the MCs, this method had too many uncertainties about what happened with the MCs. Therefore, we proceeded to explore a delivery option where we could be certain that the MCs were delivered within the GI of the fish.

The second method was inspired by the oral gavage of rodents, where MCs would be orally administered directly to sea bass to ensure consumption of the MCs. MCs were enclosed in a size 9 gelatin capsule, and a 1 mL syringe ("Inject-F 1ml luer," B Braun, Melsungen, Germany) was fitted to hold the capsule. Sea bass were isolated and anesthetized in 0.04% (v/v) benzocaine solution. The sea bass mouth was kept open with a tweezer, and the gelatin capsule containing MCs was placed at the back of the throat of the fish using the 1 mL syringe, releasing the pill with the syringe stamp (Fig 19). The fish were then placed in saline water and surveyed until awakening from anesthesia and with no visual signs of displeasure. Fish were subsequently euthanized by an overdose of benzocaine solution at time points 4 h, 6.5 h, 8 h, and 24 h postintubation.

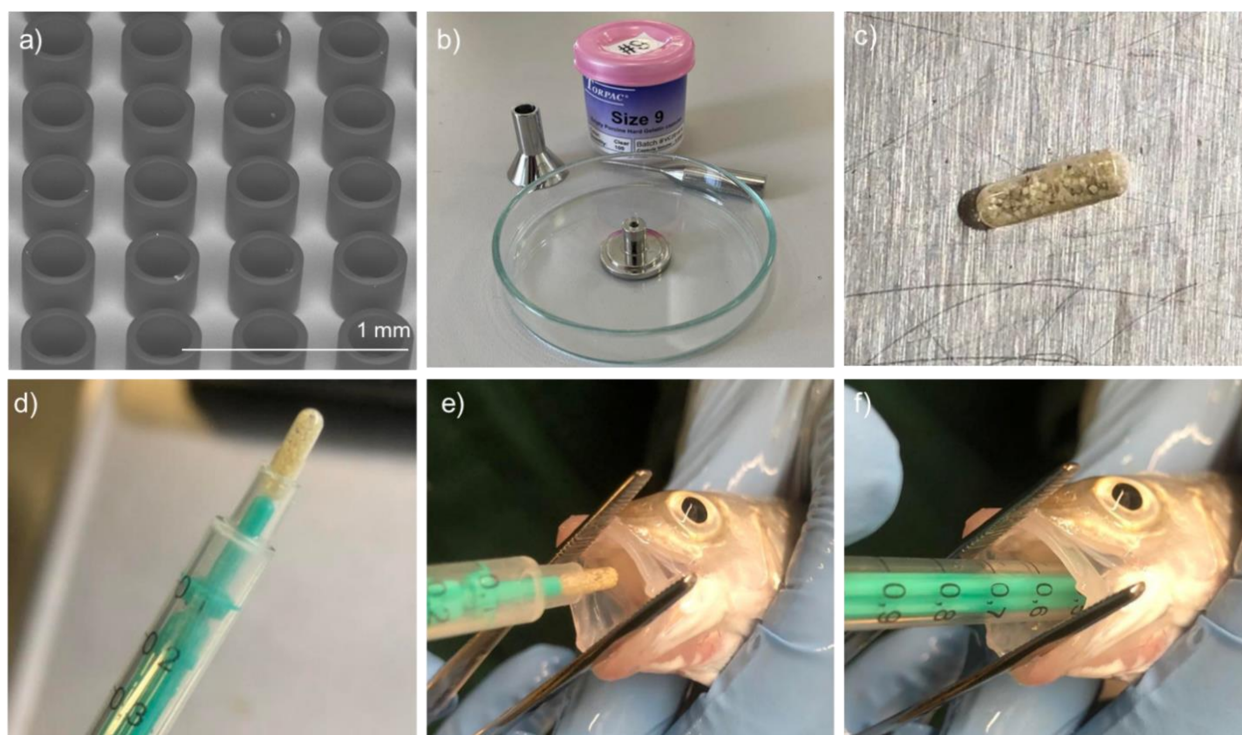



**Fig. 19.** SEM image of the produced microcontainers (MCs) (a). Process of filling MCs into size 9 gelatin capsules, using a funnel (b). Filled gelatin capsule with approximately 1,000 MCs (c). Fitted syringe used for the dosing of sea bass (d). Oral gavage procedure of anesthetized sea bass (e, f).

After euthanization, the fish were dissected, and digestive tract sections were isolated and examined by a fluorescent microscope (Fig. 20). Here, it was evident the MCs had been successfully delivered to the fish and were able to pass through the GI tract of the fish. After 4 h, MCs were observed in the stomach; after 6.5 h, MCs could be seen in the intestine (Fig. 20b, 20c, 20d); after 9 h, the MCs seemed to have cleared the fish, with only a few containers visible in the posterior intestine. The GI tract of sea bass euthanized at 24 h was likewise empty. This revealed a total transit time of approximately 8 h, with an intestinal transit time of 3–5 h. Furthermore, the MCs appear safe for use in fish, as the sea bass were observed regularly post administration, with no signs of visual discomfort or harm appearing throughout the experiment. Neither macroscopic signs of local reaction nor obstruction were seen in the intestines. Eventually, it will be necessary to figure out a more feasible way of administrating the MCs to sea bass. For instance, MCs could be directly incorporated into the fish feed, which would be the most optimal way of orally dosing the fish with the devices.

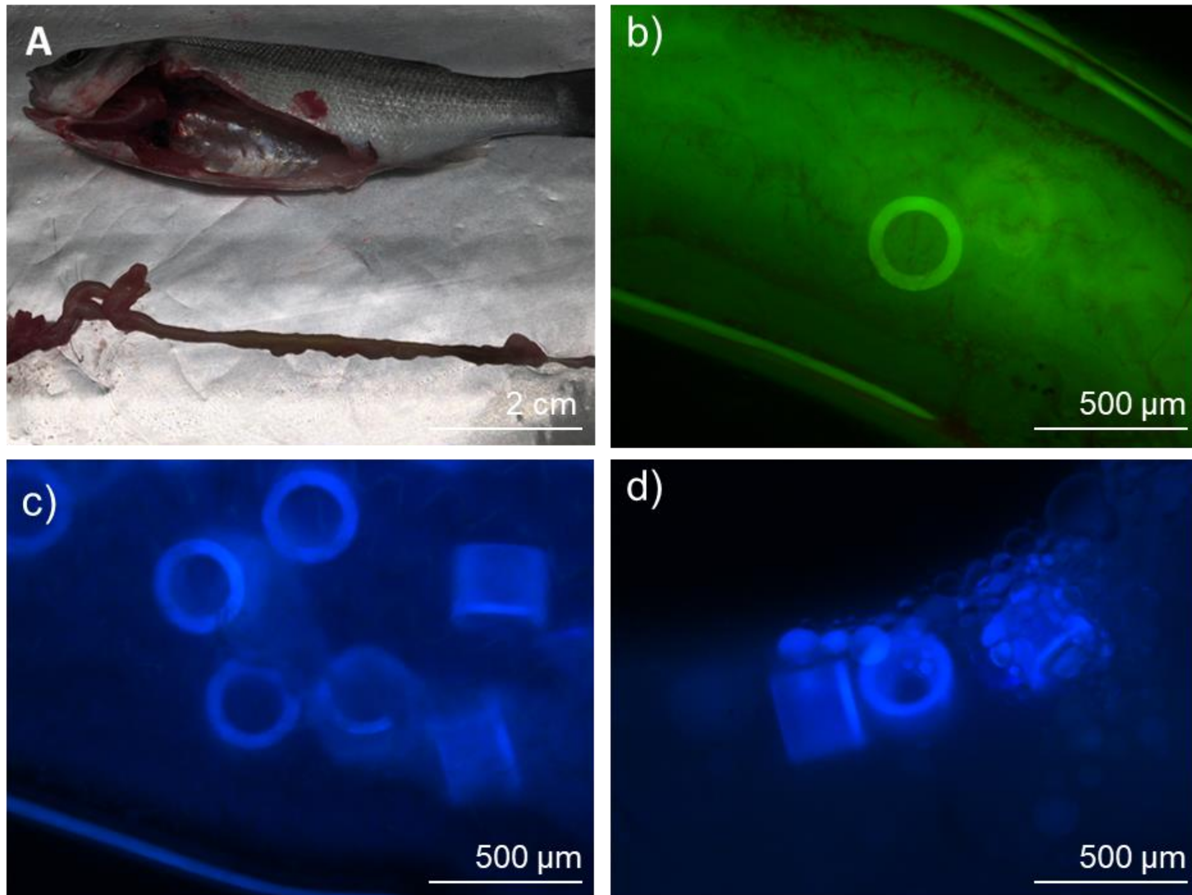



**Fig. 20.** Dissected sea bass with isolated stomach and gut (a). Green fluorescent image of the intact proximal intestine, displaying microcontainer (MC) 6.5 h after oral administration (b). The content of the intestine displayed autofluorescence in the green spectrum resulting in a high background. Therefore, violet fluorescence was used to distinguish the MCs better. Violet fluorescent images of the intact distal intestine, displaying empty MCs at two different locations 6.5 h after oral administration (c, d).

### 3.2.2 Loading and release of *P. pastoris* RGNNV VLP extract

In this study, an RGNNV VLP (designed by Ansgar Stratmann of W42 Biotechnology in Germany) was expressed in a *P. pastoris* strain. To facilitate shipment, the harvested *P. pastoris* extract was lyophilized and received as a powder. This type of powder has never been loaded into MCs previously, so various loading strategies were utilized to identify the most effective method. Three different loading methods were tested, using 10 MC arrays per method, subsequently determining the weighted average per MC (Table 2, Fig. 21a). This test demonstrated that embossing was the least efficient way for filling this powder, whereas centrifugation and manual filling with a brush were equally efficient. The manual filling method was employed in future experiments as this was the most reproducible and time-efficient approach.

**Table 2** The average weight calculated from 10 arrays of microcontainers loaded, using one of the three different filling methods. Data are presented as the mean ± the standard deviation.

| Method | Weight pr. MC |
| --- | --- |
| Embossing | 2.08 µg ± 0.37 µg |
| Centrifugation | 2.76 µg ± 0.24 µg |
| Manual filling | 2.81 µg ± 0.31 µg |

The MCs function by protecting their content through the stomach's acidic environment and by facilitating release upon reaching the intestine as a feat of pH-degradable polymerics lids and adhesive traits of the MCs.[212,213] The pH levels in the GI tract of European sea bass have been measured to be between 5.3 and 5.7 in the stomach and 6.5 and 6.8 in the intestine.[240] With this information, the pH-degradable polymer EL100 was chosen as the coating for the containers.[206] Due to the documented variance in the stomach pH and the higher pH measurement of 5.7 being very close to pH 6, a visual *in vitro* study with maleic acid was conducted to get an indication of the polymer functionality. MCs were spray-coated with EL100 lids, which were measured to be 27.3 ± 2.1 µm by contact profilometry (Fig. 21b). This lid thickness was chosen based on previous studies with rats, which have a similar transit time from the stomach and through the intestine as the sea bass.[213,241] After coating, the MCs were submerged into maleic acid with pH 5.7 for 60 min (Fig. 21c), simulating the higher stomach pH in seabass. The MCs were subsequently bathed in maleic acid with a pH of 6.5 for 60 min (Fig. 21d), mimicking the lower intestinal pH of seabass. The SEM pictures confirmed that this coating most likely could be used to target delivery in the intestine of seabass. After 60 min in pH 5.3, the EL100 polymer lids remained intact with no apparent loss of content. When exposed to pH 6.6, the EL100 polymer progressively dissolved, and the capsid protein containing *P. pastoris* was liberated from the MCs.

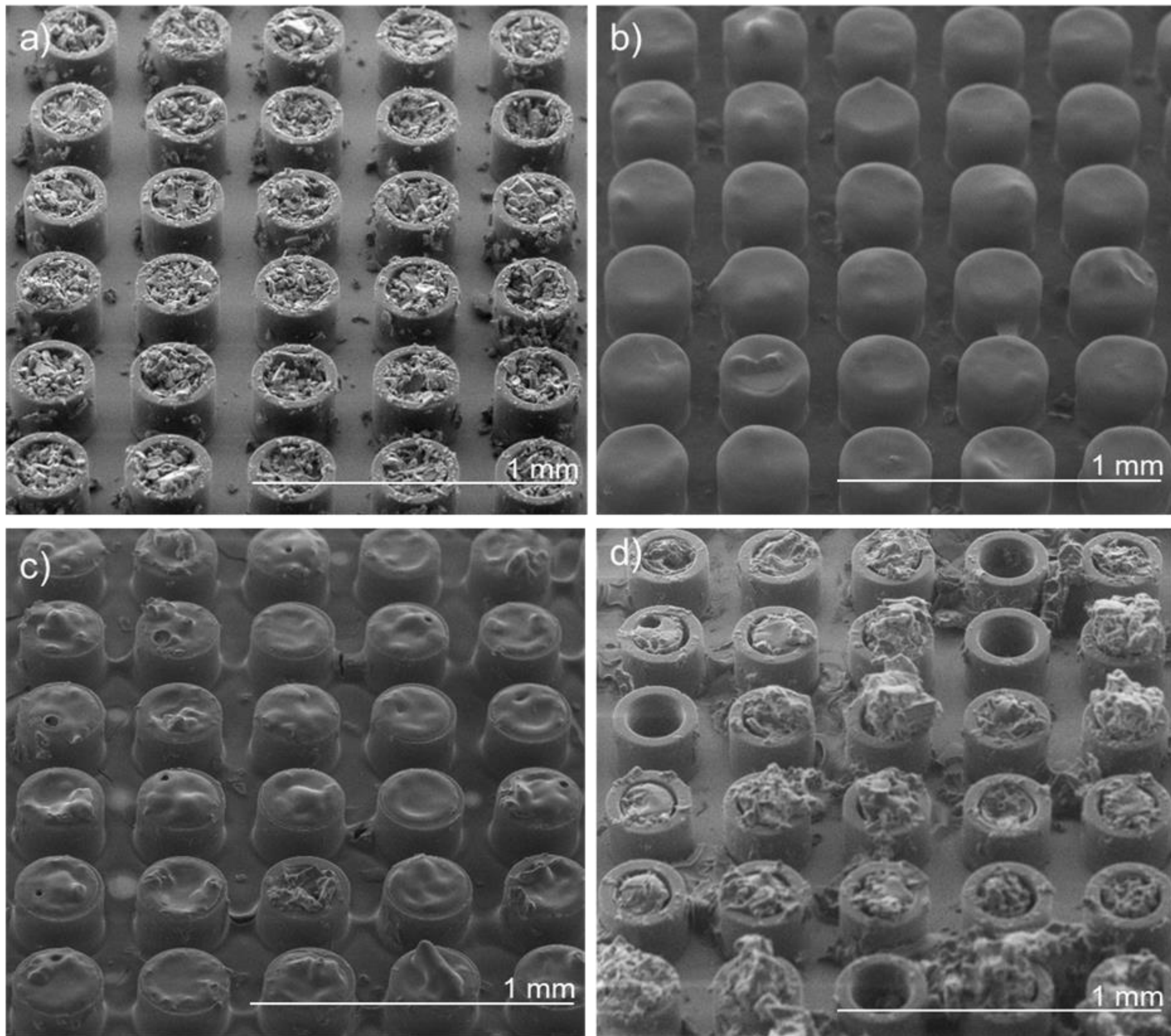



**Fig. 21.** SEM image of microcontainers (MCs) loaded with the *P. pastoris* extract containing the RGNNV VLP (a). SEM image of MCs coated with Eudragit L100 polymer (b). SEM image of MCs after being submerged for 60 min in 10 mM maleic acid at pH 5.3, corresponding to the pH found in the sea bass stomach (c). SEM image of MCs after being transferred and submerged for 60 min in 10 mM maleic acid at pH 6.6, simulating the environment in the sea bass intestine (d).

Next, it was investigated if this was also the case in an *in vivo* setting. The sea bass were administered loaded and coated MCs orally, and the fish were killed 5.25 hours later. Content from the stomach and the intestine was extracted, and identified MCs were isolated (Fig. 22). It was observed that MCs found in the stomach of the seabass still had the EL100 lid intact, verifying the observed results from the *in vitro* study (Fig. 22a, b). Also coherent with the *in vitro* results, the MCs found in the proximal intestine were mainly empty with no apparent coating left (Fig. 22c, d). It could not be identified from the SEM pictures whether the remaining substance was *P. pastoris* extract or intestinal residue. These observations confirm that the MCs and the EL100

coating function as intended, promoting the MCs as a candidate for oral delivery of vaccines to fish.

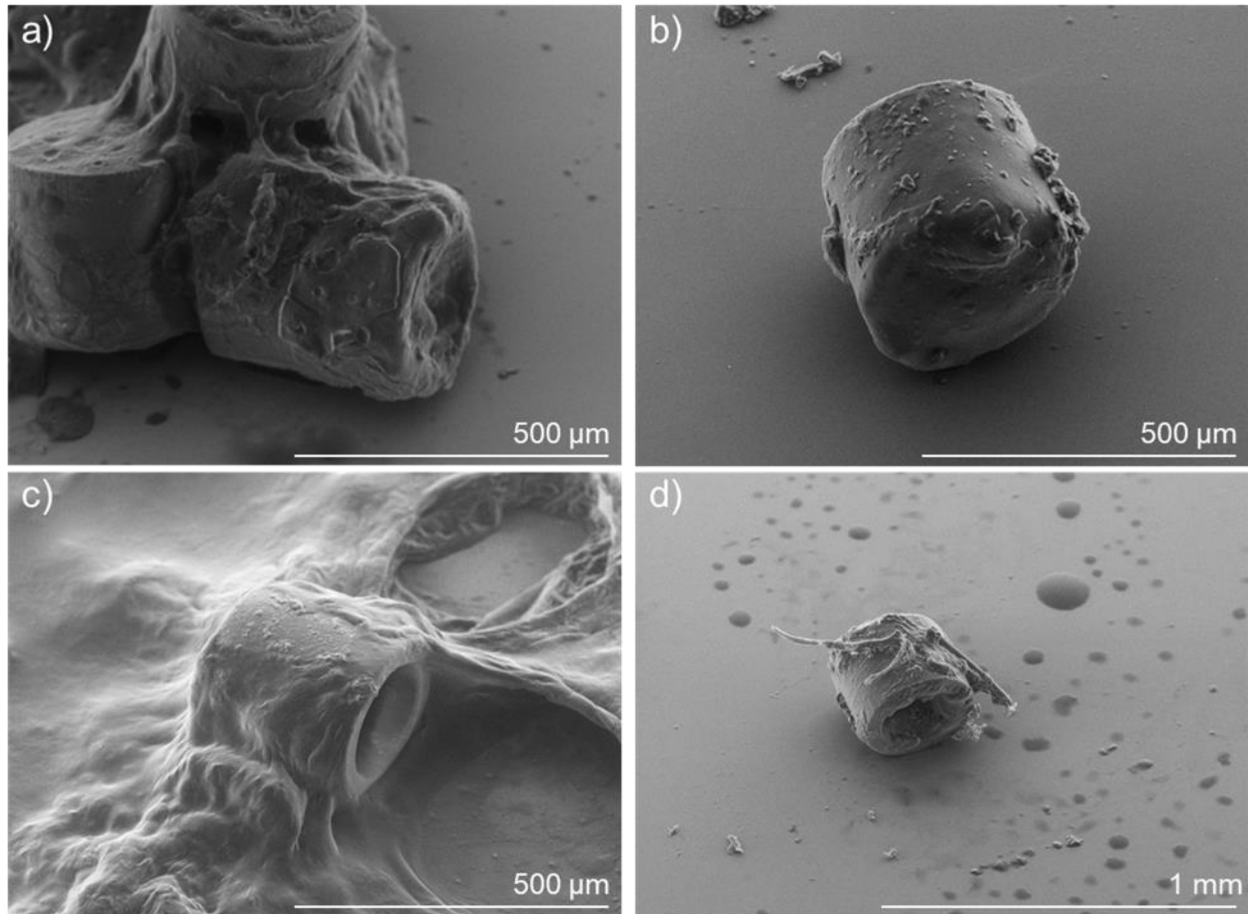



**Fig. 22**. SEM images of microcontainers extracted and isolated from the stomach (a, b) and the intestine (c, d) of sea bass, 5.25 h post oral administration

### 3.2.3 Viral nervous necrosis challenge of sea bass

The preliminary studies determined that the MCs were safe for use in sea bass and capable of facilitating release in the fish intestine. It was then hypothesized that delivery of the RGNNV VLP orally in MCs could induce an effective immune response against VNN. To investigate this, a challenge study was conducted.

Sea bass were divided into six groups (Table 3), containing 25–28 fish in each group. Two lyophilized *P. pastoris* extracts were produced (Ansgar Stratmann, W42, Biotechnology GmbH): one not expressing and one expressing the RGNNV VLP. To determine the concentration of RGNNV VLP in the *P. pastoris* extract, a western blot was conducted with known amounts of previously purified capsid protein (Fig. 23). Using the GeneTools software, the intensity of the bands developed from the *P. pastoris* extract samples was compared to the bands representing

400 ng and 200 ng capsid protein, as these bands represented unsaturated concentrations of antigen. A final concentration of 50.9 ng VLP/µg *P. pastoris* extract was calculated from this.

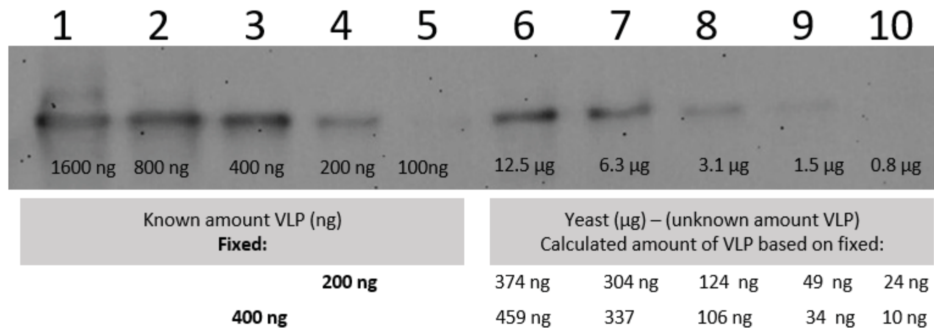



**Fig. 23.** Western blot of an RGNNV standard curve (rows 1–5) and the *P. Pastoris* extract expressing the RGNNV VLP in different amounts. 400 ng and 200 ng developed the clearest bands and were chosen as references for the *P. Pastoris* samples. *Reprinted with permission from the Ph.D. thesis of Sofie Barsøe*

Based on the measured concentration and the results from the loading optimization study, the two groups receiving MCs were dosed with a total of 300 µg of *P. pastoris* extract, corresponding to a dose of 15 µg RGNNV VLP for the group receiving the VLP expressing extract. To identify the effect of MCs, two groups were administered the same amount of extract dissolved in sterile saline via oral gavage. A mock-vaccinated group was included as a negative control, receiving saline by oral gavage. Finally, a group injected intraperitoneally (i.p.) one time with 40 µg purified VLP served as a positive control.

**Table 3.** The groups included in the challenge study, with dosing amounts of *P. pastoris* and VLP calculated from the western blot (Fig. 22). Numbers in ( ) represent the double dose given on day 29. VLP = virus-like particle, MCs = microcontainers, i.p. = intraperitoneal.

| Group | Treatment | Volume | Dose (*P. pastoris* | Dose (RGNNV VLP) | Route |
|---|---|---|---|---|---|
| **1. *Pichia* + VLP** | Lyophilized *Pichia* expressing VLP dissolved in saline (3 µg/µl) | 100 µl (200 µl) | 300 µg (600 µg) | 15 µg (30 µg) | Oral gavage |
| **2. *Pichia* ÷ VLP** | Lyophilized *Pichia* dissolved in saline (3 µg/µl) | 100 µl (200 µl) | 300 µg (600 µg) | - | Oral gavage |
| **3. *Pichia* + VLP** | Lyophilized *Pichia* expressing VLP dissolved in saline (3 µg/µl) | 100 MCs (200 MCs) | 300 µg (600 µg) | 15 µg (30 µg) | Oral gavage (with a fitted syringe) |

| | | | | | |
|---|---|---|---|---|---|
| **4. *Pichia* ÷ VLP** | Lyophilized *Pichia* dissolved in saline (3 µg/µl) | 100 MCs (200 MCs) | 300 µg (600 µg) | - | Oral gavage (with a fitted syringe) |
| **5. Saline** | - | 100 µl | - | - | Oral gavage |
| **6. VLP** | Purified VLP 800 µl/ml | 50 µl | - | 40 µg | i.p. injection |

Groups receiving oral doses were immunized in a prime-booster-booster-booster regime on day 0, 9, 16, and 29. In the final dose on day 29, the dose was doubled. The fish were distributed evenly into two replicate tanks (Fig. 24) and tagged with fluorescent elastomer (VFIE tag, North West Marine technology) on the dorsum, according to the group. With this method, the groups can be identified, and for each boosting, the fish were sorted into separate buckets prior to being dosed (Fig. 24). After the last immunization day, blood samples were taken from four fish of each group and analyzed with ELISA to determine if a systemic IgM response had been established (Fig. 25). The $OD_{450}$ values showed no indication of IgM detection in any group receiving oral administrations, except for one fish in group 4 receiving *Pichia* ÷ VLP in MCs. The i.p. The VLP group displayed a robust measure of IgM levels, which was also expected for this group. These results indicate that oral administration with MCs and RGNNV VLP could not induce a systemic IgM response. However, even though no systemic response was observed, a local response in the fish GI tract could potentially be present.

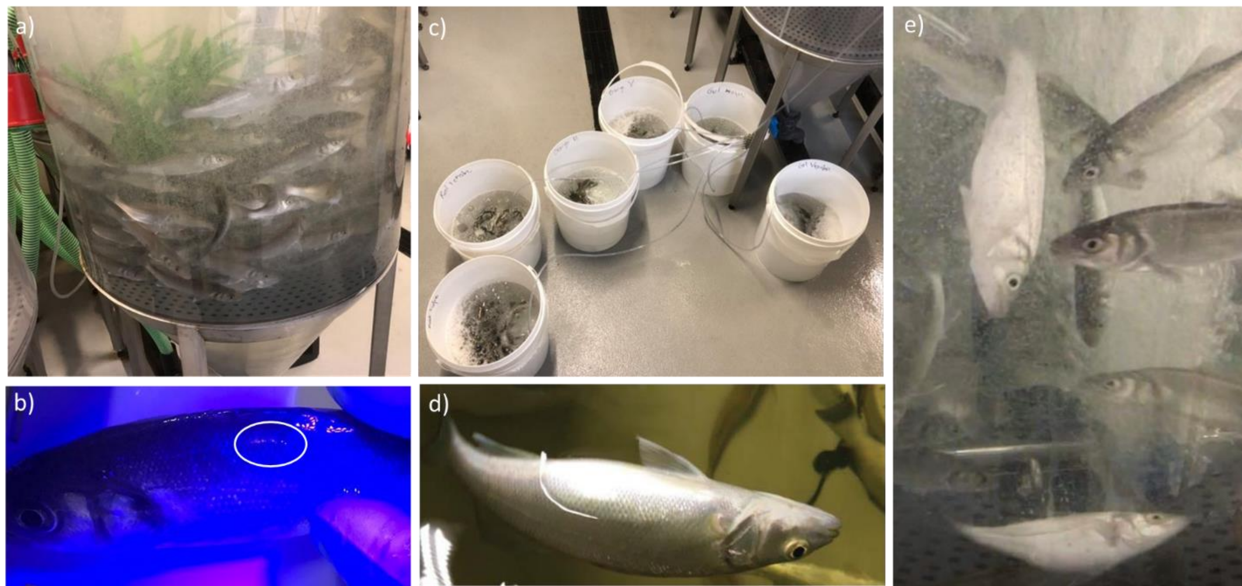



**Fig. 24.** Methodic pictures from the immunization and challenge process of sea bass. Tank with approximately 60 fish (a). The groups were divided equally into two of these tanks. Fish were fluorescently tagged according to the specific group (the tag is marked with the white circle) (b). During the immunizations, fish were divided into buckets and dosed

accordingly (c). When infected with VNN, early symptoms include apparent lethargy and vertical spinning, easily observed (d, e). *Pictures d and e are reprinted with permission from the Ph.D. thesis of Sofie Barsøe.*

On day 64 (35 days after the last oral dose), the fish were bathed with RGNNV (strain 2009.283, $1.73 \times 10^6$ $TCID_{50}$/ml, for 7 h in 13 L saltwater.[242] Early symptoms of VNN are a spiraling swimming pattern and lethargy, which are easily noticed with visual inspection (Fig. 24). Therefore, the sea bass were monitored several times daily, and fish showing symptoms were euthanized and recorded. On day 22, no signs or symptoms appeared for 7 days, and the experiment was stopped, designating all remaining fish as survivors.

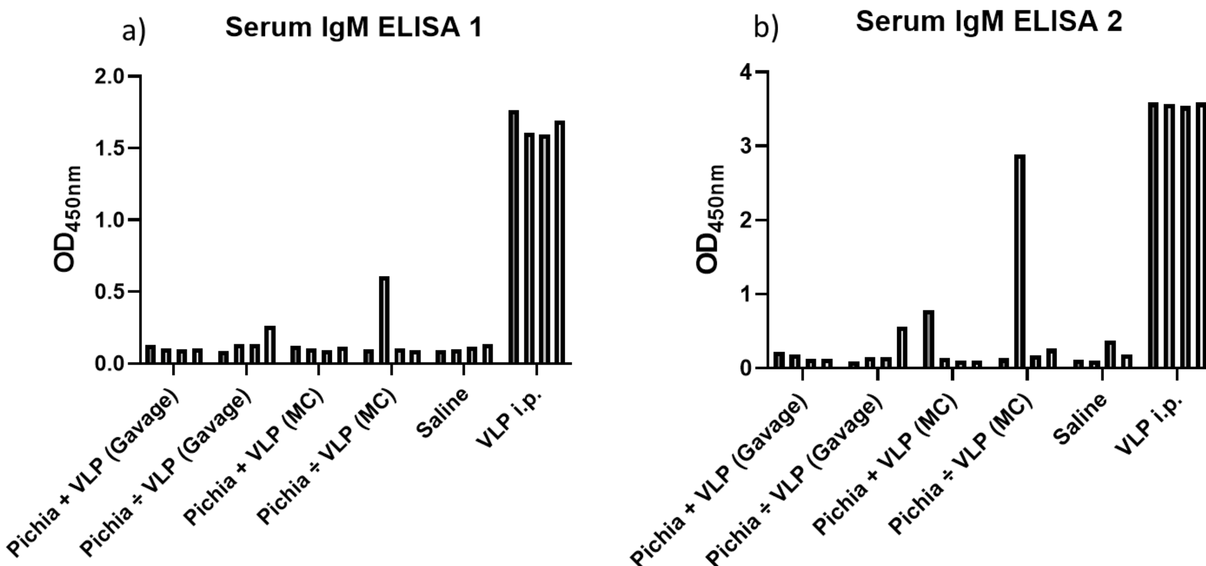



**Fig. 25.** Plotted optical density (OD) values measured at 450 nm. Serum from four fish was analyzed for antigen-specific IgM with ELISA developed at 9 min (a) and 18 min (b).

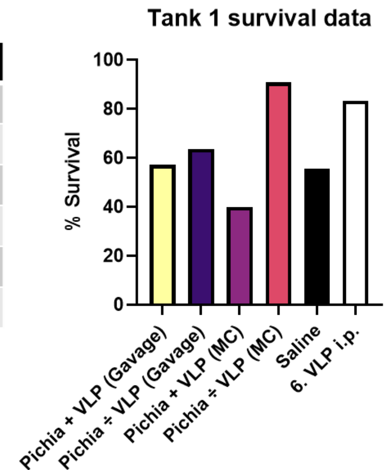
During the experiment, some incidents occurred, which could have influenced or compromised the results, primarily regarding practical issues in the caretaking of the sea bass. In one event, the caretakers accidentally loaded freshwater instead of saltwater in the tanks sometime between the second and third booster. However, the exact date was not logged. The sudden shift in salinity can induce stress for the fish, which can compromise normal immune functions.[243] Furthermore, some fish were lost due to improper securement of the lids, resulting in the fish jumping out of the tanks. Finally, during the challenge period, the air supply was compromised twice, resulting in acute deaths from oxygen deprivation. This happened in the same tank, resulting in the loss of 19 fish in this tank. In total, 23 fish were lost due to these practical issues. As the group sizes were already small, the loss of these fish greatly influenced the study's statistical power.

Therefore, the results should be interpreted with caution. Ideally, the study should be repeated in an improved experimental setup.

Due to the above-mentioned complications, the survival rates from the two tanks are presented separately. The groups receiving *P. pastoris* extracts in solution by oral gavage displayed the lowest survival rates, together with the group receiving *P. pastoris* expressing the RGNNV VLP in MCs, comparable with the mock group (Fig. 26). Interestingly, the group receiving *P. pastoris* without VLP in MCs did display high survival rates in tank 1. As noted earlier, one fish from this group also displayed higher IgM levels compared to the other orally dosed groups (Fig. 25). Due to this, it was suspected that the correct extract had been dosed to this group. However, western blot analysis of the extracts confirmed this was not the case (not shown). Further investigation is needed to determine the exact reason for this. The group receiving i.p. VLP demonstrated survival rates of approximately 90% in both tanks, in coherence with previously presented studies using this vaccine.[128,129] Despite the high survival rate of this group, they do fall a little short compared to the study from *Barsøe et al.*[128] The accidental shift from saltwater to freshwater could very well be the reason for this. Furthermore, the mock group displayed lower than expected survival rates, indicating that the fish were more sensitive to infection than previously observed.

a)

| Treatment | Total (n) | Censored (n) | Diseased (n) | Survivors (n) |
|---|---|---|---|---|
| 1. ***Pichia* + VLP** | 8 | 1 | 3 | 4 |
| 2. ***Pichia* ÷ VLP** | 11 | 0 | 4 | 7 |
| 3. ***Pichia* + VLP** | 10 | 0 | 6 | 4 |
| 4. ***Pichia* ÷ VLP** | 11 | 0 | 1 | 10 |
| 5. **Saline** | 9 | 0 | 4 | 5 |
| 6. **VLP** | 13 | 1 | 2 | 10 |




b)

| Treatment | Total (n) | Censored (n) | Diseased (n) | Survivors (n) |
|---|---|---|---|---|
| 1. ***Pichia* + VLP** | 9 | 2 | 3 | 4 |
| 2. ***Pichia* ÷ VLP** | 11 | 5 | 4 | 2 |
| 3. ***Pichia* + VLP** | 10 | 2 | 3 | 5 |
| 4. ***Pichia* ÷ VLP** | 11 | 3 | 4 | 4 |
| 5. **Saline** | 8 | 1 | 3 | 4 |
| 6. **VLP** | 11 | 1 | 1 | 9 |

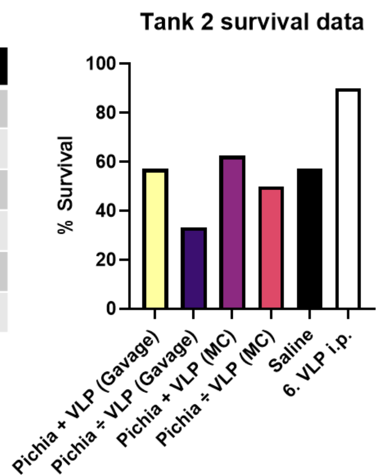



**Fig. 26.** The raw data collected after the challenge in tank 1 (a) and tank 2 (b), along with the calculated and plotted percentage survival for each tank. Censored fish were removed from the study due to practical failures.

## Summary

A method for the oral administration of MCs was developed; to my knowledge, this is the sole method documented for European sea bass. Furthermore, the MCs were deemed safe to use in this animal model. The function of the MCs with EL100 lids in sea bass was assessed *in vitro* and *in vivo* and was observed to facilitate target release in the intestine, similar to other trialed animal models. A challenge study with the VNN virus was conducted to investigate if the MCs could effectively deliver the RGNVV VLP and establish a protective response. The survival rate of fish dosed with MCs was comparable to the mock group and displayed no seeming stimulation of a protective response. However, many practical complications were experienced during this study. Ideally, the study should be repeated to investigate the abilities of the MCs properly.

## 3.3 Assessment of the MCs to orally deliver vaccines and the animal models

*In this section, the ability of the MCs to deliver vaccines orally is discussed, based on the results and outcomes from **Projects I–III**. Furthermore, a short summary of the animal models and their pros and cons regarding experiments with MCs is included.*

It is not possible to directly compare the three projects as they included different animal models and vaccine formulations, but some general technical information can be derived from them. The most promising example of the MCs' ability to deliver vaccines and stimulate a response was observed in the first study (**Project I**). Even without an s.c. prime, the MCs did manage to generate a weak, but measurable, response—which was not the case in the other studies. This study also utilized the prime-pull effect, with indications that this method might be the most promising with the MC technology, similarly observed by Laier *et al.*[207] The shift between animal models was exploratory and based on the fact that the employed vaccine components in each of the studies had been reported to elicit mucosal immune responses, through either oral or nasal administration, in the animal model in question. This was with the exception of rats, although the AP205 VLP had been observed to stimulate mucosal response nasally previously, which was likewise shown in this thesis. The choice of rats as an animal model was made on account of the fast transit of the MCs in mice along with methodological and technical problems with the mice and MC administration, which will be discussed further in section 3.3.1.

It was evident that the vaccine formulation is of high importance, especially concerning the use of effective mucosal adjuvants. In **Project II**, no immune stimulation was observed from oral dosing of the AP205 VLP, which was likely due to the absence of adjuvants. The case is somewhat different concerning **Project III** where the addition of an adjuvant could affect the safety risk while also increasing the cost of the vaccination of fish. In a fish farm mass vaccination, it is vital to take into account what the most feasible option is. This especially concerns cost and time, and the holy grail, in this case, would be to only vaccinate the fish once in an early stage, with minimal production loss risk. This criterion also limits the use of the prime-pull method in this setting. Furthermore, MCs would also need to be developed as a fully biodegradable system to be employed in a fish farm setting. This would most likely also be the case for the technology to be licensed for human use.

The search for compatible biodegradable materials to produce MCs is ongoing and has initiated several studies with various materials and production methods. The SU-8 polymer functions by cross-linking when exposed to UV light. Needless to say, if the SU-8 is replaced with a material that does not crosslink by UV exposure, the production method also needs to change. Finding a

suitable material can be challenging, and several parameters need to be taken into account, such as the versatility of the new material and production method, the stability of the developed devices, the modifiability of the design, drug/vaccine loading amount, safety, and cost. Recently, Abid et al. explored some of these parameters with the biodegradable polymer poly-ε-caprolactone (PCL) and developed a single-step hot punching approach for fabrication. Using a Nickel stamp on a substrate of poly(vinylalcohol) (PVA), containers were successfully punched in PCL with a diameter of 230 μm, height of 95 μm, and reservoir height of 65 μm. Paracetamol was used as a model drug in the study and was successfully loaded into PCL MCs in a volume of 2.4 μg. Eudragit S100 was spray coated onto the PCL MCs, which were evaluated for their release profile *in vitro* and their pharmacokinetic profile *in vivo* in rats. The results acquired from these studies indicated that sustained release was achieved by the PCL containers and displayed similar properties as the SU-8 containers in other studies. This study suggests PCL as a variable candidate to replace SU-8 along with the hot-punching fabrication method, being time efficient and scalable.

In this thesis, the studies suggested that retention was a major issue in the various animal models. The MCs did not seem to be retained in mice, which also influenced the decision to choose rats for **Project II**. Although some retention might have been established in sea bass and rats, no seemingly positive effect on the immune response was observed. This begs the question of whether retaining the formulation in the intestine will have an enhancing effect on the stimulated response. A difference between drug and vaccine delivery is that vaccines can have an impact without being absorbed into the bloodstream. This is an advantage, as extensive research to enable the efficient absorption needed for drugs might, in principle, not be necessary for vaccines. However, a disadvantage is that the immune system needs time to locate and interact with the vaccine formulation and to be established, thus requiring a longer absorption window than most drugs[56]. It is therefore likely that the implementation of more drastic design changes, which optimize the retention, could enhance the immune response. The increased retention time observed in sea bass and rats was only 1–2 h, but it might be that retaining the formulation longer, for instance, 12+ h, would establish an enhancing effect on the response. While they cannot be directly compared, the differences in response between the mice and rats are most likely because of the employed vaccine components, even though the MCs were more retained in the rats. By appearing to be more retained in larger animals, the choice of the animal model suggests to play a major role both in the function of the MCs and in the methodology of the animal experiments.

### 3.3.1 Lessons learned from the *in vivo* studies

In the projects, various technical complications arose from the *in vivo* studies regarding the MCs. In the first study, mice were used due to being a well-known model for immunization studies and have extensively been used to investigate the CTH552 antigen. This model has also previously been used for testing oral vaccine candidates.[164] However, the mice proved challenging to handle when administrating MCs, as the devices needed to be dosed in capsules and could not be traditionally administered with gavage. Furthermore, due to ethical concerns, only two capsules were allowed to be administered per immunization, which heavily limited the dosing amount. Fasting has previously been utilized in other studies with rats as a tool to slow the digestive system and increase the time the MCs reside in the GI tract.[209] However, mice are generally not an animal that can be fasted due to their high rate of metabolism.[244] As previously discussed, it also seems that the mice physiology is not optimal for testing the devices in the 100-µm range. By occupying 12.5%–15% of the intestine, MCs will most likely be pushed along by peristalsis and consumed foods and unable to imbed in the mucus to evade these factors.[235] It did not seem like the mouse model was compatible for research with the MC technology. For these reasons, rats were chosen as a model in **Project II** but did not elicit any response. Once again, a possibility is that the formulation is not retained for enough time in the intestine for a response to be established, but it is also likely that the AP205 cVLP simply is not at potent oral immunogen. It could be interesting to conduct other studies with this model and maybe other vaccine formulations or included adjuvants if employing the cVLP again. Nevertheless, does it seem that larger animal models are more compatible with the MCs, but a change in the animal model could have complications. For example, could rabbits be proposed as a model for further immunization studies. However, if further studies were conducted with for instance the α-GalCer adjuvant, it might not produce as promising results as observed in mice. α-GalCer recognizes NKT receptors in mice and humans, with no published data in rabbits. Although it would be interesting to investigate whether α-GalCer could function in rabbits, it should be taken into consideration that a change in the animal model for the benefit of the microtechnology could be a disadvantage for the formulation.

European sea bass were surprisingly easy to handle and allowed for fast, easy dosing and anesthetic procedure when needed. It was a nice change of frame to work with the animal the vaccine candidate in question was intended for. It could be interesting to conduct more studies on this model. Moreover, the idea of incorporating the MCs into the feed was captivating from a personal perspective. Another fish which could be interesting to as a model for the MCs, is the zebrafish. This fish has been used widely as an animal model in drug and immunological studies, and used for investigation of properties such as pharmacokinetics, immune cell migration, and

pathogen recognition. [67,245–247] It would be interesting to acquire such information when delivering vaccines with the MCs.

## 3.4 Additional studies

*In this section, results from two additional studies will be presented. The first study focuses on developing an in vitro cell model to evaluate the potential of mucosal vaccine candidates and release from MCs. The second study is a small in vivo study, where the kinetics of MCs and another device called "Foils" were investigated in rabbits.*

### 3.4.1 Development of an *in vitro* cell model for oral vaccine delivery by MCs

*The presented results are from the master thesis by Nicoline Andersen, whom I supervised. (Permission to include these in the PhD thesis has been granted)*

Efficient *in vitro* systems are of great interest today, as these often are cheaper and more accessible than *in vivo* studies. Additionally, they can facilitate high-throughput screening of the compounds of interest.[248] Furthermore, *in vitro* systems comply with the increasing focus on animal ethics and the 3R (refinement, replacement, and reduction) guidelines.[249] From the perspective of vaccinology, animal studies are almost unavoidable, as it is nearly impossible (or very challenging) to replicate all necessary pathways, cell types, and molecules that can impact the immune response in an *in vitro* setting. However, *in vitro* systems can, in this regard, be used to indicate which formulation candidates hold the most promise to succeed in the preclinical trials. Regarding mucosal vaccine candidates, some models have been suggested, focusing on the permeabilization of the formulations through the epithelial cell layer and targeting M cells.[250] This could be especially advantageous for delivery with the MCs, to get an indication on release profiles and to characterize some function of the formulations when delivered by the MCs.

In this study, an inverted Transwell *in vitro* model was attempted to be recreated in order to analyze and describe the release and effect of the MCs when vaccine formulations are administered. Several studies have investigated the feasibility of *in vitro* M-cell development by culturing lymphocytes with the Caco-2 cells.[197,250–252] These studies cocultivated Caco-2 with the B-lymphocyte cell line Raji B—a human lymphoblast-like cell derived from a Burkitt's lymphoma patient.[253] The studies observed that this cocultivation would drive differentiation of the Caco-2 into cells with an M cell–like morphology (i.e., no microvilli).

#### *3.4.1.1 Development of the Caco-2 and Raji B coculture*

The model was developed based on a protocol by *Rieux et al.* that includes a four-step development process after the proper cultivation of both cell lines (Fig. 27)[254]. A total of $5 \times 10^5$

Caco-2 cells were seeded onto the transwell membrane and allowed to develop for 5 days. Subsequently, the transwell membrane was inverted and a silicon tube was applied on the basolateral side to allow the seeding of 2.5 × $10^5$ Raji B cells after 9 days in the inverted position. This cocultured membrane was then incubated for another 5 days, at which point the differentiation of M-like cells should have occurred; a total of 19 days were needed to develop the complete model. After cell development, the model was inverted back to its original position for measurement and analysis. This inversion of the model allowed for a closer contact between the cells than the normally orientated model wherein the Caco-2 cells migrate to the opposite end of the membrane[250].

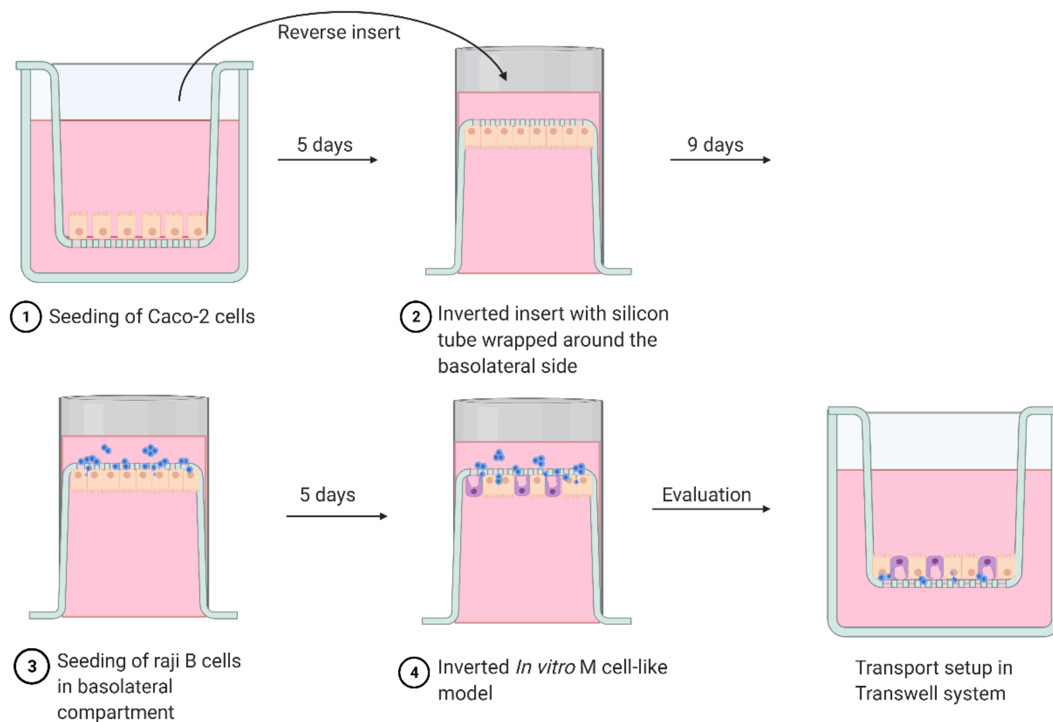



**Fig. 27.** The four-step protocol for the inverted Caco-2 + Raji B coculture model. Caco-2 cells were seeded and allowed to cultivate for 5 days (1). Then, the transwell membrane was inverted and covered with a silicon tube and incubated for 9 days (2). Thereafter, the Raji B cells were seeded onto the inverted transwell membrane and incubated for another 5 days (3). Ideally, the Raji B cells would have interacted with the Caco-2 cells and differentiated into M-like cells (4). The model is then inverted to its original position for transport studies. *Reprinted and adapted with permission from the master thesis of Nicoline Andersen.*

Co- and mono-cultures were simultaneously developed to characterize their differences and, hopefully, confirm the formation of M-like cells. To verify the integrity of the cell monolayer, transepithelial/transendothelial electrical resistance (TEER) measurements were extensively used.[255] Furthermore, the TEER values can indicate whether differentiation into M-like cells

occurred. This cell differentiation will result in the loss of tight junctions, leading to lower TEER values.[250] A drawback of this model is that the TEER values cannot be monitored in the inverted state, as the silicon tube does not allow the electrodes to be appropriately placed. Consequently, the TEER measure can only occur on day 5 before inversion and on day 19 after model development.

The measured TEER values on day 5 showed a large variation in the obtained values (Fig. 28). This was somewhat expected as other laboratories have reported obtaining different values while following the same protocol. *Hayeshi et al.* compared Caco-2 cells from 10 different laboratories and determined that minimal differences in culture conditions, passage number, or seeding density can significantly affect the growth pattern.[256] Various studies have reported TEER values in the range of 80–600 $\Omega \times cm^2$. TEER values obtained in this project were in the range of 24-345 $\Omega \times cm^2$ for the Caco-2 monolayers. Looking at the values obtained for the mono- and coculture at day 19, both of these had decreased compared to the cultures on day 5. Values for the mono-culture were slightly higher than those of the coculture, which could be because of the formation of M-like cells. However, the integrity of the cell layer as well as the presence of M-like cells cannot be determined using TEER values alone. This method is convenient and highly sensitive and can provide varying results, depending on the handler. To verify the integrity and the functionality of the tight junctions, permeability studies are warranted which can be investigated using fluorescent markers, such as lucifer yellow used in this study (data not shown). Several methods may be needed to verify the presence of M-like cells.

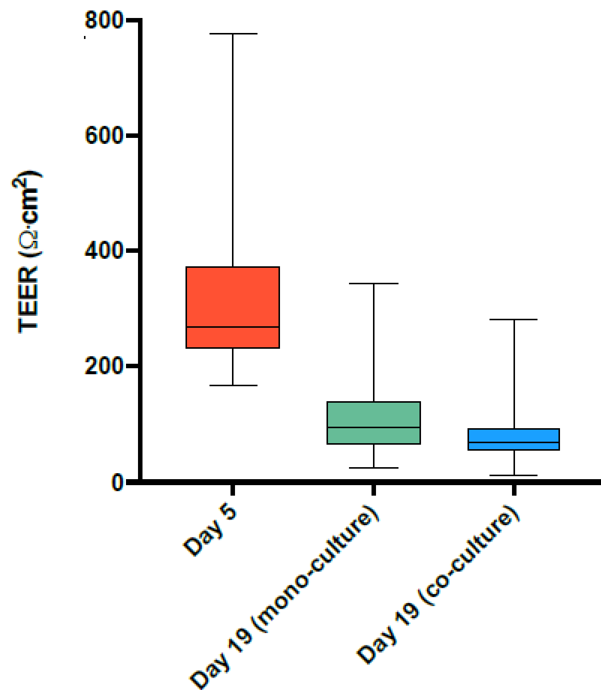



**Fig 28.** Transepithelia/transendothelial electrical resistance (TEER) values were measured 5 days after the initial seeding of Caco-2 cells and plotted in a boxplot. Because of the applied silicon tube, the TEER values could not be monitored until day 19 when the models were inverted back. On day 19, TEER was measured for the mono- and cocultures. Data are presented as means with highest and lowest quartiles, along with highest and lowest values recorded (n = 112 for day 5, n = 56 for mono- and cocultures). *Reprinted with permission from the master thesis of Nicoline Andersen.*

#### *3.4.1.2 Assessment of the presence of M-like cells*

To verify the presence of M-like cells, cell morphology is one of the most effective indicators. To date, no human-specific M-like cell markers have been identified. Instead, the presence of M-like cells has been identified by the lack of microvilli on the apical surface.[254] The surfaces of the cells in the mono- and cocultures were assessed using SEM (Helios NanoLAB 600) (Fig. 29).

SEM images indicated a tight layer of Caco-2 cells in the mono-culture but showed multiple layers of cells, even though a monolayer was desired. On comparing the mono- and cocultures, it was clear that the cocultures contained a lot of holes, explaining why no confluent monolayer was obtained in these cultures. Furthermore, multiple layers were observed in the cocultures, which could impact the formation of M-like cells as their differentiation is regulated by close contact with the Raji B cells. Our results do not suggest the presence of M-like cells. Cell cultures were further analyzed using confocal laser scanning microscopy (CLSM).

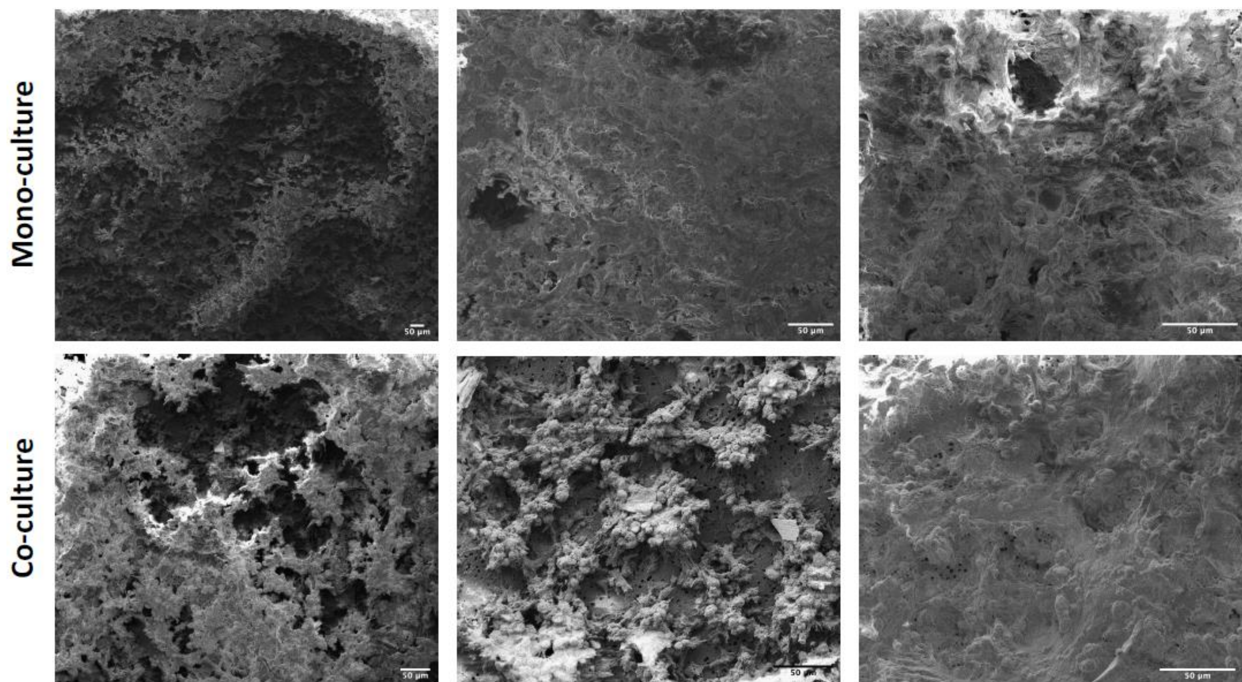



**Fig 29.** SEM images of different areas in the Caco-2 mono- and Caco-2/Raji B cocultures. In three images of the mono-cultures, a confluent layer of cells was observed. However, a trend of multiple layers was also noticed. In the three images of the coculture, holes on top of multiple layers of cells were evident, as observed with the mono-cultures. Furthermore, no apparent presence of M-like cells was noticed. *Reprinted with permission from the master thesis of Nicoline Andersen.*

A Zeiss LSM 700 microscope was used to observe the changes in the cell layers of the mono- and cocultures. Actin filaments were stained with Alexa Fluor$^{TM}$ 594 or 488 Phalloidin to obtain red or green fluorescence, and the nuclei were stained with Hoechst trihydrochloride trihydrate to obtain blue fluorescence. Some large cells were identified with diameters >20 µm (Fig. 30). Nuclei staining revealed that there might have been a cluster of cells as several nuclei were observed in the area. However, as observed in the SEM images, multiple layers of cells were visible, suggesting that the observed nuclei could have been from underlying cells. Further CLSM analysis was conducted with an anti-GP2 Alexa Flour 488 staining (Fig. 31). Glycoprotein 2 (GP2) was proposed as a marker of human M cells owing to the massive upregulation of this receptor on the cell surface.[257] However, GP2 is also present in other cells in the epithelium; this does not make it a specific marker for M cells. Nevertheless, the GP2 upregulation had been used to identify M-like cells previously by *Hase et al.*[258] The GP2 expression was therefore investigated in the developed cell model by CLSM with an anti-GP2-alexa flour 488 antibody. Very weak green fluorescence could be noticed from the images, which did not seem very adequate to suggest the formation of M-like cells. Furthermore, merging the image with actin filament and nuclei staining images did not reveal any particulate characteristics or cell pattern, which aligned with the

observed GP2 fluorescence. This indicated that M-like cell differentiation was not achieved in this study.

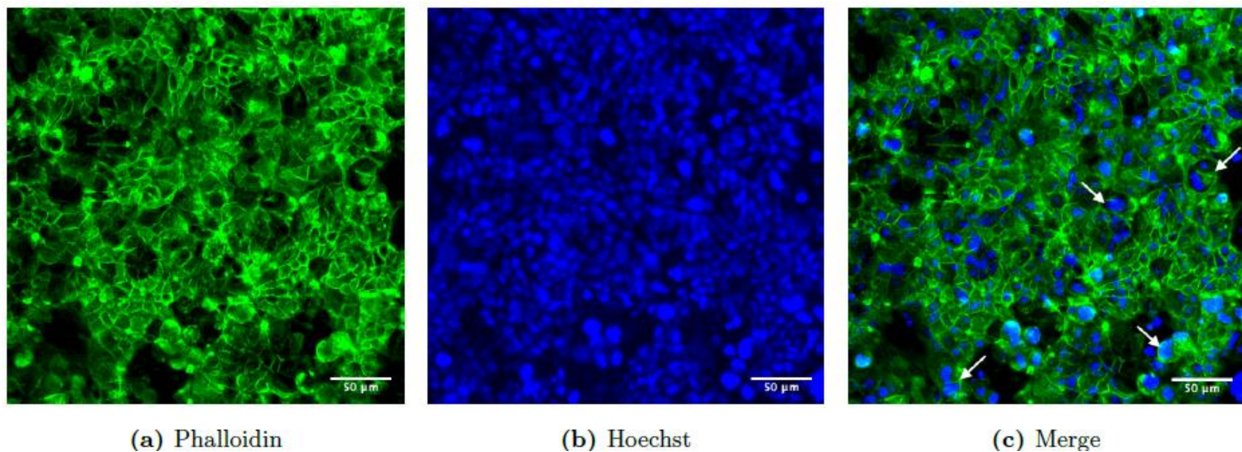



**Fig. 30.** Confocal laser scanning microscopy results of cocultures with stained actin filaments (a) and nucleus (b). Merging the images revealed cells with a different morphology (white arrows) (c). *Reprinted with permission from the master thesis of Nicoline Andersen.*

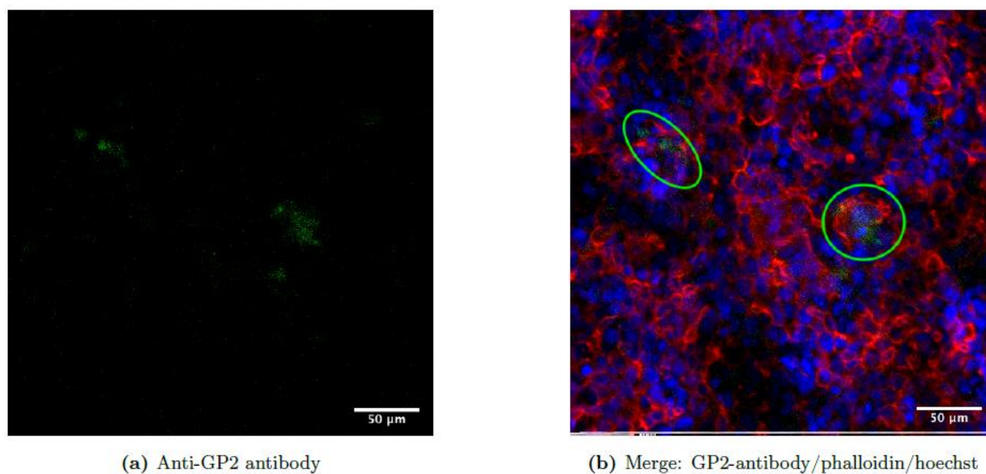



**Fig 31.** Confocal laser scanning microscope images of cocultures stained with an anti-GP2 antibody (a). Merged images depicting the actin filaments (red) and nucleus (blue) (b). The weak signals from the GP2 antibody are indicated with green circles in the merged image. *Reprinted with permission from the master thesis of Nicoline Andersen.*

Even though the cell model was not sufficiently developed to conduct actual release and characterization studies of vaccine formulations delivered using MCs, it is necessary to investigate the viability of the cells when exposed to the MCs (Fig. 32). The MCs were incubated with the mono- and cocultures for 3 hours, and the viability of the cultures was measured. Results showed that the MCs had no harmful or cytotoxic effect on the cells. This is not only beneficial in terms of the safety of the MCs but also shows their applicability in such *in vitro* assays. The cell

layers were further analyzed via CLSM, which revealed a confluent layer of cells after exposure to the MCs (Fig. 32).

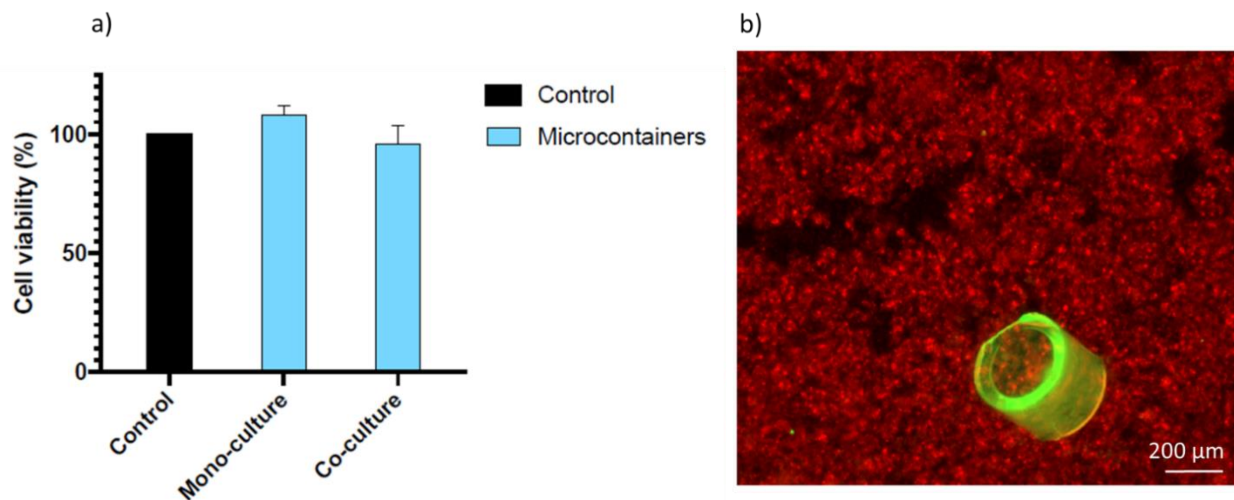



**Fig. 32**. Viability of the mono- and coculture after exposure to microcontainers (MCs) for 3 h (a). Confocal laser scanning microscope image of stained actin filaments (red) with an auto fluorescent MC (b).

Even though it did not seem as if successful differentiation of M-like cells was achieved in this study, this type of *in vitro* model could act as a primary tool to assess and characterize the ability of microdevices, such as MCs, or other aiding compounds, such as permeation enhancers, to assist with the mucosal targeting and permeabilization of the delivered vaccines.

## 3.4.2 Kinetics of MCs and Foils in rabbits

### *3.4.2.1 Visualization of MCs in rabbits*

One of the conclusions of **Project I** was that MCs are most likely to function more efficiently in a larger animal model owing to the more negligible effect of peristalsis and the presence of a thicker mucus layer to adhere to and embed within. To this end, rabbits were suggested as animal models. Rabbits have extensively been used in immunological studies as they are immune-competent and infection-susceptible.[259] Considering that MCs have never been administered to such a large animal previously, we had to verify whether the MCs can be visualized in the rabbit intestine. For this purpose, a pilot study was conducted wherein MCs were prepared with $BaSO_4$ and PLGA lids in the same manner as described in **Project I**. A size-3 capsule (Torpac) was filled with approximately 7000 MCs and orally administered to a single New Zealand White rabbit. After 6 h, the rabbit was euthanized and dissected to isolate the target regions of the GI tract. As this study focused only on the visibility of the MCs in the intestine of rabbits and only one rabbit was included, only the cecum and colon were examined (Fig. 32) by planar X-ray imaging as described

in **Project I**. The MCs were identifiable on the obtained images, appearing as small black dots similar to that in mice and rats. Furthermore, they seemed to be distributed across the entire colon. MCs in mice and rats showed a tendency to travel in clusters.[209] Nevertheless, the pilot study revealed that MCs could be successfully visualized in rabbits. Further studies should include analysis at multiple time points to investigate the exact transit time of MCs in this animal model. Furthermore, it could also be interesting to study this in both the fasted and fed state of the animal.

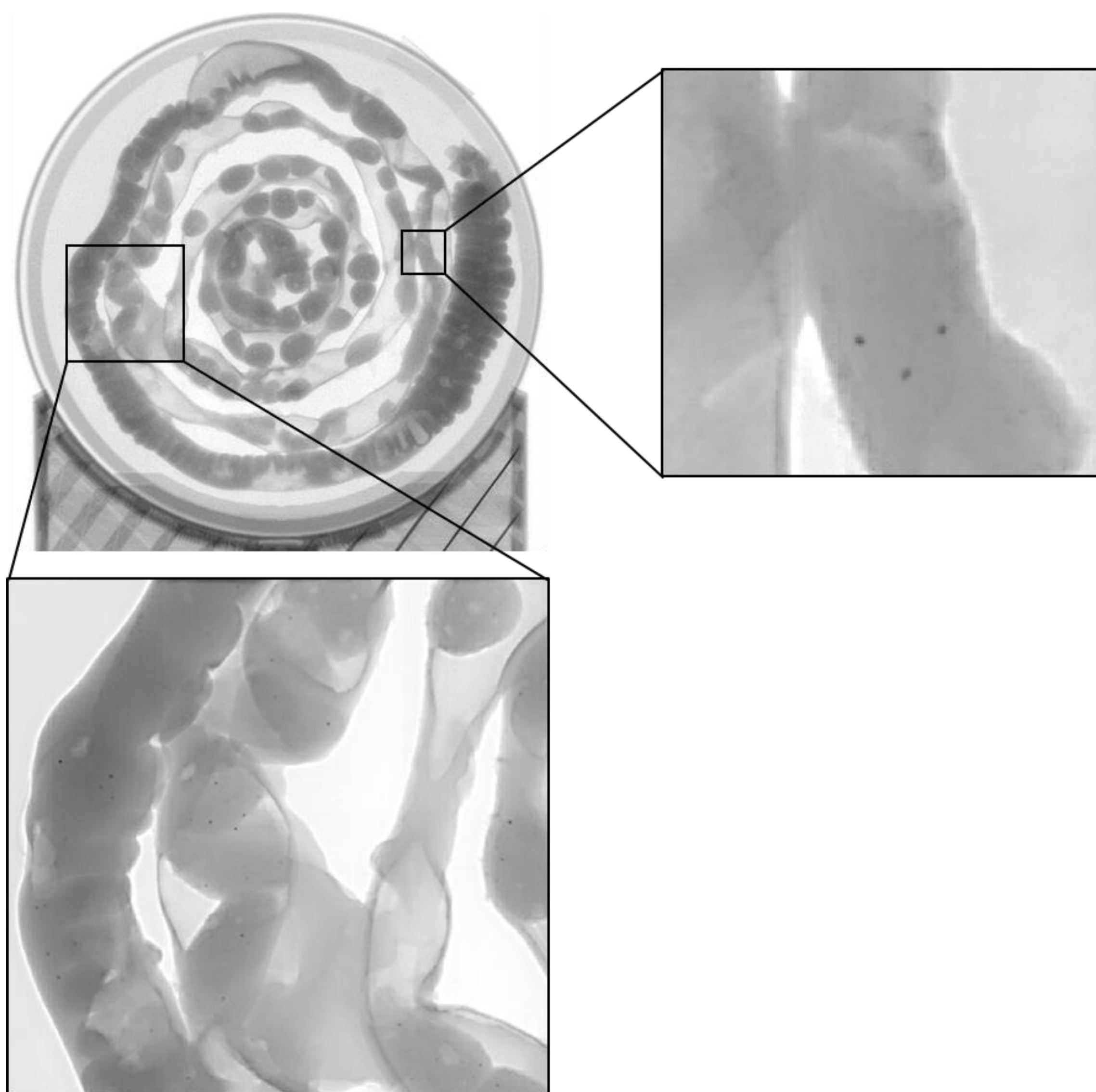


**Fig. 33.** X-ray images of the cecum and colon of one rabbit dosed with microcontainers and euthanized after 6 h. The microcontainers are visible as black dots and seem to be distributed across the entire colon.

#### *3.4.2.2 Kinetics of foils in rabbits*

Foils are an emerging class of devices that were initially developed for the oral administration of insulin.[260] They are produced by PDMS in a mold in a process that is highly reproducible and

modifiable, enabling the fabrication of any size of foils. Foils comprise a matrix of reservoirs that contain any compound similar to the MCs (Fig. 34). The foils are folded and encapsulated such that the reservoirs point outward toward the capsule. When the capsule dissolves, the foils unfold and function as a patch on the mucous membrane in the GI tract. This allows the reservoirs to be fixed in a single direction as well as remain in close proximity to the intestinal membrane; this is a major advantage of this device. Furthermore, the foils appear to be retained because of the elastic forces pressing against the barrier wall. To reach the small intestine to deliver drugs and vaccines, the capsules can be given an enteric coating, such as EL100. The coating and capsule will then dissolve upon reaching the intestine, allowing the foils to unfold and deliver their content. Foils have previously been examined in rats where problems regarding gastric emptying have been encountered. It is challenging to ensure that the enteric coated capsules undergo gastric emptying and travel to the intestine. Therefore, it was hypothesized that a larger animal could be the solution to this issue.

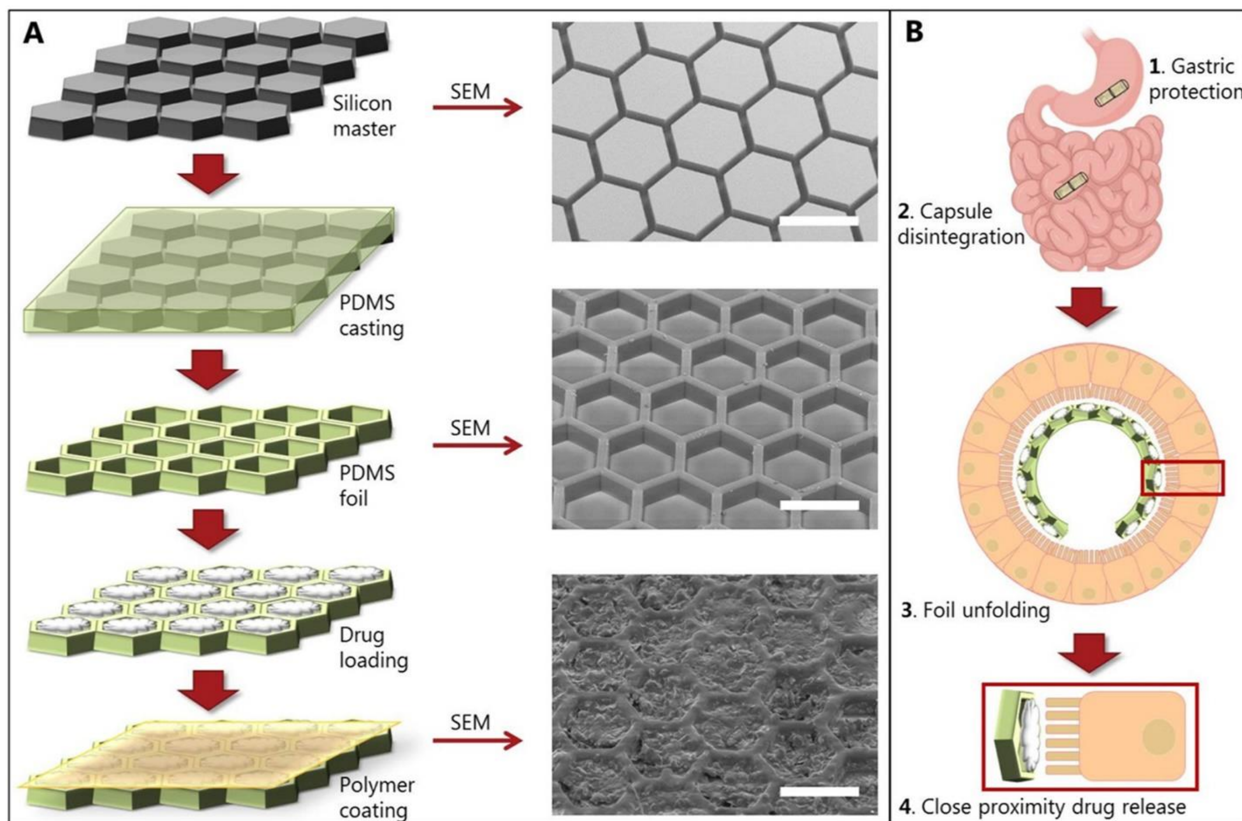



**Fig. 34.** The foil fabrication steps and SEM images depict the fabrication device (scale bars: 400 μm) (a). Concept and intended function of the foils, which is protected in the stomach by an enteric coated capsule, dissolve upon reaching the intestine (b). Once dissolved, the foil will unfold to allow unidirectional release and close proximity to the intestinal wall. *Reprinted with permission from Elsevier.*[261]

The 35 × 12 mm foils were incorporated with $BaSO_4$ to be visualized by CT scan. Four female New Zealand white rabbits were included in the study. Two of the rabbits were fasted overnight on the day before oral administration to determine whether the fed and fasted state would impact the foil kinetics. Rabbits were euthanized 9 h after administration and dissected, and the regions of the GI tract were isolated. The organs were then subjected to CT scans in the same manner as described in **Project I** (Fig. 35). It was clear visible that the foils did not undergo gastric emptying, regardless of whether the rabbits were fed or fasted upon administration. This could be because the chosen capsule size was too large to enter the intestine. The enteric coating of the capsules acted as intended by holding tight in the stomach, except in one rabbit (Fig. 35b). This was most likely the cause of a breach in the coating that occurred before or during administration. However, in this rabbit, the foil appears to have unfolded and aligned appropriately with the epithelial membrane in the stomach.

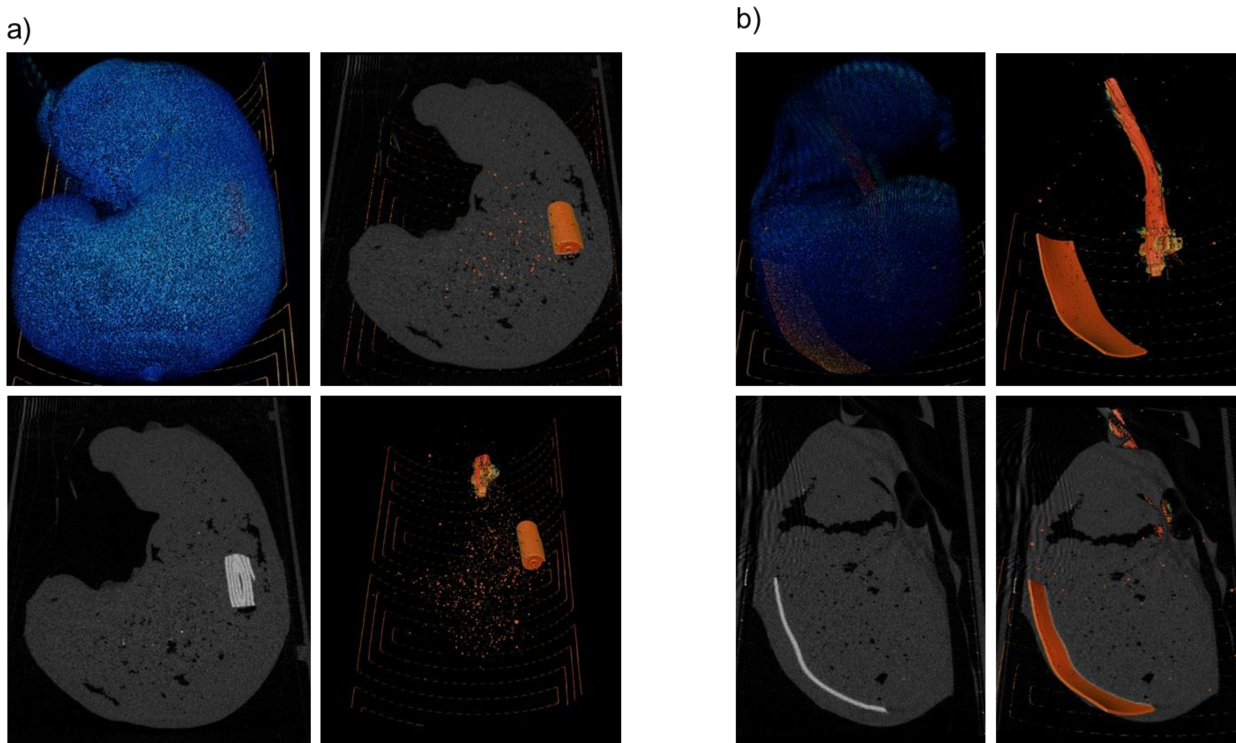



**Fig. 35.** CT images of the stomachs of rabbits dosed with foils in capsules. Three of the rabbits had intact capsules in the stomach that did not undergo gastric emptying (a). One capsule in one rabbit dissolved, exhibiting accurate alignment of the foils with the stomach wall (b).

# 4. Conclusions

In this thesis, the potential of MCs in delivering vaccines orally were assessed in three projects using different vaccine formulations in various animal models.

In the first project, the *C. trachomatis* antigen CTH522 was used. Two screening studies were conducted to investigate the mucosal adjuvants for the formulation with CTH522 and polymeric lids for the MCs to deliver the vaccine formulation effectively. These studies revealed that the glycolipid α-GalCer is the most prominent adjuvant for CTH522 and EL100-55 polymer lids that best facilitate delivery in MCs. A lyophilization protocol was also established as it was more feasible to load powder onto MCs. An optimized lyophilization cycle was developed along with buffer optimization with various excipients. A 10% trehalose + 10 mM Tris (w/v) buffer resulted in the best lyophilized product. The final experiment was conducted by combining the CTH522 formulation with α-GalCer and MCs equipped with EL100-55 lids. MCs induced a slightly higher response of systemic Th17 and intestinal Th1, Th17, and IgA following a s.c. prime with CTH522 + CAF01 compared with the included control groups. Solely oral dosing with MCs induced significantly higher Th1, Th17, and IgA levels in the intestine compared with naïve mice. These results indicate that the MCs are capable of stimulating the mucosal immune system with the CTH522 + α-GalCer formulation. However, because the measured responses were relatively weak, the kinetics of the MCs were investigated. The transit time of the MCs was comparable to the standard transit time in mice; this could be why a more robust response was not established. Therefore, further studies in larger animal models are warranted to make future observations more scalable to humans.

In the second project, the SpyC.AP205.L2 cVLP platform was used. The cVLP was lyophilized for loading onto MCs. Upon reconstitution, the quality of the particles was assessed and found to be comparable to that of non-lyophilized cVLP. Rats were immunized with cVLP in MCs, and designated control groups were included. Unfortunately, no measurable immune response was elicited in the orally dosed animals.

The third project focused on the use of MCs as potential tools for oral vaccination in the European sea bass. For this, *in vitro* studies were conducted by simulating the pH of the fish GI tract and *in vivo* studies were conducted wherein sea bass were continuously monitored for visual discomfort. Accordingly, MCs were established as functional and safe for use in fish. Furthermore, the function and kinetics of the MCs were also documented *in vivo* by visualizing the micro devices inside and outside the GI tract of the sea bass. Finally, the fish were immunized four times orally with the RGNNV VLP in MCs and subsequently challenged with the virus. The results did not

indicate any protective effect of immunization with MCs against the disease. However, several practical complications were encountered during the study, indicating that the study should be repeated.

Overall, these results do indicate that oral delivery of vaccines in the MCs can induce an immune response. However, the vaccine formulation and components have a significant impact on the stimulated response. Moreover, the function of the MCs is affected by the choice of animal model, and studies on kinetics suggest that MCs are more compatible with larger animals.

Furthermore, the development of an *in vitro* assay to evaluate release of mucosal vaccine formulations from MCs was attempted. Although unsuccessful, such an assay could prove efficient in understanding the release and function of formulations when used with the MCs. *In vivo* studies on the MCs and foils in rabbits were also conducted. MCs could be effectively visualized in the GI tract of rabbits, suggesting that further kinetic analysis in this animal model can be conducted. Foils could not undergo gastric emptying and should be fabricated to be smaller for this procedure to succeed. One of the foils unfolded in the stomach and displayed an excellent alignment with the epithelial wall. This could be of great advantage in the oral delivery of vaccines and should be investigated in future studies.

# 5. Future perspectives

To further assess the abilities of the MCs for oral delivery, studies in larger and more compatible animal models are warranted. Design changes to the microdevices to facilitate retention should also be a focus point in future studies. Furthermore, the use of commercialized oral vaccines might be a more optimal way to test the proof-of-concept for the microdevices instead of experimental novel antigens. In **Project I**, the mice were not very compatible with the MCs but could still be a viable animal model for screening oral vaccine candidates. Intra-intestinal infusion and intestinal closed-loop model can potentially prove effective in acquiring indications of promising candidates. These methods could especially benefit from the screening of new adjuvants. In this regard, the CAF adjuvants also present an opportunity to specifically tailor new candidates to induce mucosal responses in the intestines by their modifiability. It would be interesting to optimize a candidate for oral delivery of CTH522 with MCs or another microdevice. In addition, bio distribution studies might also provide essential information on the function of the formulations both in and out of microdevices.

In **Project II** the AP205 cVLP did not seem to be an inducer of mucosal immune responses and are in need of additional tools other than MCs. Identifying prominent adjuvant candidates to be used in trials with the cVLP could be vital in establishing a robust immune response. Furthermore, it would be interesting to see the effect of the prime-pull method with the cVLP technology.

In **Project III,** a natural next step would be to repeat the challenge study, due to the practical complications that occurred. Aside from that, it would be interesting to see the performance of the MCs, if loaded with purified RGNNV VLP. Furthermore, a larger gelatin capsule (size 9) than the one used in the challenge study, which would allow for a larger immunization dose, was found to be suitable for oral administration to the sea bass. Further exploring the MCs in other cultured fish species such as rainbow trout and Atlantic salmon would also be intriguing. Either the RGNNV VLP or other fish vaccines could be used for this exploration.

Several novel microdevices are in the development pipeline at the IDUN Center, such as foils, microneedles, micromotors, and self-configurable enable proximity devices. These new devices will focus on increasing the device's retention in the GI tract, which is critical for efficient oral vaccination. It would be interesting to use these for the oral delivery of vaccines. For example, even though the foil did not undergo gastric emptying in the rabbits, it seemed capable of efficient delivery in the stomach. This could be highly beneficial in delivering vaccine candidates against diseases such as *H. pylori*.

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# Appendixes

## Appendix 1

# Paper I:

# Oral vaccination using microdevices to deliver α-GalCer adjuvanted vaccine afford mucosal immunity

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# Oral vaccination using microdevices to deliver α-GalCer adjuvanted vaccine afford mucosal immunity

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## Abstract

Oral vaccination has in the recent years gained a lot of attraction, mainly due to optimized patient compliance and logistics. However, the development of oral vaccines, especially oral subunit vaccines is challenging. Micro technology can be utilized to overcome some of these challenges, by facilitating protection and effective delivery of the vaccine components in the gastro intestinal tract (GI tract). One such technology is Microcontainers (MCs), which can be realized to be mucoadhesive and to target specific regions of the GI tract via oral delivery. Here, we test MCs, for oral delivery of the *C. Trachomatis* vaccine candidate CTH522, in combination with effective mucosal adjuvants. The adjuvants alpha-galactosylceramide (α-GalCer), C-di-GMP and cholera toxin B were compared *in vivo*, to identify the most prominent adjuvant for formulation with CTH522. Formulations were administered both purely oral and as boosters following a subcutaneous (s.c.) prime with CTH522 in combination with the CAF®01 adjuvant. CTH522 formulated with α-GalCer showed to be the most efficient combination for the oral vaccine, based on the immunological analysis. Lyophilized formulation of CTH522 and α-GalCer was loaded into MCs and these were subsequently coated with Eudragit L100-55 and evaluated *in vivo* in mice for the ability of MCs to mediate intestinal vaccine delivery and increase immunogenicity of the vaccine. Mice receiving oral prime and boosters did show a significantly enhanced mucosal immune responses compared to naive mice. This indicates the MCs are indeed capable of delivering the vaccine formulation intact and able to stimulate the immune cells. Mice orally boosted with MCs following a s.c. prime with CAF01, demonstrated improved systemic and local Th17 responses, along with increased local IFN-γ and IgA levels compared to both the s.c. prime alone and the homologous oral prime-boost immunization. However, due to the relatively weak observed effect of the MC delivery on the immune responses, it was hypothesized that the MCs are proportionally too large for the GI tract of mice, and thus cleared before an effective immune response can be induced. To investigate this, MCs were loaded with $BaSO_4$, and orally administered to mice. Analysis with X-ray and CT showed a transit time of approximately 1-1.5 h from the stomach to the cecum, corresponding to the standard transit time in mice, and an extremely narrow absorption window. This indicates that mice is not a suitable animal model for evaluation of MCs. These data should be taken into consideration in future *in vivo* trials with this and similar technologies, where larger animals might be a necessity for proof-of-concept studies.

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# 1. Introduction

In the recent years, requirements for vaccines targeting mucosal pathogens such as influenza- and corona viruses, have gained a high amount of interest, as these pathogens constitute a continual global threat[1]. This is not only the case with respiratory diseases, but also other pathogens like those causing sexual transmitted diseases (STDs) which likewise gain entry through the mucosa. One example is the gram-negative bacteria *Chlamydia Trachomatis* one of the most common sexually transmitted bacterial diseases with 129 million infected people annually[2]. Untreated or repeated bacterial infection with *C. Trachomatis* can lead to pelvic inflammatory disease ultimately causing tubal factor infertility and ectopic pregnancy. Recently it has become clear that following treatment for a chlamydial genital infection, woman cured of genital infection, often get re-infected due to autoinoculation from the lower gastrointestinal (GI) tract, because chlamydia has the ability to reside in the GI tract for long periods of time in the absence of clinical disease[3]–[5]. Therefore, a vaccine against infection with *C. trachomatis* could potentially benefit from inducing genital and intestinal mucosal- in addition to systemic immunity.

Nearly all modern vaccine research is based on subunit vaccines, due to ease of modification and high safety. These advantages, however, result in reduced immunogenicity, creating the essential need for the vaccines to be formulated with adjuvants. Today, a substantial amount of adjuvants exits, and their individual function varies according to the type and intended purpose, but also on factors such as, mode of administration and antigen formulation[4],[5]. A lead vaccine candidate against *C. Trachomatis* is the protein based subunit antigen CTH522, which requires co-delivery with an effective adjuvant that supports mucosal immunity[8]. The liposomal adjuvant CAF01 has showed promise in this regard, being able to stimulate Th1 and Th17 cells, needed for effective mucosal protection[9],[10]. In a recent clinical phase 1 trial, the CTH522 vaccine was evaluated by intramuscular injection in combination with CAF01, followed by intranasal administration with CTH522 alone. Here, the CAF01 adjuvanted CTH522 achieved a promising immunogenicity profile, generating neutralizing systemic and genital IgG and IgA antibodies [8]. Currently, the only subunit antigen in a licensed mucosal vaccine is the Cholera Toxin B (CTB), which has frequently been investigated as an adjuvant. CTB have been known to induce mucosal immune stimulation via various routes of administrations, such as intranasal, sublingual and oral [1],[8]. However, the immunomodulating effect of CTB is questioned by the presence of residual cholera toxin or LPS in CTB preparations, making it a challenge to separate adjuvanticity from toxicity[1]. Some recent studies have deemed highly purified CTB as an inducer of immune tolerance, rather than a promoter of mucosal immune responses when administered orally or intranasally[12]. Another mucosal adjuvant candidate is Cyclic-di-GMP (c-di-GMP), a ligand of the STING pathway. Intranasal administered c-di-GMP has on several occasions been shown to develop protection in the respiratory tract, primarily observed to induce high Th1 and Th17 stimulation. Two studies using c-di-GMP as an adjuvant in an influenza vaccine, recorded high stimulation of Th1 IFN-genes and enhanced Th17 cytokine production, as a feat of activating STING[13], [14]. *Madhun et al.* also investigated c-di-GMP as an intramuscular adjuvant, which interestingly showed no enhancement of the immune response, compared to administration with only the antigen. Furthermore, the glycolipid α-Galactosylceramide (α-GalCer), an activator of invariant Natural Killer T (iNKT) cells, have mostly been used in cancer immunotherapy [15]. However, α-GalCer has been reevaluated as a promising adjuvant of the mucosal immune system, especially following oral administration. In recent studies, whole-cell killed *Helicobacter Pylori* and enterotoxigenic *Escherichia coli* vaccines adjuvanted with α-GalCer, demonstrated induction of intestinal IgA and Th1 cell immunity, along with serum IgG responses following oral administration [13],[14].

Oral delivery of vaccines holds potential in lowering the cost of administration and logistics of vaccine dosing significantly, especially in mass vaccination situations. In addition, oral administration could

encourage vaccination for the population due to the high patient compliance [18]. Oral immunization draws its advantage in being able to induce effective secretory IgA antibody and T-cell responses in mucosal tissues in the gastrointestinal (GI) tract, and other local mucosal sites [19]. Oral delivery of subunit vaccines, such as the CTH522 vaccine candidate, is however very sensitive to chemical and enzymatic degradation in the stomach and intestines [18]. Thus, subunit vaccines are in need of innovative oral delivery systems in addition to potent adjuvants. This system should be capable of protecting the vaccine from degradation and facilitate uptake by effective delivery to the intestinal epithelium. The mucosal tissue in the intestines, contains an abundance of immune cells to be utilized, making it a noteworthy target [20]. The concept has been widely studied, and many attempts have been made to facilitate effective oral delivery of vaccines. This includes technologies such as nano- and micro-particular systems such as PEG and PLGA particles, along with permeation enhancers for vaccine formulations such as ionic liquids [21], [22], [23]. Previously, we have tested microcontainers (MCs) as a delivery system of a spray dried vaccine formulation consisting of cubosomes, the adjuvant Quil-A and the model antigen ovalbumin (OVA) [24]. It was observed that the MCs are indeed capable of protecting their content through the GI tract, until intended release in the small intestine of mice by the pH dependent polymeric coating Eudragit L100-55 (EL100-55). Furthermore, it was shown that the humoral response could be slightly improved with oral boosters after a parental prime. However, the MCs ability to deliver an antigen candidate, targeting a mucosal infecting pathogen along with promising mucosal adjuvants, remains to be tested.

In this study, we investigated the mucosal immune inducing adjuvants, CTB, c-di-GMP and α-GalCer for the purpose of oral vaccination with the *C. Trachomatis* antigen CTH522, to boost and redirect a subcutaneous (s.c.) prime injection with CTH522 + CAF01 into the intestines. Moreover, a lyophilization procedure was developed and optimized for the vaccine formulations to enhance thermal stability. In previous studies, MCs have been coated with poly(lactic-co-glycolic acid) (PLGA) or chitosan for drug delivery purposes. We here tested their capability to both function as mucosal agents and coatings on MCs, along with the polymer EL100-55, in an *in vivo* comparison study. Findings of the most promising adjuvant along with the most optimal MC coating are combined, and their ability to induce a mucosal immune response against CTH522 via oral administration in mice is evaluated (Fig. 1).

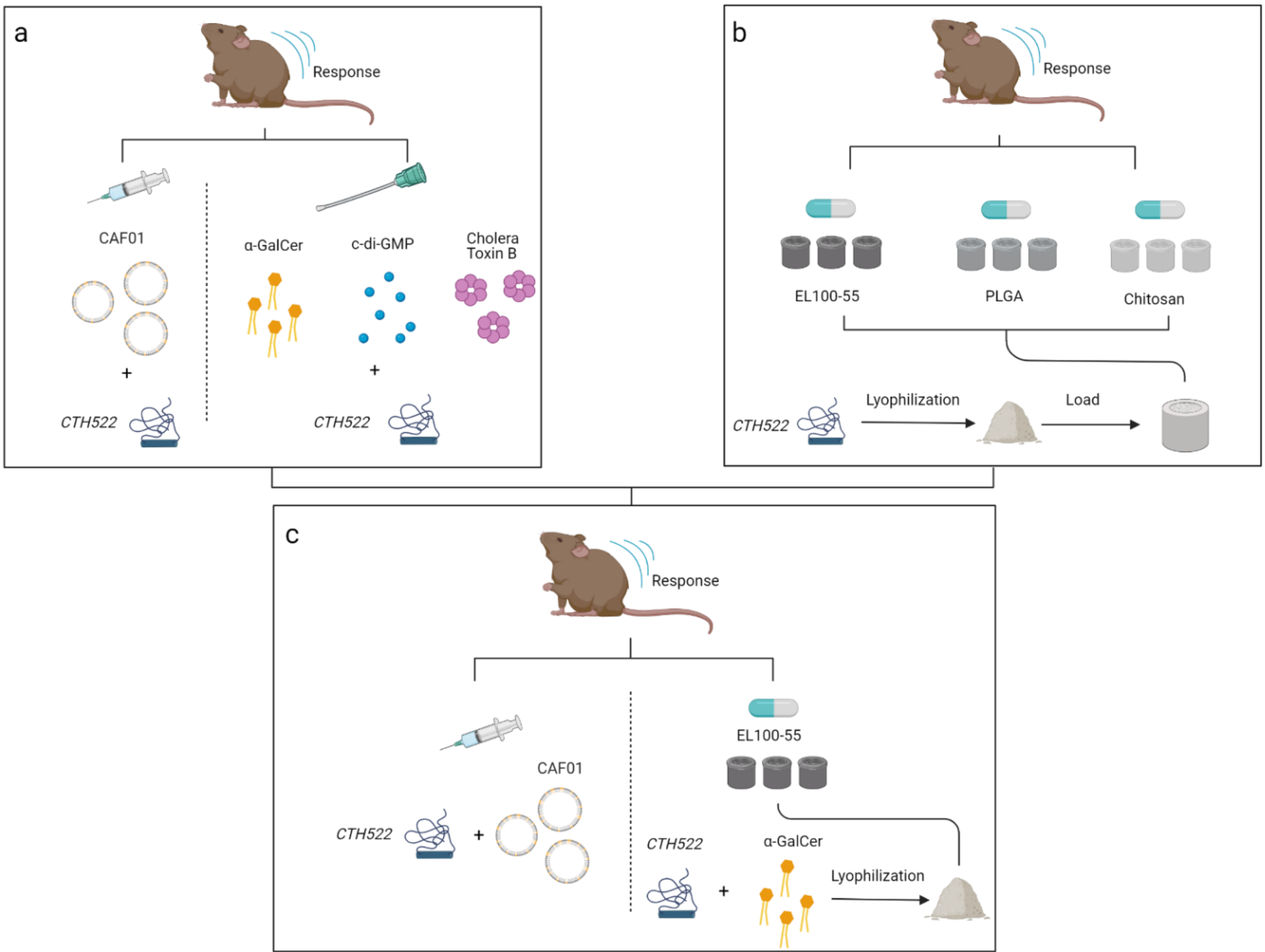



**Fig. 1.** Overview of the work flow conducted in this paper. To identify the most effective mucosal adjuvant, an *in vivo s*creening of the adjuvants α-GalCer, c-di-GMP and Cholera Toxin B, formulated with CTH522 was set up (a). Formulations were administered orally with gavage either following an s.c. prime with CAF01 + CTH522 or as oral administration only. To enhance thermal stability of the vaccine formulation, a lyophilization procedure of CTH522 was implemented (b). Lyophilized powder was loaded into microcontainers (MCs) and subsequently coated with the polymers Eudragit L100-55 (EL100-55), poly(lactic-co-glycolic acid) (PLGA) or chitosan and filled into gelatin capsules for oral immunization to mice, following an s.c. prime with CAF01 + CTH522 (b). Based on the results from these experiments, an experiment with lyophilized CTH522 adjuvanted with α-GalCer, loaded into MCs and coated with EL100-55 was conducted (c). MCs were administered orally either following an s.c. prime with CAF01 + CTH522 or as oral administration only. *Created with Biorender.com*

## 2. Materials and methods

### 2.1 Materials

C-di-GMP was purchased from Invivogen (San Diego, CA, USA), CTB was obtained from Sigma Aldrich (St. Louis, MO, USA) and α-Galactosylceramide was purchased from Avanti lipids (KRN7000, Bermingham, AL, USA). CTH522 and CAF01 were produced in-house as described in[8]. PLGA (low MW 7–17 kDa, 50:50 PLA:PGA), trehalose, L-histidine and soybean trypsin inhibitor were obtained from Sigma-Aldrich (St. Louis, MO, USA). Eudragit L100-55 was purchased from Evonik (Darmstadt, Germany). Size M gelatin capsules were bought from Torpac (SG Heerlen, The Netherlands). PBS and non-essential amino acids were obtained from Life Technologies (Roskilde Denmark). HEPES buffer, RPMI 1640, L-Glutamine and sodium pyruvate was purchased from Invitrogen (Weltham, MA, USA). Fetal calf serum was from Biowest (Nuaillé, France), TMB ready-to-use-substrate was bought from Kem-En-Tec (Taastrup, Denmark), Tween20 was from Merck (Darmstadt, Germany), $H_2SO_4$ and $BaSO_4$ (precipitated, 99%) was bought from VWR International (Radnor, PA, USA). NUNC 96 well Maxisorp plates was obtained from Thermo Scientific (Roskilde, Denmark) and Falcon 100µm nylon cell strainers were bought from Corning (Vordingborg, Denmark). HRP rabbit anti-mouse IgG was purchased from AH Diagnostics (Tilst, Denmark), Biotin goat anti-mouse IgA was obtained from Sourthern Biotech (Bermingham, AL, USA) and Streptavidin – HRP conjugate was bought from BD Pharmingen (Lyngby, Denmark). Biotin rat anti-mouse IFN-γ and purified rat anti-mouse IFN-γ was purchased from BD Pharmingen (Lyngby, Denmark). Biotin anti-mouse IL-17A and purified rat anti-mouse IL-17A was obtained from BioLegend (San Diego, CA, USA). Concavalin A was purchased from GE Healthcare (Marlborough, MA, USA). CB6F1 mice were bought from Envigo (Indianapolis, IN, USA)

### 2.2 Mice

Male CB6F1 mice were purchased weighing at least 25 g (approx. 12 weeks old) following the minimum oral dosing recommendations from Torpac. Mice were acclimatized 1 week prior to starting experiments. Mice had free access to food and water at all times. All experiments were approved by the Animal Experiments Inspectorate of Denmark, under the license 2020-15-0201-00610 and were conducted in compliance with the Danish laws regulating experiments on animals and the EC Directive 2010/63/EU.

### 2.3 CTH522 formulation with CAF01, C-di-GMP, CTB and α-GalCer

CTH522 (1.4 mg/mL) were thoroughly vortexed with 1 mg/mL CAF01 every 10 min for 30 min prior to immunization. CTB was rehydrated to a concentration of 5 mg/mL and mixed with CTH522 by pipetting. C-di-GMP was similarly rehydrated to a concentration of 5 mg/mL and mixed with CTH522 by pipetting. α-GalCer was mixed, as suggested by Avanti, in 5.7% trehalose, 0.75% L-histidine, and 0.5% Tween 20, making a 5 mg/mL solution. After mixing, the solution was heated at 80 ℃ and sonicated every 10 min for 1 min, until the material was completely dissolved. Dissolved α-GalCer was then mixed with CTH522 and thoroughly vortexed.

## 2.4 Lyophilization of vaccine formulation

CTH522 alone or adjuvanted with α-GalCer were lyophilized in a Christ Delta 2-24 LSCplus freeze-dryer (Christ, Osterode am Harz, Germany) with the program depicted in Table 1. Antigen and adjuvants were lyophilized in a 10% trehalose + 10 mM Tris-base formulation.

**Table 1.** The parameters for the program used to lyophilize the CTH522 + α-GalCer vaccine formulation.

| Phase | Freeze | Primary Drying | Primary Drying | Primary Drying | Secondary Drying | Secondary Drying |
|---|---|---|---|---|---|---|
| Time | 3:00 h | 0:15 h | 0:15 h | 36:00 h | 5:00 h | 10:00 h |
| Temp. | -42 °C | -42 °C | -30 °C | -30 °C | 20 °C | 20 °C |
| Vacuum | - | 0.120 mbar | 0.120 mbar | 0.120 mbar | 0.120 mbar | 0.120 mbar |

## 2.5 SDS-gel of hydrated vaccine formulation

Gel electrophoresis was performed in a Mini-PROTEAN Tetra system (Bio Rad, Hercules, CA, USA) with a Mini-PROTEAN TGX Precast 12 well gel (Bio Rad, Hercules, CA, USA). For the denatured proteins, 2 µg of rehydrated lyophilized CTH522, and 2 µg non-lyophilized CTH522 were formulated with sample buffer Tris/Glycerol, Bromphenol Blue) + SDS and DTT in a 1:1 ratio and loaded on the gel. For the native proteins 2 µg of rehydrated lyophilized CTH522, and 2 µg non-lyophilized CTH522 were formulated with sample buffer ÷ SDS and DTT in a 1:1 ratio and loaded on the gel. The gel was run at 300 V for 16 min using Power Pac 300 (Bio Rad, Hercules, CA, USA). The gel was removed and washed with deionized water and then emerged in Bio-Safe Coomassie G250 Stain (Bio Rad, Hercules, CA, USA) for 60 min, followed by wash with dionized water for 2x30 min.

## 2.6 Fabrication, drug loading, polymeric coating and capsule filling of microcontainers

MCs were fabricated with the negative epoxy photoresist SU-8 by a two-step photolithography process as described previously [25]. The MCs were produced on top of a titanium|gold (Ti|Au) coated silicon wafer to allow easy removal from the wafer. The wafer was then cut into 12.8 by 12.8 $mm^2$ chips containing 25 by 25 arrays of MCs using a dicing saw (DISCO, München, Germany). MCs on chips were loaded with vaccine formulation powder of CTH522 + α-GalCer using an embossing method as described previously [26]. A shadow mask was used to cover the gaps between the MCs, thus, filling the MCs without filling the space between them with powder. The average powder load in the MCs was estimated by weighing 10 chips before and after loading and calculating the average of 1 microcontainer. After loading, the MCs were sealed with either EL100–55, PLGA or chitosan through a spray coating process, using an ExactaCoat spray coater (Sono Tek, Milton, Canada) equipped with an ultrasonic nozzle actuated at 120 kHz (Accumist, Sono Tek, Milton, Canada). Acetic acid containing 0.5%(w/w) chitosan (low molecular weight, 75-85% deacetylated) was sprayed with the spray coating parameters for chitosan depicted in Table 2. Dichloromethane (DCM) containing 0.5%(w/w) PLGA (7-17 kDa, 50:50 PLA:PGA) was sprayed with the spray coating parameters for PLGA depicted in Table 2. Isopropanol containing 1% (w/v) EL100-55 and 5% (w/w in relation to EL100–55) dibutyl sebacate was sprayed with the spray coating parameters for EL100-55 depicted in Table 2.

**Table 2.** Spray coating parameters used for the coating of the polymers chitosan, PLGA and EL100-55 on microcontainers (MCs).

| | Feed flow | Generator power | Air pressure | Temperature | Nozzel distance to MCs | Speed | Passages |
|---|---|---|---|---|---|---|---|
| **Chitosan** | 0.1 mL/min | 1.3 W | 0.030 kPa | 50 °C | 7.5 cm | 25 mm/s | 110 |
| **PLGA** | 0.1 mL/min | 2.2 W | 0.030 kPa | Room temp. | 5 cm | 10 mm/s | 55 |
| **EL100-55** | 0.1 mL/min | 2.2 W | 0.028 kPa | 35 °C | 5 cm | 10 mm/s | 25 |

After the coating procedure, MCs were directly removed from the chips using a scalpel. MCs were filled into size M gelatin capsules using a size M funnel (Torpac, SG Heerlen, The Netherlands). The amount of MCs in the capsules were determined by weighing the capsules before and after filling. Based on the filling of 10 capsules, a size M capsule could contain 82 ± 3.7 MCs.

### 2.7 Microcontainer coating characterization and release study

Microcontainer chips were placed in acid baths subsequent to loading with lyophilized CTH522 formulation and coated with chitosan, PLGA or EL100-55. Chips was submerged in 36.6 °C 2 mM pH 4.7 maleic acid for 60 min and visualized with a table top scanning electron microscope (SEM) (Hitachi TM3030plus, Tokyo, Japan), using 15 kV acceleration voltage at 40× and 50× magnification. Chips were then submerged in 36.6 °C 10 mM pH 6.6 maleic acid for 30 min and 60 min, and visualized after each time point with SEM using the same settings as described above.

### 2.8 *In vivo* studies

Four *in vivo* studies were conducted in this paper (Table 3, supporting information). Mice were immunized according to Table 3, and dosed either s.c. or orally with gavage or MCs. MCs were administered in size M gelatin capsules. Mice were euthanized and harvested on day 56.

**Table 3.** Scheme of the setup for the four *in vivo* studies conducted in this paper, depicting formulation, dosage amount and administration form on the immunization days of the individual groups in each of the studies. Coating of microcontainers (MCs) is included for the groups, which were administered orally with them. Prime immunization is set at day 0, first booster immunization was set at day 21 and second booster immunization was set at day 42. Naive groups were not dosed with anything.

| *In vivo* study nr. | Group nr. | Prime (day 0) | 1. Booster (day 21) | 2. Booster (day 42) | MC coating |
|---|---|---|---|---|---|
| **1** | 1 | Naive | - | - | - |
| | 2 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | - | - | - |
| | 3. | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 + 10 µg c-di-GMP (oral gavage) | 10 µg CTH522 + 10 µg c-di-GMP (oral gavage) | - |
| | 4 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 + 10 µg α-GalCer (oral gavage) | 10 µg CTH522 + 10 µg α-GalCer (oral gavage) | - |
| | 5 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 + 10 µg CTB (oral gavage) | 10 µg CTH522 + 10 µg CTB (oral gavage) | - |
| | 6 | 10 µg CTH522 + 10 µg c-di-GMP (oral gavage) | 10 µg CTH522 + 10 µg c-di-GMP (oral gavage) | 10 µg CTH522 + 10 µg c-di-GMP (oral gavage) | - |
| | 7 | 10 µg CTH522 + 10 µg α-GalCer (oral gavage) | 10 µg CTH522 + 10 µg α-GalCer (oral gavage) | 10 µg CTH522 + 10 µg α-GalCer (oral gavage) | - |
| | 8 | 10 µg CTH522 + 10 µg CTB (oral gavage) | 10 µg CTH522 + 10 µg CTB (oral gavage) | 10 µg CTH522 + 10 µg CTB (oral gavage) | - |
| **2** | 1 | Naive | - | - | - |
| | 2 | Lyophilized 5 µg CTH522 + 5 µg α-GalCer (s.c.) | Lyophilized 5 µg CTH522 + 5 µg α-GalCer (s.c.) | - | - |
| | 3 | 5 µg CTH522 + 5 µg α-GalCer (s.c.) | 5 µg CTH522 + 5 µg α-GalCer (s.c.) | - | - |
| **3** | 1 | Naive | - | - | - |
| | 2 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | - | - | - |
| | 3 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 (oral MCs) | 10 µg CTH522 (oral MCs) | EL100-55 |
| | 4 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 (oral MCs) | 10 µg CTH522 (oral MCs) | PLGA |
| | 5 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 (oral MCs) | 10 µg CTH522 (oral MCs) | Chitosan |
| **4** | 1 | Naive | - | - | - |
| | 2 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | - | - | - |
| | 3 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 + 10 µg α-GalCer (oral gavage) | 10 µg CTH522 + 10 µg α-GalCer (oral gavage) | - |
| | 4 | 5 µg CTH522 + 5 µg CAF01 (s.c.) | 10 µg CTH522 + 10 µg α-GalCer (oral MCs) | 10 µg CTH522 + 10 µg α-GalCer (oral MCs) | EL100-55 |
| | 5 | 10 µg CTH522 + 10 µg α-GalCer (oral MCs) | 10 µg CTH522 + 10 µg α-GalCer (oral MCs) | 10 µg CTH522 + 10 µg α-GalCer (oral MCs) | EL100-55 |

### 2.9 Sample and organ preparation for ELISA analysis

Whole blood was separated by centrifugation at 10.000 G for 10 min. Serum was removed and stored in 96 well plates. Fecal pellets were collected in cold feces buffer (PBS + 0.1 mg/mL Soybean trypsin inhibitor + 1% w/v BSA + 25 mM EDRA + 50% v/v glycerol + 1mM PMSF). Pellets were then broken to form a suspension and incubated for 4 h on ice. Solids were separated from liquid by centrifugation for 10 min at 15.500 G at 4 $^{o}$C. Supernatants were transferred to microfuge tubes, and blocked over night with PBS + 1% (w/v) BSA. Isolated spleens and PPs were filtered through a cell strainer, suspended in RPMI media without FBS and centrifuged for 5 min at 1800 rpm. Then resuspended in 1mL using complete RPMI (cRPMI) (supplemented with HEPES, penicillin–streptomycin, sodium pyruvate, l-glutamine and non-essential amino acids) with 10% FBS (v/v) and counted on a NucleoCounter NC-200 (Chemometec, Allerød, Denmark). Cell cultures were seeded ($2\times10^5$/well) and stimulated with the CTH522 (1.4 µg/mL); media cRPMI (as negative control) and concavalin A (1 µg/mL) (as positive control). The supernatants were harvested after 72 h incubation and stored at -20 $^{o}$C.

### 2.10 ELISA

For the IgG antibody ELISA, 96 well plates were coated with CTH522 and then blocked with 2% w/v BSA in PBS. Sera were diluted 1:100 and 3-fold diluted across well plates. Samples were then incubated for 2 h with HRP-conjugated IgG anti-mouse monoclonal antibody. For IgA antibody ELISA, wells were coated with CTH522 and then blocked with 2% skim milk in PBS. Fecal supernatants or sera were loaded on well plates and titrated in a 2-fold dilution across the plates and detection was done using biotin conjugated anti-mouse IgA (Southern Biotech) for 1 h followed by streptavidin-HRP (BD Biosciences) for 30 min. IgG and IgA were color developed using TMB. Development was stopped with 0.2M $H_2SO_4$ after 10 min and absorbance was measured at 450 nm (correction at 570/620 nm) using a Polarstar Omega Microplate Reader (BMG Labtech). The absorbance values were plotted as a function of the reciprocal dilution of serum samples. Antibody titers were determined as the highest serum dilution corresponding to a cut-off of ≥0.2 OD450 (supporting information). For the cytokine ELISA, 96 well plates were coated with purified anti-mouse IFN-γ or IL-17A in PBS at 4°C overnight. Free binding sites were blocked with 1% BSA. Spleen and PP culture supernatants were tested in triplicates, and detection was done by biotin-labeled rat anti-mouse IFN-γ or IL-17A. Samples were then incubated for 30 min with Streptavidin HRP. Color was developed and measured as described above. Standards of IFN-γ and IL-17A were used to determine the amount of cytokine in the samples.

### 2.11 CT-scanning and X-ray imaging of mice

To study the transit time of MCs in mice, MCs were loaded with the contrast agent $BaSO_4$ and coated with PLGA as described in 2.8. MCs were given to mice orally in size M gelatin capsules. Mice were anesthetized at time points 0.5 h, 1 h, 1.5 h and 2 h. Mice were frozen and then visualized with 3D visualizations made by CT scanning (Nikon XT H 225, Nikon Metrology, Tokyo, Japan). X-rays were generated using a voltage of 70 kV and a power of 30 W (current of 0.43 mA). The 3D visualizations were created from single planar scans using 1572 projections with 2 frames per projection and an exposure time of 0.5 s. Within the final scan time of approximately 27 min, the frozen mice did not seem to thaw. The voxel size, which corresponds to the spatial resolution, was kept constant at 114.41 µm by having the same distance between the x-ray probe and the sample for all scans even though the size of the frozen mice varied slightly. The following reconstruction was made in the software provided with the CT scanner system (CT Pro 3D, Nikon Metrology, Tokyo, Japan) using a Feldkamp, Davis and Kress filtered back-projection algorithm [27]. As a final step, a 3D visualization and analysis software (Avizo, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for the CT scan data to be processed and investigated. Subsequently, the GI tract were isolated from the mice for further analysis with X-ray imaging. Planar X-ray imaging of MCs loaded with $BaSO_4$ in the removed GI tracts was carried out, using a CT scanner, to obtain quantitative

information about their location. The distance between the x-ray probe and the samples was adjusted to get a magnification of 4 and X-rays were generated using a voltage of 70 kV and a power of 30 W (current of 0.43 mA). Image acquisition with 8 frames and an exposure time of 1 s was used for the planar X-ray images and a background signal for shading correction. The shading corrections and subsequent manually counting of the MCs loaded with $BaSO_4$ throughout the entire GI tracts were made using an image processing software (ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA).

**2.12 Statistics**

GraphPad Prism 9 software (San Diego, CA, USA) was used for data handling, analysis, and graphic representation. Statistical analysis was performed using the Kruskal–Wallis test followed by a post-hoc Dunn's multiple comparison test, where p-values below 5% were considered significant. Data is presented as mean ± standard error of the mean (SEM).

# 3 Results and discussion

**3.1 Screening of mucosal adjuvants for formulation with CTH522 and oral administration**

Adjuvants are necessary to induce a proper and efficient immune response against infectious pathogens, especially in regard to subunit type antigens. However, studies have shown that the function of an adjuvant can vary depending on how it is administrated and which kind of antigen is included [7], [28]. In this study, the adjuvants were thus tested according to how well they performed being administered orally in formulation with CTH522. CTH522 was formulated with c-di-GMP, α-GalCer or CTB and dosed orally to mice, either receiving oral prime and boosters, or as boosters following a subcutaneous (s.c.) prime with CTH522 adjuvanted with CAF01 (Fig. 2). In order to evaluate the ability of the oral administered adjuvant formulations to enhance immune responses, a naive group and a group receiving only s.c. prime of CTH522 and CAF01 were included as controls. The IgG specific antibodies in the serum, along with the cytokines elicited in the spleen, were measured as representatives of the systemic response (Fig. 2a, 2b, 2e). A trend towards higher levels of IFN-γ and IL-17A, indicators of Th1 and Th17 induction, was seen in all groups receiving oral gavage boosters compared to the s.c. priming group (Fig. 2a, 2b). Notably, α-GalCer did achieve higher levels of cytokine secretion compared to the other adjuvants, although not significantly. Low to inconsistent levels of IFN-γ and IL-17A comparable to the naive mice, were measured in the groups receiving oral prime and boosters. The CTH522 IgG specific titers, showed no significant difference between the s.c. group and the oral boosted groups (Fig. 2e), suggesting the measured IgG response is developed on account of the s.c. prime injection. Further evidence of this was seen in titers elicited by the oral dosed groups, which, like the cytokine analysis of these groups, were comparable to the naive mice. Cytokine levels in Peyer's Patches (PPs) along with CTH522 specific IgA antibody titers in feces were analyzed in order to represent the local mucosal immune response elicited in the intestine (Fig. 2c, 2d, 2f). Here, the same pattern in cytokine levels was observed as in the systemic response analysis. Mice receiving s.c. prime and oral boosters showed increased secretion of IFN-γ and IL-17A in PPs compared to the s.c. group, however not significantly (Fig. 2c, 2d). α-GalCer excelled slightly in stimulation of Th17 cells in the PPs compared to the other adjuvants. A change was however seen in the oral primed and boosted groups, where α-GalCer did achieve significantly higher levels of IFN-γ compared to the naive group ($p < 0.0286$, Fig 2c). Measured CTH522 specific IgA titers in fecal pellets showed no difference between the s.c. primed groups, suggesting that the response is an effect of the CAF01 injection (Fig. 2f). However, all groups receiving oral prime and boosters generated significantly higher IgA titers compared to the naive mice ($p = 0.0346$, Fig. 2f), indicating the adjuvants are capable of eliciting local IgA response when administered orally and in formulation with CTH522. The results observed in this study, points towards α-GalCer, as the most prominent adjuvant for formulation and oral administration with CTH522. A reason for this could be that being a glycolipid, α-GalCer is more stable and less prone to

immediate degradation by the low pH in the stomach. α-GalCer have also before shown to boost immunogenicity of HIV antigens peptide by simple oral immunization[29].

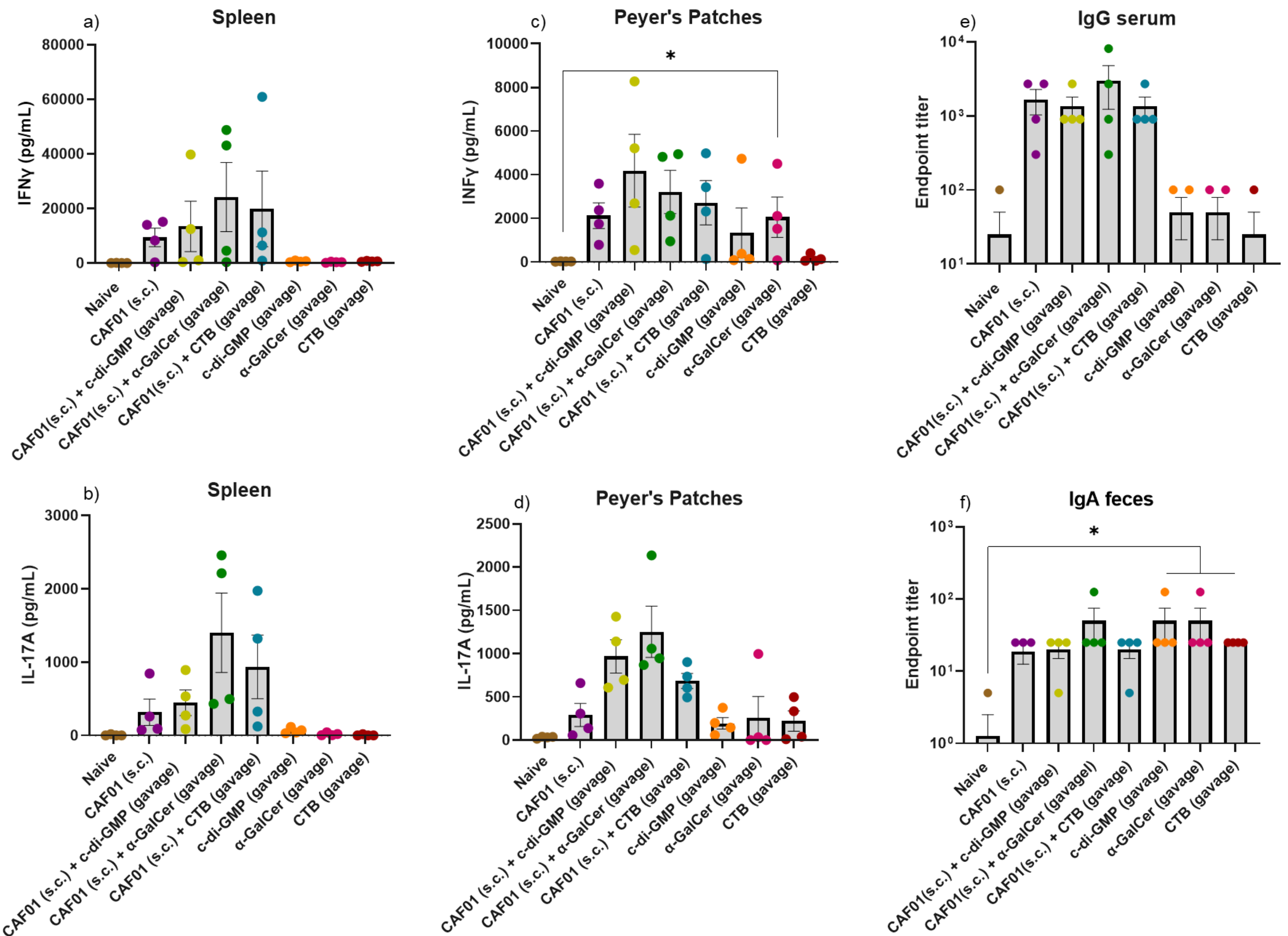



**Fig. 2.** Levels of secreted IFN-γ and IL-17A measured in spleen (a, b) and Peyer's Patches (c, d). CTH522 specific serum IgG and fecal IgA antibody titers (e, f). Mice, except the naive and CAF01(s.c.) group, were immunized 3 times in a prime-booster-booster regime, with either a sub cutaneous (s.c.) prime injection followed by oral boosters with gavage or oral prime followed by oral boosters with gavage. Immunizations were given at day 0, 21 and 42. Data are shown from individual mice and bars represent mean ± SEM (n = 4). * $p < 0.05$.

### 3.2 Lyophilization procedure for the CTH522 + α-GalCer formulation.

Since vaccine formulations are often thermally labile, lyophilization is frequently used to stabilize vaccine for distribution and storage [30]. This is especially of advantage in places where cold chain management is difficult. During the lyophilization process, solvent is removed, and replaced by a stabilizing substance which also functions as a lyoprotectant[30]. The method is complex and requires optimization of both process parameters and buffers, to achieve optimal drying of the formulation and no loss of immunogenicity in the case of vaccines[31]. The lyophilization program described in 2.4 was used with various excipients at different ratios, and evaluated by the consistency and visual appearance in reference to [32] (Table 4). 10%(w/v) Trehalose + 10 mM Tris were deemed the most prominent excipient and additive, achieving the best dried state post-lyophilization.

| Antigen | Adjuvant | Stabilizer | Buffer | Visual attribute of sample | Consistency score |
|---|---|---|---|---|---|
| CTH522 (10 µg) | α-GalCer (10 µg) | 5% Trehalose | 10 mM Tris | Uniform | + |
| | | | 10 mM Tris + 2% Glycerol | Meltback | - |
| | | | PBS | Cracked | + |
| | | 10% Trehalose | 10 mM Tris | Uniform | +++ |
| | | | 10 mM Tris + 2%Glycerol | Meltback | - |
| | | | PBS | Uniform | + |
| | | 15% Trehalose | 10 mM Tris | Uniform | ++ |
| | | | 10 mM Tris + 2%Glycerol | Meltback | - |
| | | | PBS | Uniform | ++ |
| | | 10% Sucrose | 10 mM Tris | Uniform | ++ |
| | | | PBS | Cracked | + |
| | | 10% Lysine | 10 mM Tris | Collapse | - |
| | | | PBS | Collapse | - |
| | | 10% Mannitol | 10 mM Tris | Cracked | + |
| | | | 10 mM Tris + 2% Glycerol | Meltback | - |

**Table 4.** Buffer formulations for optimization of lyophilization procedure. Post-lyophilization visual attributes were referenced to [32] and the consistency score were based on the ease of loading the powder into microcontainers.

Lyophilized CTH522 was then rehydrated and analyzed by SDS-PAGE to determine that no degradation was happening as a result of the lyophilization or rehydration process (Fig. 3a). To investigate if the lyophilization caused any loss of immunogenicity to the antigen, the formulation was evaluated in an *in vivo* study. Lyophilized CTH522 + α-GalCer was rehydrated and given s.c. to mice in a prime-booster regime (Fig. 3b, 3c, 3d). As controls, a naive group was included along with a group receiving the same formulation but non-lyophilized. From the study, it was evident that no loss of immunogenicity was seen, as no difference was observed between the lyophilized group and non-lyophilized group on any of the measured antibody or cytokine responses. The developed lyophilization process and optimized buffer formulation are thus capable to effectively lyophilize CTH522 while in formulation with α-GalCer, without causing damage to the antigen and successfully retain the immunogenicity. Antigens and adjuvants are usually not lyophilized while in formulation, as it can have implications on the process[33]. The developed protocol could potentially be used for other such formulations in future studies.

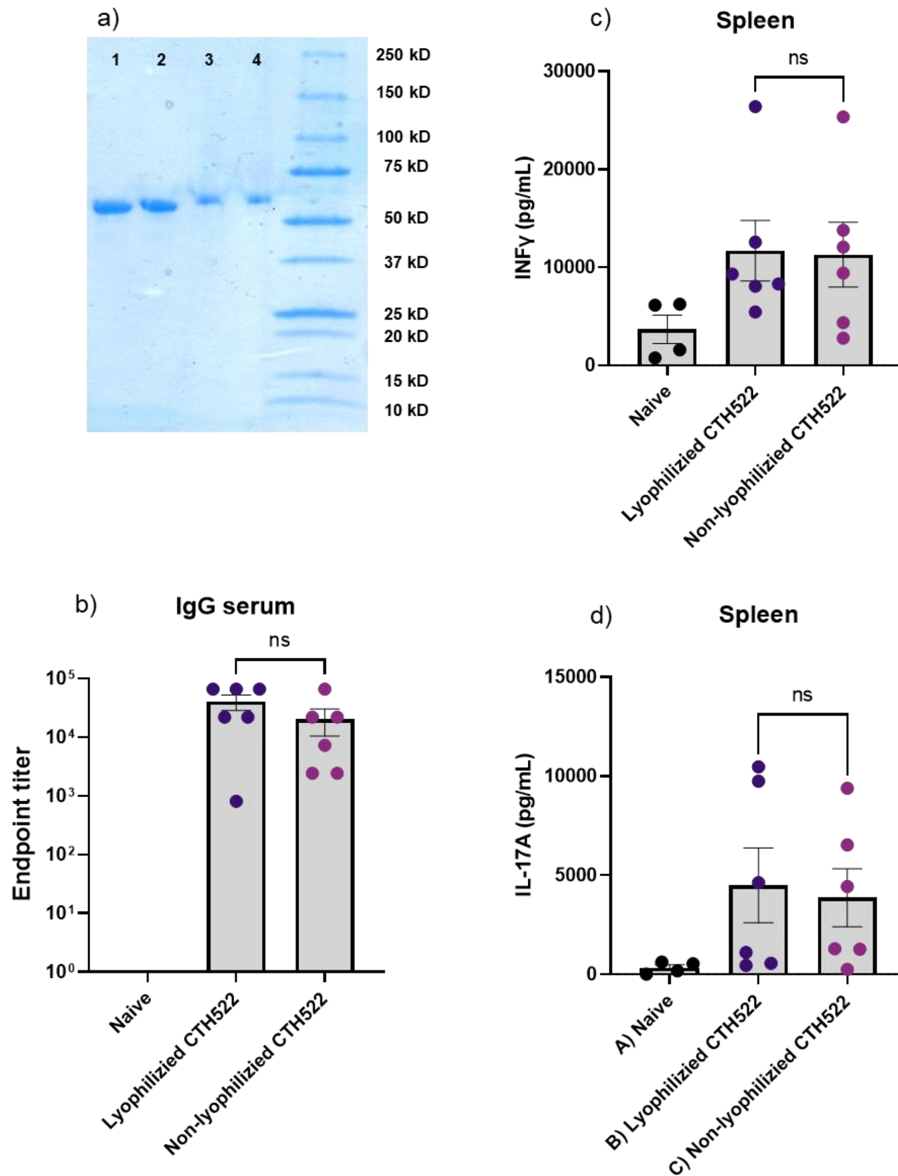



**Fig. 3**. SDS-gel of rehydrated lyophilized CTH522 + SDS (row 1), non-lyophilized CTH522 + SDS (row 2), rehydrated lyophilized CTH522 ÷ SDS (row 3) and non-lyophilized CTH522 ÷ SDS (row 4) (a). CTH522 specific serum IgG antibody titers (b). Levels of secreted IFN-γ and IL-17A measured in spleen (c, d). Mice, except the naive group, were immunized subcutaneously 2 times in a prime-booster regime at day 0 and 21. Data are shown from individual mice and bars represent mean ± SEM (n = 6).

### 3.3 Fabrication, loading and coating of microcontainers

MCs were fabricated with an outer diameter of 313.4 ± 1.7 µm and height of 289.4 ± 5.3 µm, and with an inner diameter of 262.5 ± 0.9 µm and height of 235.8 ± 4.4 µm. MCs were loaded with 3.1 ± 0.6 µg/microcontainer of powder and then sealed with either chitosan, PLGA or EL100–55 lids. The average thickness of the lid coating was measured by contact profilometry to be 27.3 ± 2.1 µm for chitosan, 28.7 ± 4.5 µm for PLGA and 25.7 ± 1.5 µm for EL100-55.

PLGA and chitosan have both been documented to have adjuvant properties and are widely used for mucosal delivery of antigens and active pharmaceutical ingredients (APIs), often as particle carriers[13],

[34], [35]. Here, it was investigated if coatings with PLGA or Chitosan on MCs could benefit the delivery of the CTH522 antigen. Additionally, the pH-dependent EL100-55 coating was also included. This polymer has the ability to stay solid at pH 4.7, corresponding to the pH in the stomach of mice, and dissolve at pH 6.6 corresponding to the pH in the small intestine of mice[36], [37]. These properties can be used to protect the content in the stomach and effectively target the small intestine for the release[24]. This was compared to naive mice and a group only receiving a s.c. prime to distinguish the oral response as in 3.1 (Fig. 4). Additionally, the local immune response in the intestine was also investigated for all three coatings, which have not been done before. Systemically no difference in response was seen (Fig. 4a, 4b, 4e), however, CTH522 specific IgA antibody levels measured in feces from MCs coated with EL100-55 were significantly increased (p = 0.0488, Fig. 4f). This suggests that the EL100-55 coating can be used for effective delivery of CTH522 with MCs, and that no improvement is gained from coatings with PLGA and chitosan.

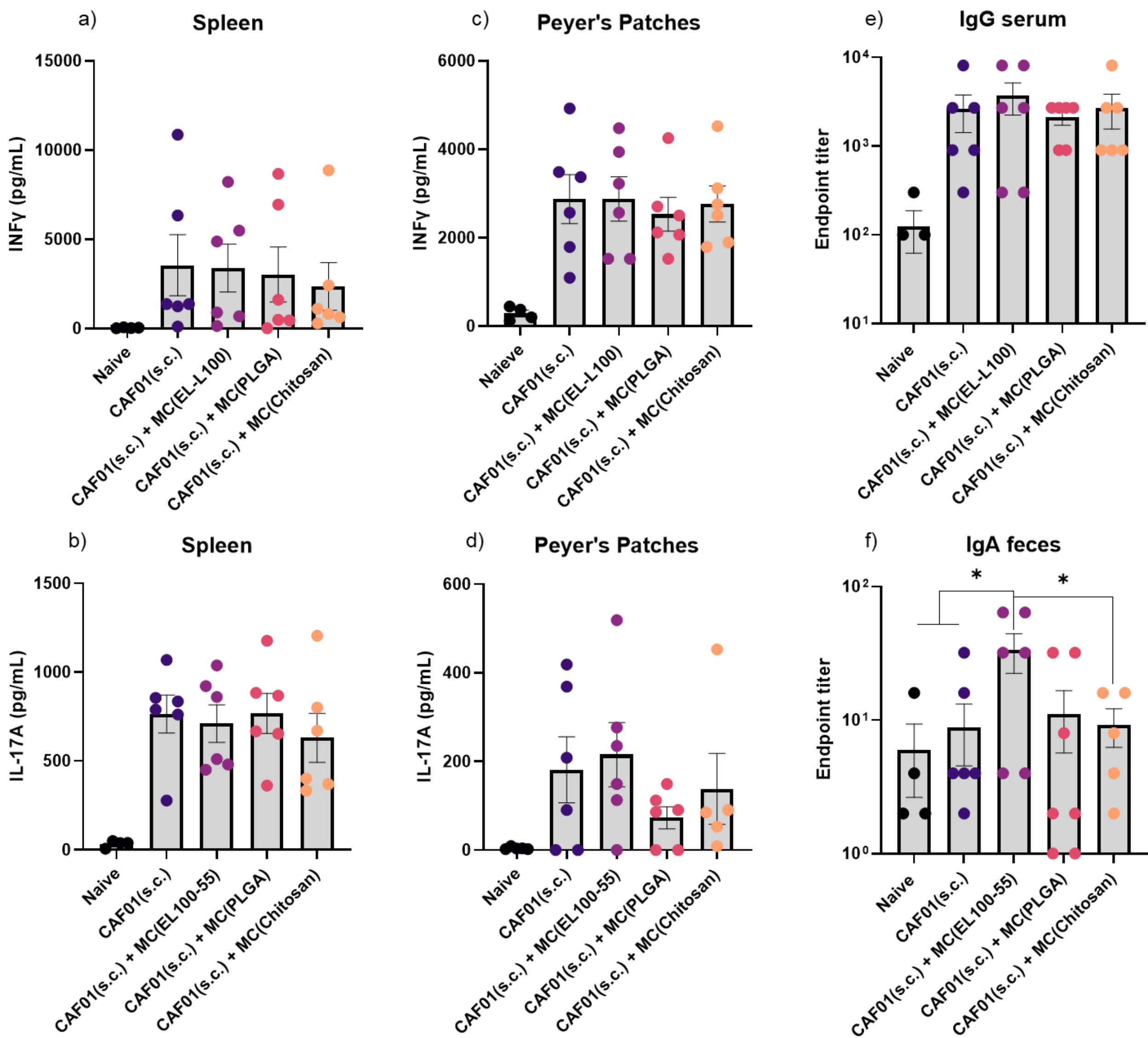



**Fig. 4.** Levels of secreted IFN-γ and IL-17A measured in spleen (a, b) and Peyer's Patches (c, d). CTH522 specific serum IgG and fecal IgA antibody titers (e, f). Mice, except the naive and CAF01(s.c.) group, were immunized 3 times in a prime-booster-booster regime, with either a sub cutaneous (s.c.) prime injection followed by oral boosters with

gavage or oral prime followed by oral boosters with gavage. Immunizations were given at day 0, 21 and 42. Data are shown from individual mice and bars represent mean ± SEM (n = 6). * $p < 0.05$.

The reason for this was further investigated, by visually tracking the release of the vaccine formulation from the MCs. Chips with MCs were coated with EL100-55 (Fig. 5a), PLGA (supporting information) or chitosan (supporting information) subsequent to vaccine loading, and emerged in maleic acid of pH 4.7 and 6.6 at 36.6 $^{o}$C simulating stomach and intestinal conditions of mice, in the same manner as previously presented [24]. After 60 min in pH 4.7, lids of EL100-55 (Fig. 5b) and PLGA were still intact, however the majority of the chitosan lids had disappeared along with the content of the containers. A property of chitosan is a swelling behavioral trait, which can be utilized to achieve a slow sustained release if correctly engineered[38], [39]. The coating formulation used in this experiment does however, not seem to be compatible with the MCs and has probably detached itself upon swelling. This is most likely the reason why no effect was seen in the immunological analysis, due to an early release of the vaccine formulation in the stomach, rendering it ineffective. After 60 min, the MC chips were moved to pH 6.6 for 2x30 min. It is evident that the EL100-55 gradually disappears along with the content of the MCs (Fig. 5c, 5d). PLGA does still appear to be intact at these conditions, indicating that no release of vaccine occurs, making it the probable cause to why no effect was observed in the immunological analysis. PLGA has numerous times been employed as a particular delivery vehicle, and does seem to have adjuvant effects when used in this format, due to the particle morphology[34], [40]. As a coating however, this trait is obviously not exploited, and the PLGA formulation used in this study does not seem very well suited for the purpose of proximal intestinal release. However, it has been reported that PLGA can be tailored for colon-directed release, by modifying the lactic and glycolic ratios in combination with pH degradable polymers[41]. A PLGA formulation optimized for the purposes of this study could then most likely be manufactured. From another perspective, a colon targeting PLGA formulation, could be useful for vaccine-related purposes to stimulate local responses against colonic infecting pathogens. In this study, the immunological analysis and subsequent troubleshooting of the release of CTH522 formulation from MCs, revealed EL100-55 as the most proficient coating, for the purpose of oral vaccine delivery with MCs. A significant higher IgA response was observed in fecal samples from this group, most likely due to intended release of CTH522 in the intestine.

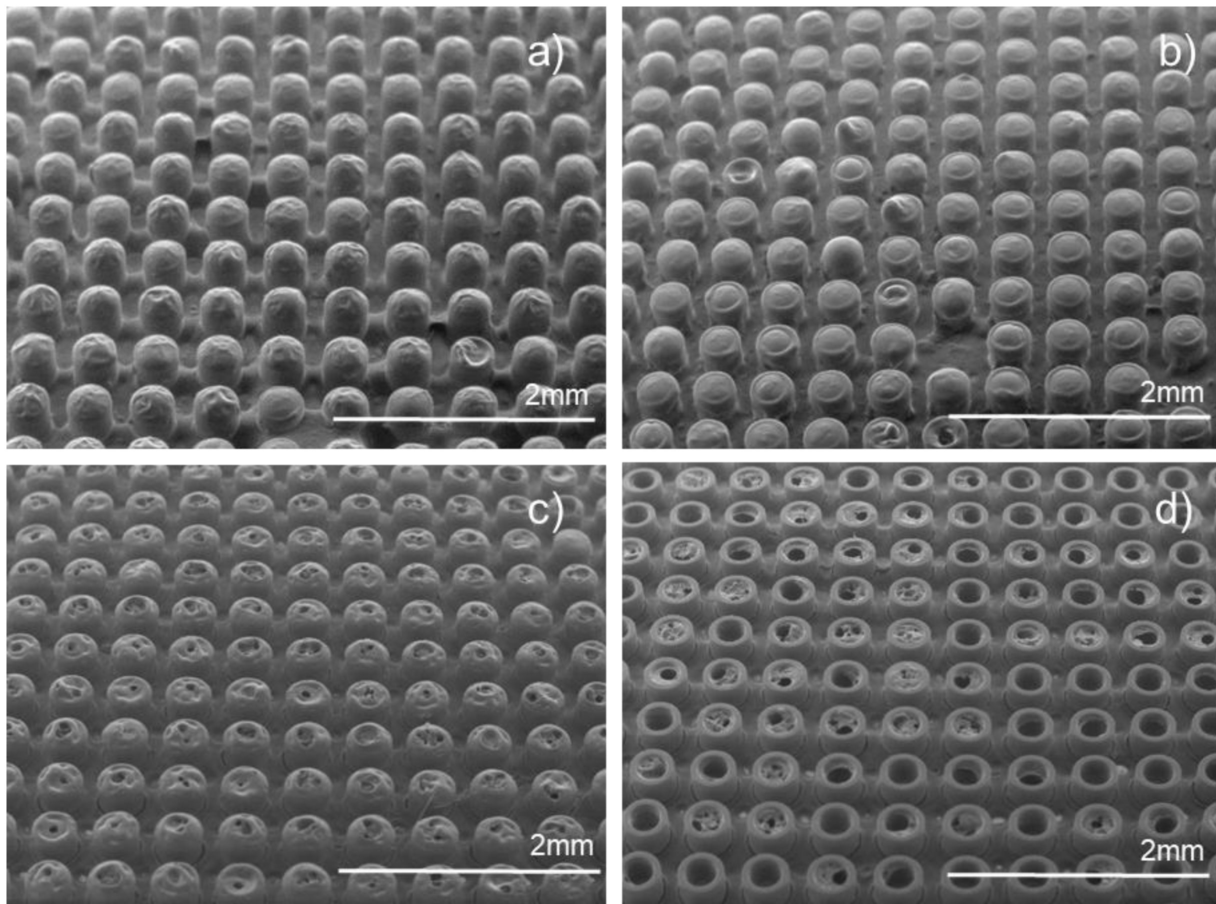



**Fig. 5**. SEM images showing dry microcontainers (MCs) on a chip loaded with CTH522 + α-GalCer coated with EL100-55 (a). MCs were soaked in 36.6°C 2mM maleic acid at pH 4.7 simulating the environment of the mouse stomach and imaged after 60 min (b). MCs were then transferred to 36.6°C 10mM maleic acid at pH 6.6 simulating the environment of the mouse intestine and imaged after 30 min (c) and 60 min (d).

**3.4 Immunological analysis of oral delivery of CTH552 + α-GalCer with microcontainers coated with EL100-55.**

Based on the results obtained from the screening of adjuvants and MC coatings, CTH522 was formulated with α-GalCer and dosed in MCs with EL100-55 lids, both as oral prime and boosters and as oral boosters following an s.c. prime with CTH522 + CAF01 (Fig. 6). Increased systemic IL-17 levels were observed in the groups receiving oral boosters with either MCs or gavage, compared to the group only receiving a s.c. prime indicating that the response is stimulated by the oral boosters, although not significantly enhanced (Fig. 6b). Furthermore, the IL-17 cytokine levels were slightly higher in the group boosted with MCs compared to the gavage group. The local immune response in PPs and IgA levels in feces, also showed a trend towards enhanced levels in the MC group compared to the s.c. and oral gavage groups, but not significantly higher (Fig. 6c, 6d, 6f). *Davitt et al.* demonstrated a significantly enhanced systemic and local response of cholera specific IgA antibodies, along with increased INFγ levels in the PPs, from dosing Dukoral adjuvanted with α-GalCer orally[42]. These findings are in line with the trends observed in the local mucosal responses in the present study, however the stimulated responses were not statistically enhanced. A likely cause for the different findings is that Dukoral contains a killed whole-cell antigen, thus benefitting from intrinsic adjuvant traits as opposed to CTH522 [43], [44]. An interesting observation from this study is the stimulation of Th17-cells in PPs, which is not seen in the study by *Davitt et al*. CAF01 is known to induce systemic Th17 responses, that can be pulled into mucosal tissues after local mucosal

vaccination. The Th17 responses observed in this study could thus be an example of this prime-pull effect, where the Th17 cells, generated by the s.c. prime injection, are migrating to the intestinal tissue upon oral boosting [10], [45], [46]. Elevated levels of Th1 and Th17 responses in PPs as well as IgA antibody titers were also observed in the oral MC prime-boost group compared to the naive mice, indicating that the CTH522 + α-GalCer formulation is indeed capable of inducing mucosal immune responses when delivered orally in MCs ($p = 0.0436, 0.0273, p = 0.0281$, Fig. 6c, 6d, 6f). Albeit the oral MC prime-boost group did not reach the same level of immune induction as the prime-pull groups.

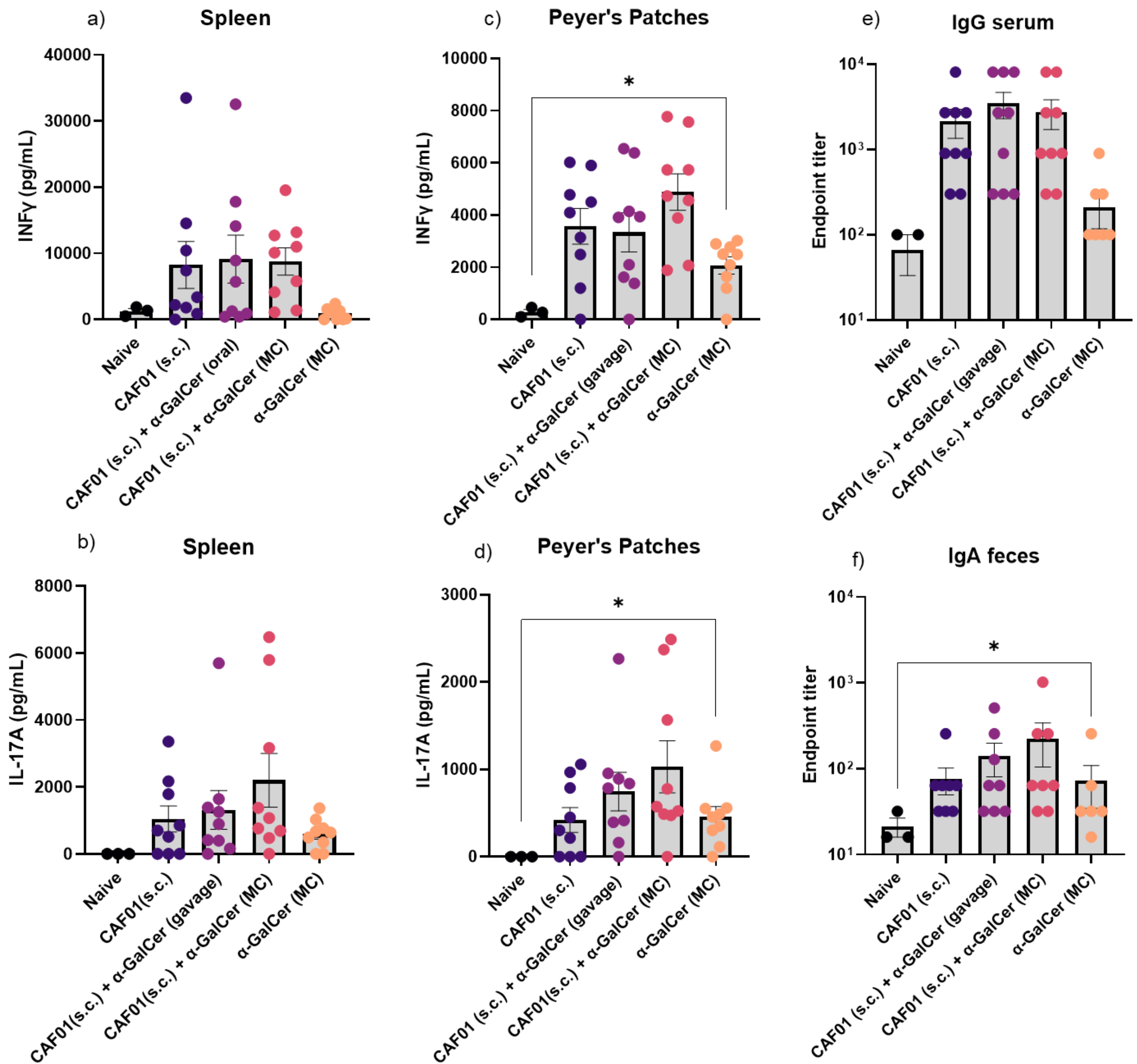
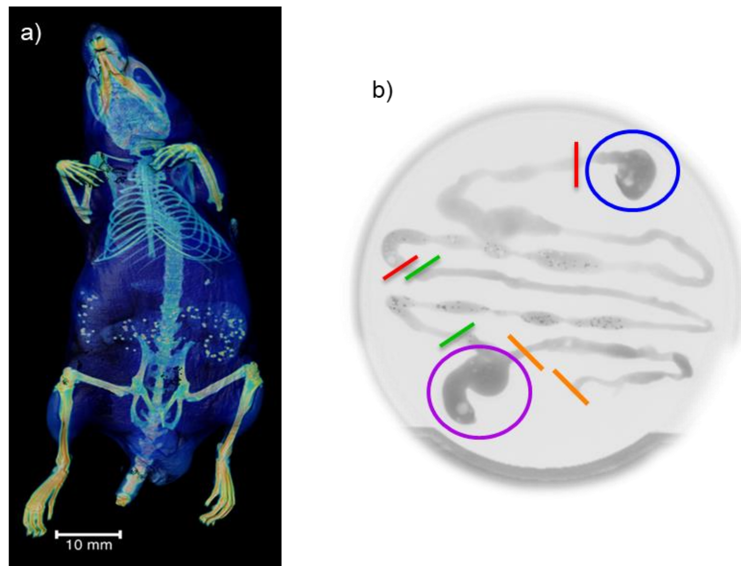



**Fig. 6.** Levels of secreted IFN-γ and IL-17A measured in spleen (a, b) and Peyer's Patches (c, d). CTH522 specific serum IgG and fecal IgA antibody titers (e, f). Mice, except the naive and CAF01 (s.c.) group, were immunized 3 times in a prime-booster-booster regime, with either a subcutaneous (s.c.) prime injection followed by oral boosters with gavage or microcontainers (MCs). One group received oral prime followed by oral boosters with MCs. Immunizations were given at day 0, 21 and 42. Data are shown from individual mice and bars represent mean ± SEM ($n = 9$). * $p < 0.05$.

### 3.5 CT-scan and X-ray imaging of microcontainer transit kinetics in mice

Even though we observed measurable immune responses upon delivery of adjuvanted CTH522 in MCs, levels were lower than expected. It was therefore speculated that the MCs transit is either too fast or that they deliver the vaccine to the wrong intestinal compartment for stimulation of the immune cells to occur. A recent study by *Esterházy et. al.* thus demonstrated that draining lymph nodes in the distal intestine, promoted effector T-helper cells, whereas proximal lymph nodes promoted $T_{reg}$ responses[47]. These findings indicate that release of vaccine in the proximal intestine may not be optimal, whereas the distal intestinal compartment could be highly relevant to target[1], [47].

X-ray and CT-scanning have before been used to thoroughly investigate transition in rats but never in mice[25]. Combined analysis of these methods showed that the transit time from the stomach to the cecum of MCs in mice was about 1-1.5 h (Fig. 7). This corresponds to the standard transit time of the GI tract in mice, and could be the reason why a higher immune response was not achieved[48]. It is therefore possible that the immunological performance could be enhanced, by tailoring the polymeric lid to release in the distal part of the intestine. The MCs are made of the mucoadhesive material SU-8 and have previously been proved to increase retention of MCs in the intestine of rats [25], [49]. This effect was thus expected to be utilized in mice as well for the purpose of oral vaccine delivery. However, the data from rats taken together with the observed results in this study, indicate that the proportional size of the MCs to mice is too large, and the MCs are effected by peristaltic movements and moved regardless of being mucoadhesive. A solution could be to change the animal model to one proportionally larger and with a longer transit time, such as rabbits or pigs. This would additionally improve the scalability, and give vital information on the MCs kinetics in an environment genetically and metabolically closer to that of humans[50]. Ideally, a process should be put in place, designed to first get indication of promising formulation candidates in smaller animal models, which then should continue to testing with the microdevices in larger animals. Methods to employ to get indications of the formulations potential, could be intra-intestinal infusion, in combination with an intestinal closed-loop model, where the infused material is prohibited from transit through the intestine [51], [52]. This would in theory evaluate the MCs ability to deliver the vaccine to immune cells of the distal part of the intestine, should it be retained long enough in addition to vaccine formulations immunological capabilities when properly delivered.



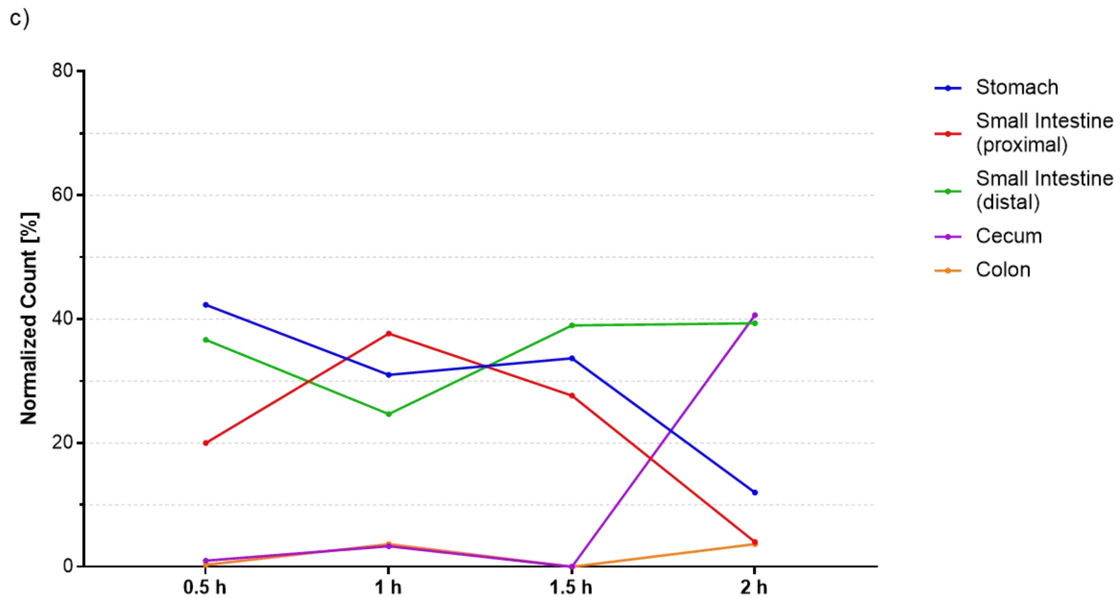




**Fig. 7.** CT-scan of mouse 2 h post-administration with $BaSO_4$ loaded microcontainers (MCs). X-ray of isolated GI tract of mouse 1.5 h post-administration with $BaSO_4$ loaded MCs with indication of stomach (blue), proximal small intestine (red), distal small intestine (green), cecum (purple) and colon (orange) (b). Graph showing amount of BaSO4-loaded MCs found in each section of the isolated GI tracts for each time point of euthanasia (c). The counts were found combining CT-scan and planar X-ray imaging and are here plotted as single points, with lines linking each point (n = 3).

## 4. Conclusion

In this work, we tested MCs ability to orally deliver the *C. trachomatis* vaccine candidate CTH522 in combination with a mucosal adjuvant. α-GalCer was found to be the most prominent adjuvant to be formulated with CTH522. A procedure to successfully lyophilize the vaccine formulation, without degrading the antigen or losing immunogenicity, was developed. MCs coated with EL100-55 elicited a significantly higher local CTH522 specific IgA response, compared to MCs coated with PLGA and chitosan, deeming EL100-55 the best choice for MC lids. CTH522 + α-GalCer administered in MCs orally following an s.c. prime, showed an increase in the mucosal immune response locally and to a degree systemically, demonstrating a prime-pull effect. Solely oral dosing with MCs also managed to generate significantly enhanced mucosal immune responses compared to naive mice. Some optimization is however needed, as the measured immune responses are relatively low, and not significantly enhanced compared to just receiving an s.c. prime. A possible reason for this could be the fast transit time in mice. CT-scan and X-ray imaging showed that the transit time of MCs delivered orally is only 1-1.5h and that they are not retained despite mucoadhesive traits. This is probably partly due to the proportional size ratio between the MCs and the intestinal tract of mice. In a mouse, the diameter of the intestinal tract is approximate 2mm, and the mucus layer is around 20-25 µm thick. Thus, the microcontainers will not be completely embedded in the mucus layer and will then easily be moved along with consumed food and peristaltic movements. Our results indicate that the mouse is not an optimal animal model, when dealing with oral delivery of devices in the 100 µm range. In future studies with such devices, larger animals may be needed to study the effects of MCs.

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**Conflicts of interest**

The authors have no conflict of interest to declare

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## Supporting information

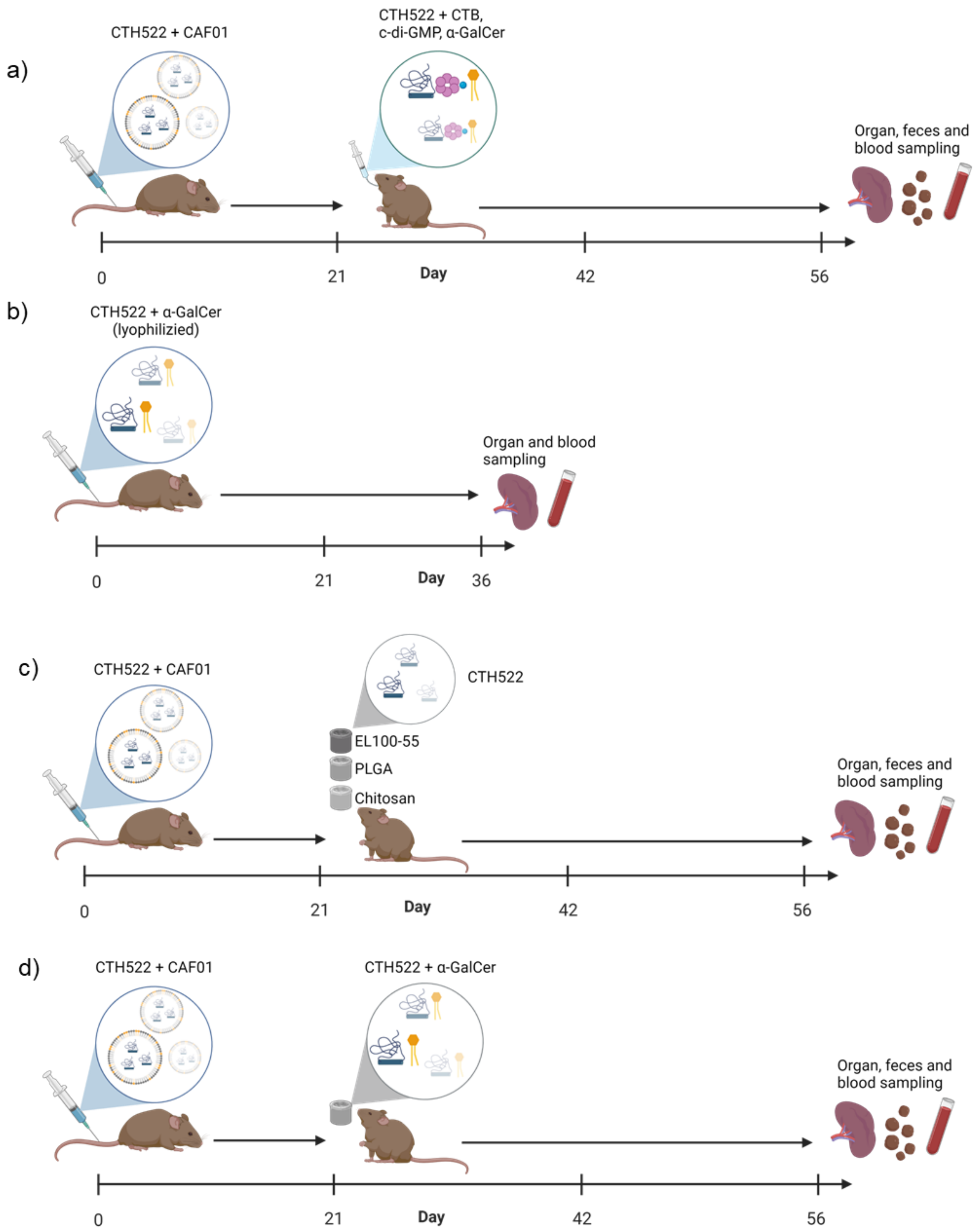


**Fig S1.** Immunization regimes of the four *in vivo* studies. Screening of adjuvants in mice receiving an s.c. prime with CTH522+CAF01 on day 0, followed by two oral boosters with gavage of CTH522 formulated with CTB, c-di-GMP, or α-GalCer on day 21 and 42 (groups only dosed orally with the adjuvant formulations were also included) (a). Mice were euthanized and organs were harvested on day 56. Test of immunogenicity of lyophilized CTH522+α-GalCer, with mice receiving an s.c. prime on day 0 and booster on day 21 (b). Mice were euthanized and organs were harvested on day 36. Screening of coating, with mice receiving an s.c. prime with CTH522+CAF01 on day 0, followed by two oral boosters with MCs coated with EL100-55, PLGA, or chitosan and loaded with CTH522 on day 21 and 42 (c). Mice were euthanized and organs were harvested on day 56. Oral delivery of the CTH522 + α-GalCer formulation in MCs, with the mice receiving an s.c. prime with CTH522 + CAF01, followed by two oral boosters with MCs coated with EL100-55 and loaded with CTH522 + α-GalCer (groups receiving oral boosters with gavage, and only dosed orally with MCs were also included) (d). Mice were euthanized and harvested on day 56. *Created with Biorender.com*

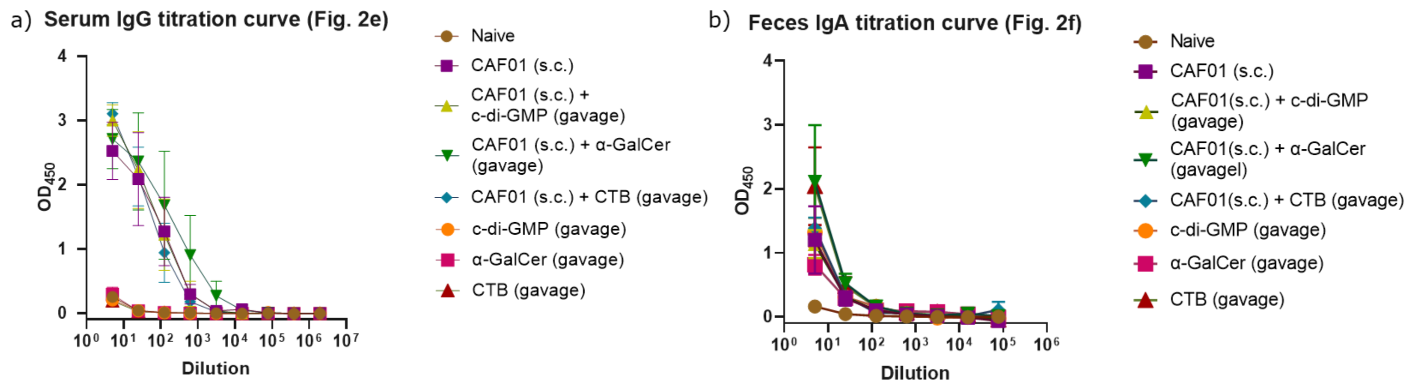



**Fig. S1**. Graphs depicting the antibody titration curves obtained by ELISA of the CTH522-specific IgG in serum (a) and CTH522-specific IgA in feces (b). The titers plotted in Fig. 2e and 2f were calculated from these graphs. Antibody titers were determined as the highest serum dilution corresponding to a cut-off of ≥0.2 OD4

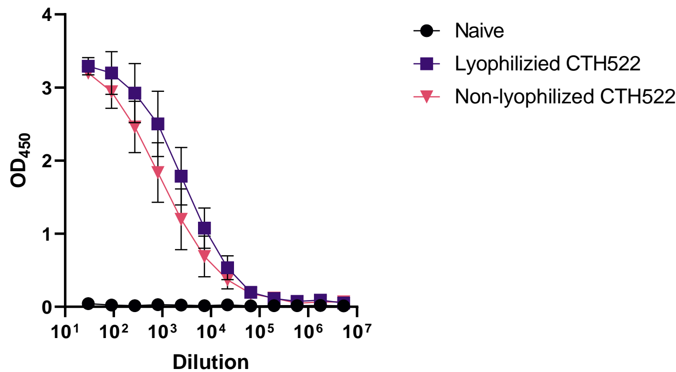
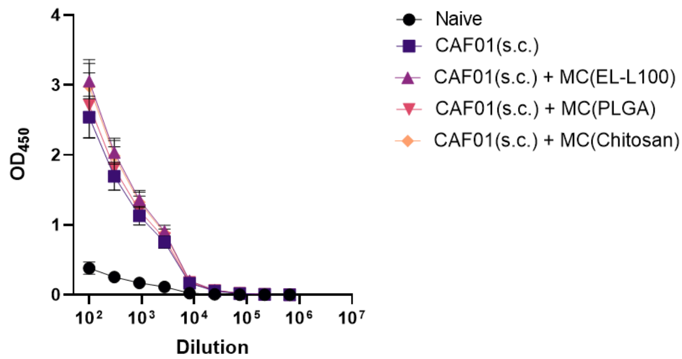



**Fig. S2**. Graph depicting the antibody titration curves obtained from ELISA of the CTH522-specific IgG in serum. The titers plotted in Fig. 3d were calculated from this graph. Antibody titers were determined as the highest serum dilution corresponding to a cut-off of ≥0.2 OD450



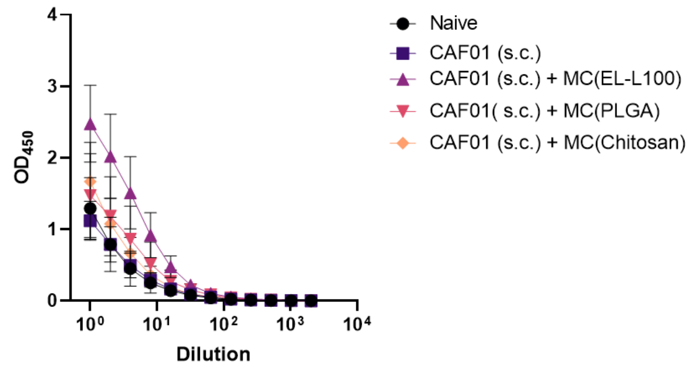




**Fig. S3.** Graphs depicting the antibody titration curves obtained from ELISA of CTH522-specific IgG in serum (a) and CTH522-specific IgA in feces (b). The titers plotted in Fig. 4e and 4f were calculated from these graphs. Antibody titers were determined as the highest serum dilution corresponding to a cut-off of ≥0.2 OD450

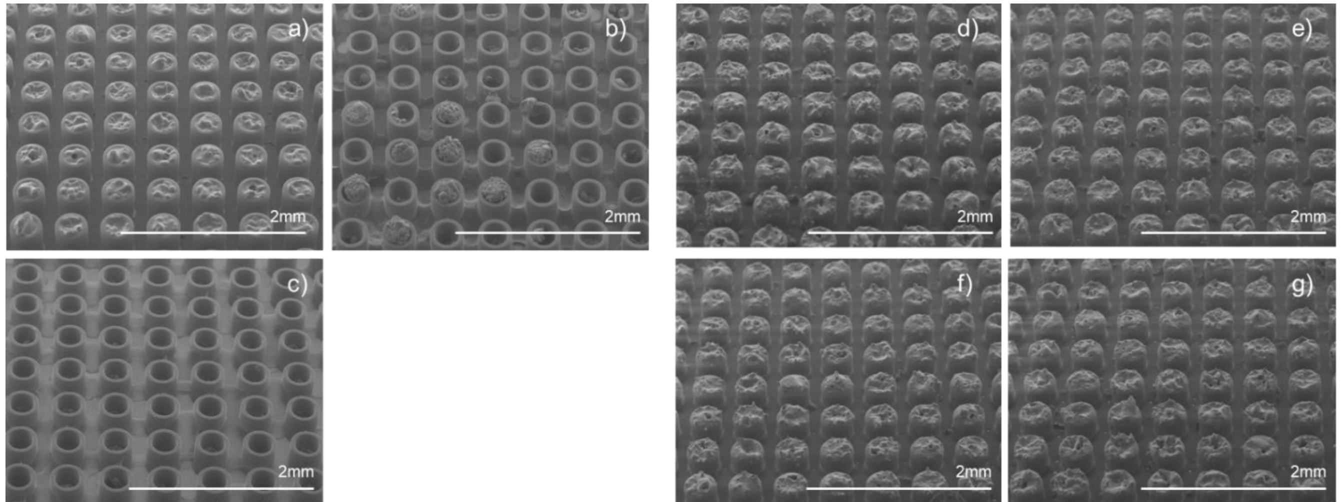
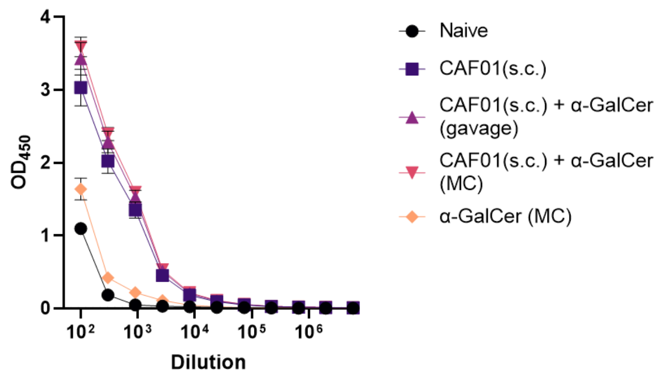



**Fig. S4.** SEM images of microcontainers coated with chitosan (a), and after being submerged in maleic acid pH 4.7 for 30 min (b) and 60 min (c). SEM images of microcontainers coated with PLGA (d), and after being submerged in maleic acid pH 6.6 for 60 minutes (e). Subsequently, PLGA coated microcontainers were transferred to maleic acid pH 6.6 for 30 min (f) and 60 min (g).

a) **Serum IgG titration curve (Fig. 6e)**




b) **Feces IgA titration curve (Fig. 6f)**

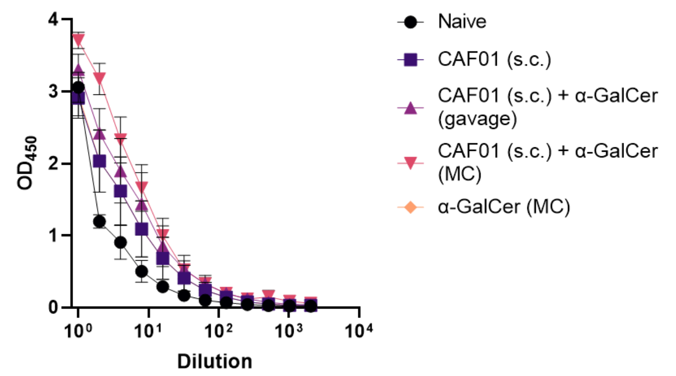



**Fig. S5.** Graphs depicting the antibody titration curves obtained from ELISA of CTH522-specific IgG in serum (a) and CTH522-specific IgA in feces (b). The titers plotted in fig. 6e and 6f were calculated from these graphs. Antibody titers were determined as the highest serum dilution corresponding to a cut-off of ≥0.2 OD450

# Paper II:

# Oral delivery of the AP205-SpyCatcher capsid virus-like-particle using microdevices

Manuscript in preparation

# Oral delivery of the AP205-SpyCatcher capsid virus-like-particle using microdevices

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## Abstract

Vaccination through the oral route is highly sought after due to the massive benefits, such as high patient compliance, logistic advantages, and local mucosal immunity. Current oral vaccines are all based on live or attenuated pathogens, but because of to safety concerns, modern vaccine research is more focused on recombinant antigens; however, these are associated with decreased immunogenicity. Nonetheless, the immunogenicity can be boosted by, for example, effective presentation to the immune system. The capsid virus-like-particle AP205 has been previously used as a scaffold for the presentation of various unrelated antigens. By conjugating the antigen to the platform using a tag/catcher protein system, robust systemic immune responses have been achieved. Similar to licensed oral vaccines, cVLP have a particular morphology, which could potentially aid in the stimulation of the mucosal immune system in the intestine. However, the environment of the GI tract presents several challenges, such as degrading acids, enzymes, and bacteria. Therefore, the cVLPs were delivered in microcontainers (MCs), which have previously shown promise in protecting drugs and antigens in the stomach, along with facilitating release in the intestine. Protocols of efficient lyophilization of the cVLPs were established along with successful loading into MCs followed by coating with the Eudragit L100 polymer. Rats were orally immunized with cVLPs in MCs, but no measurable immune stimulation were observed. In future studies, adjuvants and a prime-pull immunization regime, are warranted to effectively induce a response.

## Introduction

The current trend of vaccine refusal and hesitancy owing to the potential risks and fear of needles from parental vaccines, indicates the need for vaccinations with higher patient compliance[1]. The route of administration with the highest compliance is the oral route. In addition, prevention of several enteric pathogens such as Shigella, enterotoxigenic *E. coli* (ETEC), *Helicobacter pylori,* and *Vibrio cholerae* , require a strong IgA antibody and local immune response in the gut, which is mainly achievable via the oral route[2]–[5]. However, the nature of the digestive system poses various challenges in the form of biological and chemical barriers[6]. Consequently, the antigens must be protected from the harsh environment of the upper gastrointestinal (GI) tract and effectively delivered in the intestine[6], [7]. Developments of micro- and nano-technologies have shown promise in overcoming these challenges [8]–[10]. We have previously used microcontainers (MCs) for delivering model antigen ovalbumin (OVA), indicating that the MCs are capable of protecting the protein through the acidic environment of the stomach and facilitate their release in the intestine of mice via pH-degradable lids[11]. However, immunological analysis revealed the stimulated immune responses to be relatively weak. Currently, all licensed oral vaccines are based on attenuated or inactivated pathogens, which are beneficial, based on their intrinsic adjuvant traits, to facilitate absorption and immune stimulation in the mucosal tissue of the GI tract[2]. These advantages can be linked in part to the particulate morphology of these antigens, a trait that proteins like OVA do not benefit from. However, modern day vaccine research are focused on the use of recombinant vaccines due to the ease of modification and increased safety.

Recombinant capsid virus-like particles (cVLPs) offers a very versatile and efficient vaccination platform[12]. Owing to their structural resemblance to live viruses, particularly their size (20 nm–200 nm), they can undergo direct drainage into lymph nodes, making them highly immunogenic. In addition, the repetitive surface structure promotes uptake and cross presentation by antigen-presenting cells and facilitates efficient B cell receptor crosslinking[13]. An innovative use for cVLPs, are as scaffolds for the presentation of unrelated antigens. Display on cVLPs will present the antigen in a particulate, multivalent, and repetitive form to increase the immunogenicity of the antigen. Several cVLP-based vaccine platforms have emerged, making it possible to use a standard cVLP backbone for the delivery of a variety of vaccine antigens[14]–[18]. Previously, cVLPs have been developed using the AP205 bacteriophage, as a backbone for covalently attaching diverse antigens on the surface through a tag/catcher split-protein system, most recently displayed in the COVID-19 spike protein[19]. Recently, two studies involving chimeric enveloped virus-like particles (eVLPs) demonstrated efficient immune responses that could protect against influenza infection and HA-expressing tumors following oral immunization[20], [21]. The eVLPs were equipped with a surface protein from *Giardia lamblia*, a bacteria capable of colonizing in the intestine. These studies demonstrate the ability of VLPs to orally induce immune responses, if modified and delivered properly. Therefore, it was hypothesized that protection and effective delivery of the cVLP with the MC technology could enable their use for efficient oral vaccination.

In this study, a SpyC-AP205-L2 cVLP was heterologous expressed in *E. coli*, displaying both a vaccine peptide (human papilloma virus [HPV] 16 L2) and a bacterial protein (SpyCatcher) as model antigens. The cVLPs were lyophilized to ease their loading into MCs and increase the dosing amount. Quality controls of postlyophilized cVLPs were included to insure proper formation of the particles upon reconstitution. Lyophilized cVLP formulation was then loaded into MCs and subsequently coated with the pH-degradable polymer Eudragit L100 (EL100). An *in vivo* comparison study was conducted in rats to evaluate the immunological properties of the MC and cVLP technology in combination.

## Methods

**Rats**

All animal experiments were approved by the Danish Animal Experiments Inspectorate, approval number: 2018-15-0201-01541, and were conducted in accordance with national Danish guidelines. Rats were housed in an AAALAC-accredited facility in accordance with good animal practice as defined by FELASA. Male Wistar rats (6–8 weeks old) were obtained from Janvier Labs and housed in a specific pathogen-free facility.

**Expression and purification of cVLPs and L2-Ag85a**

SpyCatcher-AP205-L2 cVLPs were expressed and purified as described previously[14]. Briefly, the RG1 epitope of HPV16 L2 protein (QLYKTCKQAGTCPPDIIPKVEG) was genetically fused to the C-terminus of the SpyCatcher-AP205 cVLP subunit. SpyC-AP205-L2 cVLPs were expressed in BL21 (DE3) *E. coli* cells (Invitrogen) and purified by ultracentrifugation using an Optiprep™ (Sigma-Aldrich, MO, USA) density step gradient. L2-Ag85a was expressed in BL21 *E. coli* cells and purified by immobilized-metal affinity chromatography (IMAC) via its C-terminal His tag. Endotoxin was removed from the purified SpyC-AP205-L2 and L2-Ag85a via phase extraction using triton X-114 (doi: 10.1016/j.vaccine.2013.07.052), followed by overnight dialysis into 20 mM sodium phosphate (pH 7.2) + 10% sucrose buffer. Samples were diluted to a total protein concentration of 68 µM (2 mg/ml for SpyC-AP205-L2 and 2.31 mg/ml for L2-Ag85a respectively) prior to lyophilization.

**Lyophilization and quality control of lyophilized CVLPs**

SpyC-AP205-L2 cVLPs were lyophilized in a Christ Delta 2-24 LSCplus freeze-dryer (Christ, Osterode am Harz, Germany) with the program depicted in Table 1. The particles were lyophilized in a 10% sucrose + 20 mM sodium phosphate formulation.

**Table 1.** Parameters of the lyophilization program for SpyC-AP205-L2 cVLPs

| Phase | Freeze | Primary Drying | Primary Drying | Primary Drying | Secondary Drying | Secondary Drying |
|---|---|---|---|---|---|---|
| **Time** | 3:00 h | 0:15 h | 0:15 h | 36:00 h | 5:00 h | 10:00 h |
| **Temp.** | -42 °C | -42 °C | -30 °C | -30 °C | -20 °C | -20 °C |
| **Vacuum** | - | - | 0.120 mbar | 0.120 mbar | 0.120 mbar | 0.120 mbar |

After lyophilization, the cVLPs were reconstituted and analyzed with a CM 100 BioTWIN electron microscope (Phillips, Amsterdam). Particles were stained with 2% uranyl acetate (pH 7.0) prior to transmission electron microscopy (TEM).

For size distribution analysis (DLS), SpyC-AP205-L2 samples taken before and after lyophilization and reconstitution, were loaded into disposable Eppendorf Uvette cuvettes (Sigma-Aldrich, St Louis, MO, USA) and measured on a DynoPro NanoStar (WYATT Technology, USA) equipped with a 658 nm laser. Measurements were taken at 25 ˚C and consisted of 20 acquisitions of 5 s each. The average particle radius and percentage polydispersity (%Pd) was estimated using Dynamic software (version 7.5.0).

### Production, loading and coating of MCs

MCs were fabricated with the negative epoxy photoresist SU-8 through a two-step photolithography process as previously described [22]. The MCs were produced on a titanium|gold (Ti|Au) coated silicon wafer to allow for easy removal from the wafer. The wafer was then cut into 12.8 × 12.8 $mm^2$ chips containing 25 × 25 arrays of MCs using a dicing saw (DISCO, München, Germany). The MCs on chips were loaded with lyophilized cVLP powder using a previously described embossing method [23]. A shadow mask was used to cover the gaps between the MCs, thus filling the MCs without filling the space between them with powder. The average powder load in the MCs was estimated by weighing 10 chips before and after loading and calculating the average of 1 MC. After loading, the MCs were sealed with EL100-55 using an ExactaCoat spray coater (Sono Tek, Milton, Canada) equipped with an ultrasonic nozzle actuated at 120 kHz (Accumist, Sono Tek, Milton, Canada). Isopropanol containing 1% (w/v) EL100 and 5% (w/w in relation to EL100-55) dibutyl sebacate was spray-coated with the parameters presented in Table 2.

**Table 2.** Spray coating parameters used for the coating of EL100 on microcontainers

| | Feed flow | Generator power | Air pressure | Temperature | Nozzel distance to MCs | Speed | Passages |
|---|---|---|---|---|---|---|---|
| **EL100** | 0.1 mL/min | 2.2 W | 0.028 kPa | 35 °C | 5 cm | 10 mm/s | 25 |

### *In vivo* study

Rats (n = 4) were administered with a dose of 38 µg of SpyC-AP205-L2 (in MCs or in liquid formulation) or 44 µg L2-Ag85a in MCs by oral gavage in a prime/boost/boost regimen at 3 week intervals. As a positive control, rats (n = 2) were given 38 µg of SpyC-AP205-L2 with an intramuscular prime and intranasal boosts. Blood and fecal pellets were collected 2 weeks after each vaccination. The rats were given fresh bedding on the day of fecal collection, and 6 hours later, three samples of two pellets each (six pellets in total) were collected from the cage floor of each group and snap-frozen in liquid nitrogen. Fecal pellet extracts were prepared by homogenizing the pellets in 1x PBS + 1% BCA + protease inhibitor cocktail (750 µl per 100 mg feces), followed by incubation for 4 hours at 4 ˚C with agitation. The samples were centrifuged at 10,000 ×g for 10 min and the supernatant was collected.

### Serum and fecal immunoglobulin levels

Enzyme-linked immunosorbent assay (ELISA) was conducted to assess vaccine-induced immunoglobulin levels in the rat serum and feces. For serum IgG analysis, 96-well plates were coated with 0.1 µg/well L2-MBP or AP205 cVLPs and incubated overnight at 4˚C. The wells were blocked with 1x PBS + 0.5% skim milk powder and then incubated for 1 hour with two-fold serial dilutions of serum starting at 1:50. The plates were then washed thrice with 1x PBS + 0.05% Tween20 and incubated for 1 hour with peroxidase-conjugated goat anti-rat IgG HRP (Jackson ImmunoResearch). The plates were washed again thrice and developed with TMB X-tra (Kem-En-Tec, 4800 A). Total IgA levels in the fecal pellet extracts was measured via ELISA using a goat anti-rat IgA (Sigma)-capture antibody and detected using goat anti-rat IgA-HRP (Abcam). To measure the antigen-specific IgA levels, the fecal extracts were diluted to an equal total IgA starting concentration and detected using goat anti-rat IgA-HRP (Abcam).

## Results and Discussion

**Quality control of the cVLPs pre- and postlyophilization**

The SpyC-AP205-L2 cVLPs technology is a potential generic tool for antigen presentation with many possibilities. However, correct assembly and conformation is paramount for the function of the particles. cVLPs were produced as previously described [19], and confirmation of successful expression was determined by SDS, along with TEM which enables visual verification of formed particles. Furthermore, DLS was used to analyze the size distribution and polydispersity of the particles, to further asses the quality and characteristics of the cVLPs. As these particles had never undergone the developed lyophilization process previously, thorough quality control of reconstituted lyophilized cVLPs was necessary. SDS and DLS were as in the production of cVLPs, similarly used to determine if the lyophilization process had any negative effect on the particles when reconstituted (Fig. 1). Comparison of the cVLPs on SDS before and after lyophilization did not reveal any degradation of the proteins (Fig. 1a). Furthermore, the addition of a centrifugation step can help determine whether the aggregates are in the solution, in which case the centrifuged sample will exhibit a less intense band compared with the noncentrifuged sample. However, this was not the case as no loss of intensity was observed before or after lyophilization. DLS measurements of the samples further confirmed that the cVLPs were in the solution and did not seem to aggregate after reconstitution. The lyophilized cVLPs were 39.9 nm in diameter and had a 14.02% polydispersity, whereas these values for the control group were 38.0 nm and 11.14%, respectively (Supporting material).

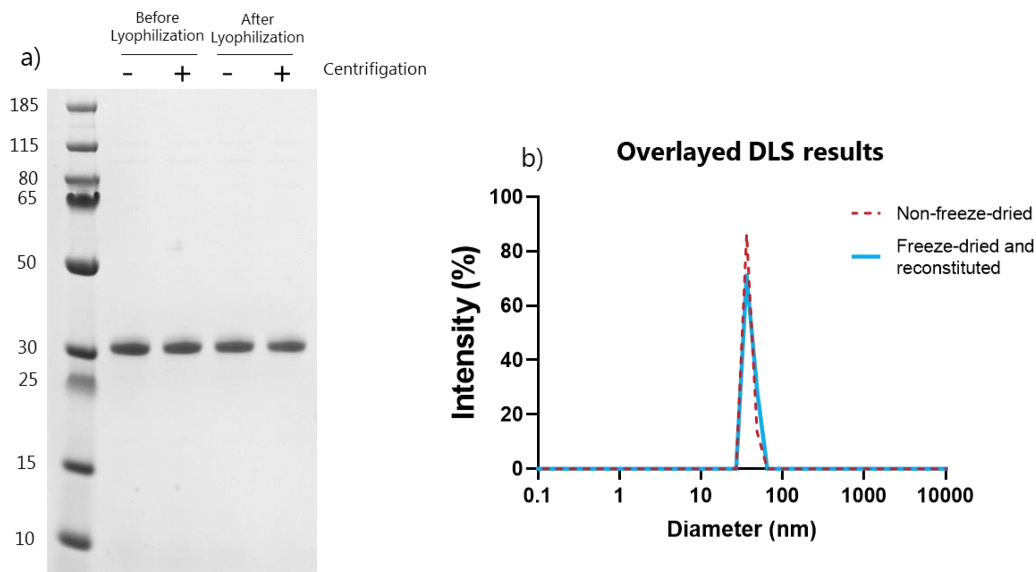


**Fig. 1.** SDS-PAGE results of SpyC-AP205-L2 cVLPs before and after lyophilization, with an additional sample undergoing centrifugation to determine potential aggregates in the solution (a). Overlayed DLS analysis of the cVLPs before and after lyophilization. The plotted graphs present the diameter of the measured particles in the population (b).

In addition, TEM was used to analyze the samples to obtain a visual confirmation that particles had properly formed after reconstitution (Fig. 2). On comparing the TEM images displaying pre- and postlyophilized cVLP samples, no noticeable visual differences were observed. Based on these results, it was concluded that the lyophilization process did not have any harmful or negative effect in terms of the reconstitution of the cVLPs.

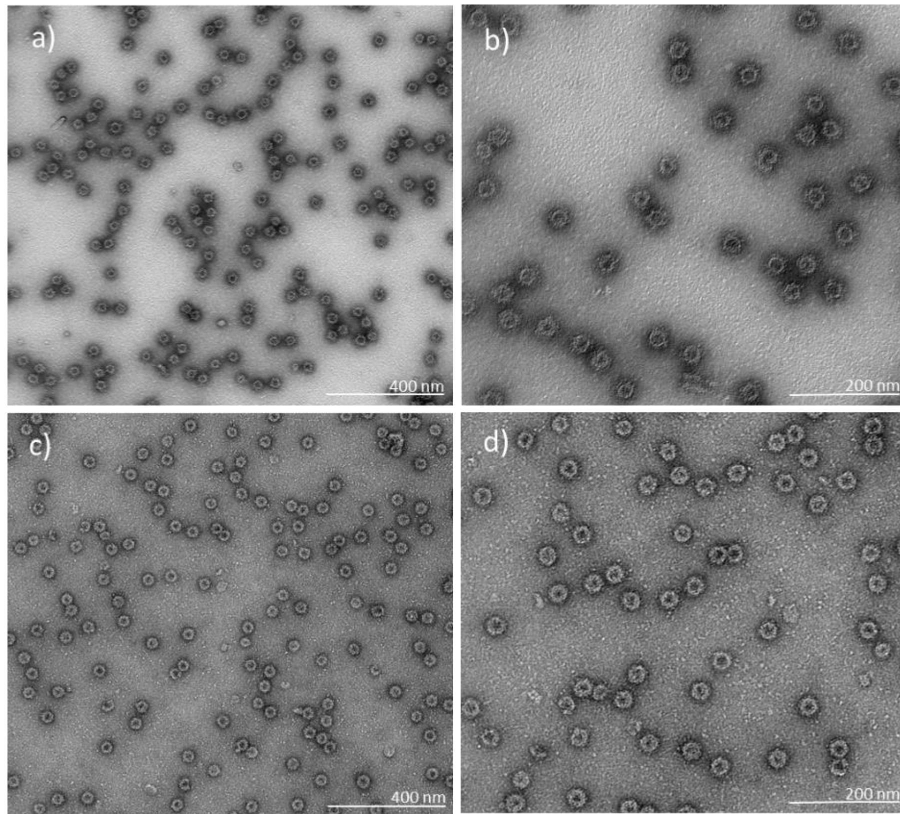


**Fig. 2.** Captured TEM images exhibitingSpyC-AP205-L2 cVLPs before (a, b) and after (c,d) lyophilization.

## Loading and coating of microcontainers

The successful lyophilization of the cVLPs enables placing a higher load in the MCs and a more concentrated vaccine dose. The lyophilized powder was loaded onto MCs and imaged via SEM to ensure complete filling of the MCs (Fig. 3a, 3b). Subsequently, the MCs were coated with the Eudragit L100 polymer (EL100) (Fig 3c). This polymer has been previously used in *in vivo* drug delivery studies with rats, documenting its function in this animal model when applied as a MC coating[24].

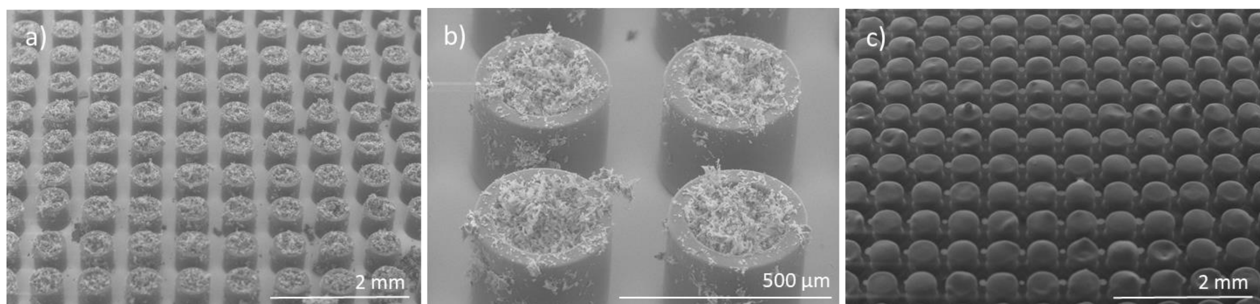


**Fig. 3.** SEM images of microcontainers (MCs) loaded with the lyophilized SpyC-AP205-L2 cVLP at 40× (a) and 120× magnification (b). Loaded MCs were subsequently coated with the EL100 polymer by spray coating. MCs containing cVLPs after coating with the EL100 polymer (c)

**Oral dosing of cVLPs in rats using microcontainers**

To investigate whether the cVLPs could be effectively delivered orally with MCs and induce an immune response, an *in vivo* study was conducted in rats. To evaluate the effect of the MCs, a group receiving the SpyC-AP205-L2 cVLPs in solution with oral gavage was examined. Furthermore, a group receiving L2 bound to the carrier protein Ag85a in MCs was also examined, to evaluate if the display on the VLP platform was affected. L2 is too small to be efficiently purified by itself and need of a carrier protein, in this case Ag85a. As a positive control, rats were injected intramuscularly (i.m) with the cVLP, followed by intranasal (i.n.) boosters. IgG level was measured in the serum to evaluate the systemic antibody response, whereas IgA level was measured in fecal pellets extracts as an indication of the local mucosal immune response. IgG levels specific for the L2 antigen were measured along with the AP205 backbone (Fig. 4a, 4b). From the results, it was evident that only the positive control group achieved a robust antigen specific IgG response against L2 and AP205. No detectable specific IgG antibodies were observed in the orally administered groups. Measurements in these groups were comparable to the background levels of the blank samples included in the assay (data not shown). The IgA antibodies were measured against L2 and AP205 as well, from which the same pattern of antibody stimulation could be observed (Fig. 3c, 3d). Seemingly, no response was observed in the oral administered groups, except in the third fecal pellet extract sample of group 1. Here, a slight increase in the IgA levels was observed comparable to the one seen in the positive control group. However, the observed IgA values were low and not convincing. Regarding the positive controls, this highlights that i.m. prime followed by i.n boosters does not afford a response in the gut with the cVLPs.

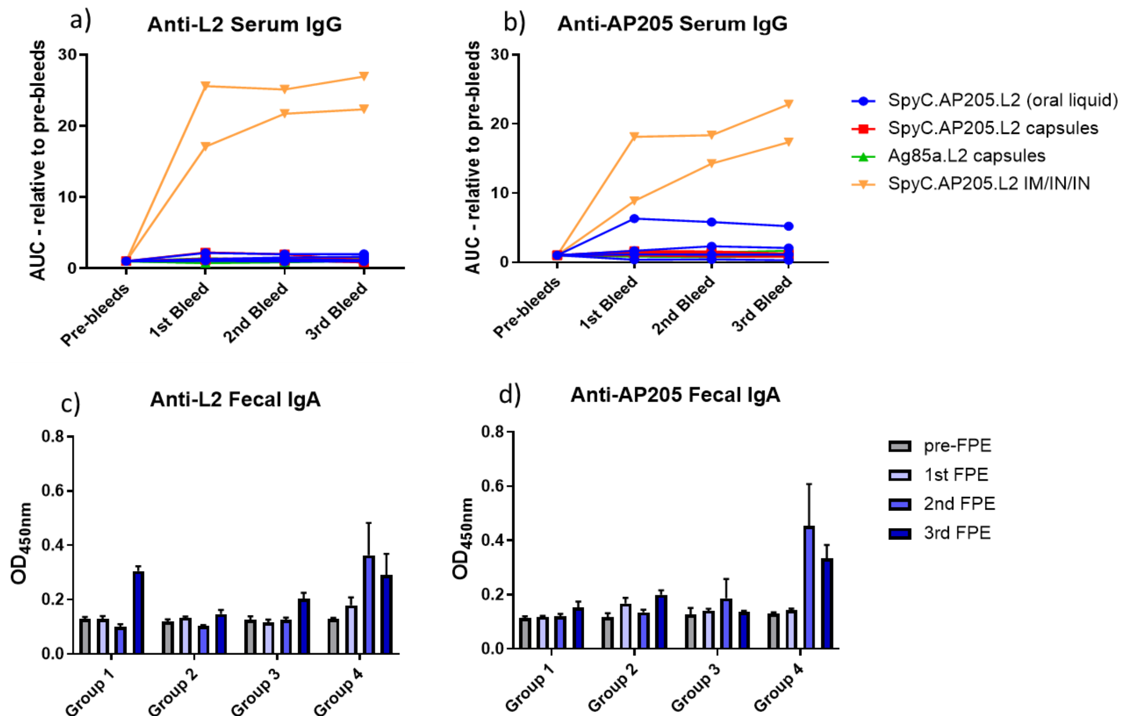


**Fig. 4.** L2 and AP205 specific IgG antibody levels measured in the serum from rats (a, b). Samples were acquired before immunization (prebleed) and two weeks after each immunization until end of experiment. Data is presented as the area under the curve (AUC) values, calculated from the ELISA titration curves. Each line represent an individual rat. L2 and AP205 specific IgA antibody levels measured in fecal pellet extracts (FPE) (c ,d). Samples were acquired before immunizations (pre-FPE) and

two weeks after each immunization until end of experiment. Data is presented as mean + SEM of optical density values measured at 450 nm ($OD_{450nm}$), as AUC could not be calculated due to too weak measurements.

The measurements of antibody responses following oral immunization with MCs or oral gavage revealed low to inconsistent levels of IgG and IgA in the serum and feces, respectively. Several reasons could be the cause to why no measurable immune responses were achieved. Of note is the lack of adjuvants. In previous studies, the SpyC-AP205-L2 cVLP have demonstrated stimuli of protective immunity without the need of adjuvants, which is a most benefitting trait of this platform as adjuvant can increase the risk of side effects and increase production cost [14], [25]. However, the obtained results do not indicate that a mucosal adjuvant could be necessary when administering the cVLPs orally. Several adjuvants have been identified to enhance the immune response against the cVLP when administered i.m., but these would not necessarily be effective when administered orally for the mucosal tissues [13]. Optimally, a screening study should be conducted to identify promising candidates for this vaccine method. The published data on potential mucosal adjuvants for VLPs is limited, but some candidates have been identified. In a comparison study by Quan *et al.*, the authors tested the effects of clinically known adjuvants with an Influenza derived VLP[26]. The study included Alum, CpG DNA, monophosphoryl lipd A (MPL), poly (I:C), gardiquimod and cholera toxin (CT). The responses were evaluated on the induction of IgG and IgA in the bone marrow and lungs along with a neutralization assay. CpG, MPL, CT and Alum displayed promising immune profiles, with CT and Alum excelling as the most effective adjuvants. Another study administered a norovirus VLP based vaccine formulated with chitosan, an organic polymer known for its mucoadhesive and mucosal adjuvant traits [27]. In this study, the vaccine formulation provided effective immunization against Norwalk viral gastroenteritis and infection. Notably, in these studies, mice were immunized i.n. in addition to the VLPs being derived from pathogens, which infects the mucosal tissues in their wild type form. The SpyC-AP205-L2 is derived from a phage and could therefore be a disadvantage in terms of being recognized by the mucosal immune system. Moreover, the adjuvants from the previous studies may not have the same effect when administered orally [28]. Nevertheless, future research can examine whether these studies provide potential mucosal adjuvant candidates for VLPs. Another method could be to modify the cVLP scaffold so as to make it more equipped for mucosal interaction, similar to the studies by *Serradell et al.* and *Bellier et al.*[20], [21]. The variant-specific surface proteins (VSPs) from *Giardia lamblia* utilized in these studies have been tried in the same manner on the SpyC-AP205-L2 particles. However, the cVLPs were still sensitive to degradation by low pH and proteases (data not shown). This indicates that the nature of the VLP plays a role as a retrovirus-derived eVLP that were used in the studies by *Seradell et al.* and *Bellier et al*. However, the concept does provide opportunity, and molecules to aid the SpyC.AP205.L2 cVLP for mucosal interaction could very well still be identified.

Regarding the practical aspect of the *in vivo* studies, some factors could be optimized, such as the immunization regime. It is possible that a prime-pull strategy would be necessary for the MCs to induce better responses[29]. As documented by *Laier et al.,* at slight enhancement of the immune response was observed by administering MC boosters following a subcutaneous prime, whereas solely oral administration with the MCs did not elicit a convincing response[11]. This could also be the case in this study, and a group undergoing such immunization should be included in future studies. Other factors that could have had an impact are the transit and intestinal medium of the GI tract. MCs have extensively been used for drug delivery in rats but never to deliver a vaccine. Kinetic studies have been conducted that documenting the transit time in rats in 2–4 h[22]. It is possible that this is not enough time for proper absorption and interaction with the immune system, necessitating longer retention time in the intestine. In relation to this, a biodistribution study might be warranted to investigate whether the release actually

is initiated in the intestine. In that case, information about where the particles migrate to and how long they stay in the gut could be vital. Regarding the intestinal medium, it is possible that the environmental conditions here could cause the particles to fall apart or completely aggregate during the reconstitutions process. This could be investigated using an *in vitro* dissolution model wherein the particles would be reconstituted in fluids simulating the regions of the rat GI tract[30].

## Conclusion

In this study, it was hypothesized that the particular morphology of the SpyC-AP205-L2 cVLP can induce a mucosal immune response after administration through the oral route. As a delivery vehicle to protect the particles from the gastric environment, MCs were used to target release in the intestine of rats using the pH-degradable polymeric lid EL100. The particles were lyophilized to facilitate the loading of the cVLPs into the MCs. Quality analysis after lyophilization revealed no negative effect upon reconstitution. cVLPs were orally administered to rats in MCs along with designated control groups to investigate the effect of the cVLP platform and the MCs. Antibody measurements indicated that no immune stimulation was achieved by any of the orally immunized groups. Introduction of adjuvants and a prime-pull immunization regime in further studies examining these technologies might be able to induce an immune response.

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## Supporting information

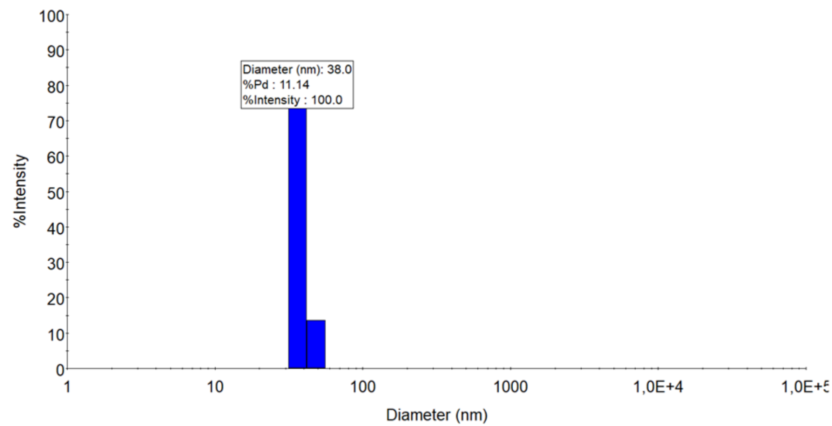


**Fig. S1** Results of dynamic light scattering of non-lyophilized SpyC-AP205-L2 cVLP depicting the diameter, polydiversity, and intensity

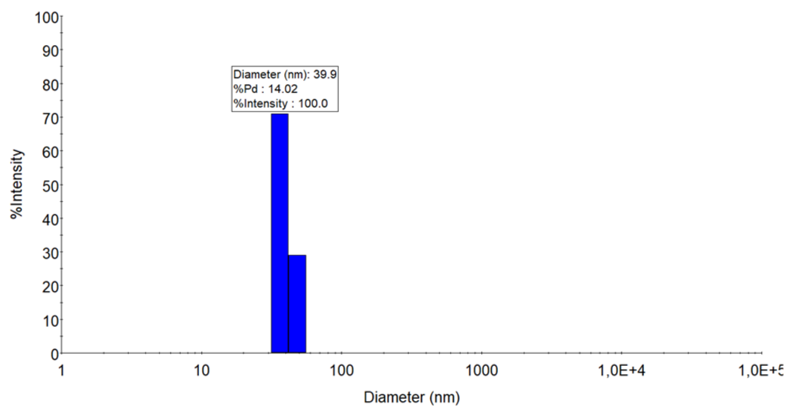


**Fig. S2** Results of dynamic light scattering of lyophilized SpyC-AP205-L2 cVLP depicting the diameter, polydiversity, and intensity