Ph.D Thesis

Application of Chitosan Preparations as Vaccine Adjuvants

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Abstract

Vaccines serve as vital tools in safeguarding humans and animals against diverse pathogens. Alongside the development of antigenic forms like attenuated and subunit vaccines, adjuvants play a pivotal role in enhancing vaccine efficacy. In recent years, vaccine adjuvant systems have witnessed rapid evolution, transitioning from aluminum hydroxide to a spectrum of emulsions capable of bolstering immune responses. However, traditional adjuvants, which predominantly activate humoral immune pathways, have faced limitations in biomedical applications, notably tumor vaccines, and are associated with adverse effects such as injection site pain.

Chitosan, a naturally occurring cationic polysaccharide, boasts properties like non-toxicity, antimicrobial activity, biocompatibility, and biodegradability, rendering it versatile in the biomedical realm. Its applications span drug delivery, wound healing, tissue engineering, dental applications, and vaccine adjuvants. As an adjuvant, chitosan exhibits immunostimulatory properties, enhances immune responses, improves antigen delivery, modulates immune mechanisms, and holds promise for needle-free vaccination approaches.

Ongoing research endeavors focus on refining chitosan vaccine adjuvants through exploration of diverse formulations, dosages, and delivery methods to optimize effectiveness. Despite its potential, further research is imperative to elucidate chitosan's mechanism of action, ensure safety, and unleash its full potential in the development of safer and more efficacious vaccines. Our focus lies in chitosan nanoparticle and Pickering emulsion formats, probing their potential as adjuvants in mammalian and fish vaccines, with the aim of advancing vaccine technology for broader biomedical and veterinary applications.

The dissertation studies were unfolded as follows:

1. Acid-responsive immune-enhancing chitosan Pickering emulsion as an adjuvant in mammalian vaccine. Chitosan, celebrated for its sensitivity to pH variations and compatibility with biological systems, was selected as the primary

material for crafting chitosan nanoparticle-stabilized Pickering emulsion (CSPE). CSPE's inherent pliability allows it to undergo stress-induced deformation upon interacting with cell membranes, mimicking the flexibility observed in natural pathogens and thus facilitating efficient cellular uptake. In the acidic environment of lysosomes, the amino groups of chitosan molecules undergo protonation, thereby increasing their solubility in water. This transition of CSPE from particle stabilization to the stabilization of polymer chains results in swelling and the accumulation of protons, ultimately leading to the rupture of lysosomes. Experimental investigations assessing CSPE's effectiveness as an adjuvant revealed its proficiency in loading antigens, promoting endocytosis, and facilitating antigen cross-presentation. Furthermore, CSPE demonstrated its capability to recruit antigen-presenting cells to injection sites and enhance T cell activation, thereby amplifying both humoral and cell-mediated immune responses. In preventive and therapeutic tumor models for lymphoma and melanoma, CSPE exhibited notable inhibition of tumor growth and prolonged survival in mice. In summary, the evasion of antigenic lysosomes facilitated by the transition in chitosan molecular states underscores the potential of CSPE to augment cell-mediated immunity. Thus, CSPE emerges as a promising candidate for a vaccine adjuvant, offering new avenues for enhancing vaccine efficacy and therapeutic outcomes.

2. A mass-producible oral vaccine system based on chitosan and its derivative against *Vibrio anguillarum* in fish. Fish mortality resulting from pathogen infections poses significant economic challenges for aquaculture. Vaccination stands out as an effective solution, and the addition of adjuvants enhances the immune protection provided by vaccines, even when slightly weakened. Chitosan emerges as an ideal adjuvant for oral administration due to its mucoadhesive and immunomodulatory properties. In this study, we introduced a composite adjuvant formulation and developed a chitosan-based oral fish vaccine formulation conducive to mass production. Subsequently, we explored a suitable immersion challenge model for flounder, incorporating attenuated *Vibrio anguillarum*. Additionally, we investigated

the immunoprotective effects of chitosan and its derived adjuvants. Our findings revealed that the combination of N-[(2-hydroxy-3-trimethylamino)propyl] chitosan chloride nanoparticles and sodium alginate exhibited remarkable protective effects against the immersion challenge model involving *Vibrio anguillarum*. This underscores the potential of chitosan-based adjuvants in enhancing immune responses and mitigating pathogen-induced mortality in aquaculture settings.

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Chapter 1 General Introduction

1.1 Vaccines and adjuvants

Vaccines are substances that help the immune system recognize and fight certain pathogens, such as viruses and bacteria. They usually contain weakened or killed parts of bacteria or their molecules, allowing the body to mount an immune response without causing disease[1]. This response allows the immune system to remember the pathogen and quickly recognize and fight it if the body is exposed to an actual disease in the future. Vaccines are essential for preventing infectious diseases and have contributed significantly to reducing the impact of many diseases around the world.

Adjuvants are non-specific immune-enhancing agent, which have no immunogenicity of its own, but can assist the function of the vaccine to enhance the body's immune response or change the type of the body's immune response[2].

1.1.1 Historical evolution and challenge of vaccines

The concept of vaccination has evolved over centuries. Below is a brief historical timeline.

Variolation: In ancient times, especially in China and India, people practiced a type of vaccination called variolation. This involved deliberately inoculating a person's skin with smallpox material to cause a mild illness and confer immunity[3]. Edward Jenner and the smallpox vaccine: In the late 18th century, British physician Edward Jenner developed the first successful vaccine. He noticed that milkmaids who had contracted cowpox appeared to be immune to smallpox. In 1796, he tested his theory by intentionally infecting a boy with cowpox and then exposing him to smallpox, but the boy was not infected. This laid the foundation for the smallpox vaccine, which ultimately led to his worldwide eradication of smallpox in 1980[4]. Development of other vaccines: Based on Jenner's research, advances in microbiology and medicine led to the development of vaccines against a variety of diseases. Louis Pasteur's research on rabies in the late 19th century was important[5]. Later, vaccines were developed for diseases such as cholera, typhoid, tetanus, diphtheria, and

whooping cough. Mass immunization campaigns: Vaccinations became more widespread throughout the 20th century. Massive vaccination campaigns have been launched to protect people from various diseases and to significantly reduce illness, disability, and death associated with these infectious diseases. Advances in vaccine technology: Over time, advances in technology have led to the use of live attenuated vaccines, inactivated vaccines, subunit vaccines, conjugate vaccines, and more recently, vaccines developed for the novel coronavirus disease (COVID-19)[6]. It has become possible to develop various types of vaccines, such as mRNA vaccines. Global immunization efforts: Organizations such as the World Health Organization (WHO) and UNICEF aim to increase access to vaccines in developing countries and eradicate or control diseases such as polio, measles, and rubella. It has played an important role in the global vaccination campaign. Ongoing research and innovation: Vaccine research continues to evolve, focusing on new technologies, improved efficacy, and broader coverage against infectious diseases. The development of vaccines against emerging infectious disease threats remains an important area of research.

Throughout history, vaccines have been instrumental in saving lives, preventing diseases, and significantly impacting public health by providing immunity to infectious diseases. Vaccines are highly effective in preventing disease, but in some cases, they can pose the following challenges:

Side effects: Most side effects of the vaccine are mild, such as pain at the injection site and slight fever. However, some people may experience more severe reactions, although they are generally rare. Managing and communicating these potential side effects is important to maintaining public confidence in vaccines[7]. Rare adverse events: Very rare adverse events may occur after vaccination. These events will be closely monitored by health authorities and researchers to assess any link to the vaccine. Although the benefits of vaccination often far outweigh these risks, such events can raise concerns and influence public perception. Concerns about vaccine safety: Reports and rumors about side effects, even if not scientifically confirmed, can influence public perception and acceptance of vaccines. It is essential to address

safety concerns, provide transparent information, and continually monitor and evaluate vaccine safety[8]. Long-term effects: Some people may have concerns about the long-term effects of vaccines, especially the potential of new technologies such as mRNA vaccines[9]. Continuous monitoring through post-market surveillance is essential to detect and assess long-term effects. Individual differences: People's responses to vaccination may vary depending on factors such as age, genetics, and underlying health conditions[10]. Ensuring vaccines are effective in different populations remains a challenge. Communicating risks and benefits: It is important to effectively communicate the risk-benefit ratio of vaccines. Increasing public confidence requires a balance between recognizing the potential risks and emphasizing the significant benefits of vaccination. Vaccine effectiveness and duration of protection: Some vaccines may not provide complete protection or may require additional doses to maintain immunity[11]. Continued research is needed to improve vaccine efficacy and ensure durable protection.

Addressing these challenges requires rigorous scientific research, transparent communication, continued monitoring of vaccine safety, increased public health literacy, and fostering trust between health authorities and the public. It is important to maintain a balance between ensuring vaccine safety and emphasizing the important role of vaccines in preventing disease and protecting public health.

1.1.2 Importance of improved adjuvants

Several important developments have occurred in the history of vaccine adjuvants (substances that enhance the body's immune response to vaccines). Early use of adjuvants: Adjuvants have been used in vaccines for over 90 years. One of the first adjuvants, aluminum salts (alum), were used in vaccines against diphtheria and tetanus in the 1930s[12]. Aluminum-based adjuvants: Alum compounds, especially aluminum hydroxide and aluminum phosphate, have been the predominant adjuvants used for decades. They enhance the body's immune response by creating a cumulative effect at the injection site, thereby prolonging the immune system's exposure to the antigen. Advances in adjuvant research: Research efforts aimed at developing novel

adjuvants that can improve vaccine efficacy, reduce the amount of antigen required, and elicit broader and longer-lasting immune responses. Adjuvants such as MF59 and AS03, which contain oil-in-water emulsions, are used in vaccines against influenza and other diseases to boost immune responses[13]. Toll-like receptor agonists: Certain adjuvants stimulate the Toll-like receptors (TLRs) on immune cells, mimicking the presence of pathogens[14]. For example, monophosphoryl lipid A (MPLA) is a TLR4 agonist used in some vaccines to elicit a stronger immune response[15]. Squalene-based adjuvants: Squalene-based adjuvants, such as MF59 and AS03, have received attention due to their ability to enhance immune responses. These adjuvants are used in pandemic influenza vaccines and some COVID-19 vaccines. Advances in mRNA vaccine adjuvants: With the development of mRNA vaccines, such as the one against coronavirus disease (COVID-19), the need for adjuvants is increasing. These vaccines use the mRNA itself as an immune stimulant, potentially reducing the need for traditional adjuvants[16]. Research into safer and more effective adjuvants: Ongoing research is focused on identifying effective and safe adjuvants. When developing next-generation adjuvants, it is important to balance efficacy with minimal side effects.

Adjuvant development continues to be an active area of research in vaccine development. Adjuvant science is continually advancing with new technologies, a deeper understanding of the immune system, and the search for safer and more effective vaccines. Improved adjuvants play an important role in vaccine development and public health for several reasons.

Improved immunogenicity: Adjuvants enhance the immune response to vaccines, allowing for stronger and more robust responses against target pathogens[17]. This often means that less antigen is needed, potentially further expanding vaccine supply. Reducing vaccine doses: By enhancing the immune response, adjuvants can reduce the vaccine doses needed to achieve immunity[17]. This is especially important in situations where vaccine availability is limited. Protection against variants: Advanced adjuvants may extend the immune response and provide protection against different strains and variants of pathogens. This adaptability is critical given the rapidly

evolving infectious pathogens. Improving vaccine effectiveness in vulnerable populations: Certain groups, such as older adults and people with weakened immune systems, may have a weaker response to standard vaccines. Improved adjuvants may help boost immunity and provide better protection[2]. Accelerating vaccine development for difficult diseases: For diseases for which traditional vaccines have limited efficacy, more effective adjuvants may overcome these challenges and enable the development of effective vaccines. Reducing vaccine reactogenicity: Some adjuvants reduce vaccine reactogenicity (side effects) by allowing the use of smaller amounts of antigen, potentially reducing side effects while maintaining efficacy[18]. Paving the way for new vaccine technologies: Advances in adjuvant technology can support the development of new vaccine platforms, such as mRNA and vector-based vaccines, by improving immunogenicity and efficacy.

Continuing research and innovation in adjuvant development is critical to improving vaccine efficacy, expanding coverage, and addressing the challenges of emerging infectious diseases. Improved adjuvants will aid in the development of more effective vaccines and ultimately contribute to improved public health outcomes around the world.

1.2 Chitosan

Chitosan is a naturally occurring polysaccharide derived from chitin, a substance found in the exoskeleton of crustaceans such as shrimp, crabs, lobsters, and certain insects. It is formed by deacetylation of chitin, a process in which acetyl groups are removed from chitin molecules[19]. This biopolymer, chitosan, is composed of repeating units of glucosamine and N-acetylglucosamine. Its structure and properties make it a versatile compound that can be used for a variety of applications in industries such as medicine, agriculture, food, and cosmetics[20].

1.2.1 Properties of chitosan

Chitosan has many properties that make it a versatile and valuable material in a variety of industries. Biocompatibility: Chitosan is biocompatible and well tolerated by living organisms. This property is very important in biomedical applications where

materials interacting with biological systems must not cause undesired reactions[21]. Biodegradable: It is biodegradable and breaks down into non-toxic components, making it advantageous for applications where gradual decomposition is preferred, such as medical devices and environmental applications[22]. Antibacterial activity: Chitosan exhibits antibacterial properties and inhibits the growth of certain bacteria, fungi, and yeasts[23]. This property is valuable in wound dressings, food preservation, and other applications where microbial control is essential. Mucoadhesive properties: Chitosan has mucoadhesive properties that allow it to adhere to mucosal surfaces. This makes them useful in drug delivery systems for targeted delivery to specific areas such as the gastrointestinal tract or nasal passages[24]. Film-forming ability: Chitosan can form films or coatings to create barriers that can be used in wound dressings, edible coatings for food preservation, or pharmaceutical formulations[25]. Absorption and adsorption: Chitosan can absorb or adsorb a variety of substances, making it useful in water treatment applications to remove pollutants and in drug delivery systems to protect and release drugs[26]. Hemostatic properties: It promotes blood coagulation and is used in hemostatic agents to control bleeding during surgical procedures and trauma. Tissue regeneration: Chitosan supports tissue regeneration by providing a scaffold for cell growth and attachment, making it valuable for tissue engineering and regenerative medicine[27]. Non-toxic and low immunogenicity: Chitosan is generally considered to be non-toxic and low immunogenic, reducing the potential for side effects in biological systems[28].

These properties make chitosan a versatile material with applications in medicine, pharmaceuticals, food, agriculture, environmental protection, and various other industries. Its unique combination of properties continues to drive research and innovation in many fields.

1.2.2 Biomedical application of chitosan

The properties of chitosan make it a valuable material for various biomedical applications. Wound Healing: Chitosan's hemostatic properties promote blood clotting and promote wound healing. Used in wound dressings, dressings, and topical

preparations to promote tissue regeneration and prevent infections[29]. Drug Delivery Systems: Chitosan's mucoadhesive properties lend itself to oral, nasal, ocular, and transdermal route drug delivery systems. Aids in controlled and targeted release of drugs, protects drugs from degradation and improves absorption[30]. Tissue engineering: Chitosan scaffolds provide a structure that supports tissue growth and regeneration. They are used in tissue engineering to create artificial organs, bone grafts, cartilage repair, and other tissue engineering applications[31]. Dental Uses: Chitosan is used in dental products such as mouthwash, toothpaste, and dental implants because its antibacterial properties promote oral health and prevent infections[32]. Surgical hemostasis: Chitosan-based hemostatic agents are used in surgical procedures to control bleeding and promote blood clotting, thereby reducing blood loss and supporting faster recovery[33]. Antimicrobial coatings: Chitosan coatings are applied to medical devices and implants to prevent infections and biofilm formation, thereby increasing the safety and longevity of these devices[34]. Gene delivery: Chitosan nanoparticles are being investigated for gene delivery applications. They encapsulate genetic material, protecting it from degradation and are useful for delivering genes for therapeutic purposes[35]. Burn Treatment: Chitosan-based dressings or membranes are used to treat burn wounds because they can promote healing, reduce pain, and prevent infection of burn wounds[36]. Tissue Adhesives: Chitosan-based adhesives are being investigated for tissue adhesion and wound closure during surgery due to their biocompatibility and ability to adhere to tissues[37].

Chitosan's biocompatibility, biodegradability, and diverse functional properties make it a versatile biomaterial for biomedical applications and contribute to advances in medical and regenerative medicine.

1.3 Chitosan as adjuvant

1.4.1 Limits of existing adjuvants

Although beneficial, existing adjuvants have certain limitations that impact their use in vaccine development. Limited modulation of immune responses: While some

adjuvants can elicit strong immune responses, they cannot specifically tailor the type of response required for a particular vaccine[38]. Achieving precise immune responses appropriate for different diseases and populations remains a challenge. Safety concerns: Adjuvants may cause local or systemic side effects in some people. Although most adjuvants are safe, rare serious side effects have been reported, raising safety concerns and requiring careful evaluation. Need for optimization: Adjuvants may not be universally effective against all antigens or vaccines. Optimizing an adjuvant formulation for a specific pathogen or vaccine type is important to maximize its effectiveness[39]. Storage and stability issues: Some adjuvants require specific storage conditions (such as cold chain storage) or have stability issues that can pose logistical challenges during vaccine distribution and storage[40]. Limited mechanistic knowledge: Despite widespread use, the exact mechanism of action of some adjuvants is not fully understood. A deeper understanding of these mechanisms may contribute to the development of more effective and safer adjuvants[41]. Adjuvant-specific responses: Responses to adjuvants can vary among individuals, impacting the consistency and predictability of immune responses in different populations. Adapting adjuvants to different populations remains a challenge[42]. Need for new approaches: The current repertoire of adjuvants may not be sufficient to combat emerging infectious diseases or develop vaccines against specific pathogens. Novel adjuvants that can induce broader and longer-lasting immune responses are needed.

Addressing these limitations requires continued research in adjuvant development, with a focus on improving safety profiles, understanding mechanisms of action, optimizing formulations for specific vaccines, and exploring new adjuvant technologies. is. Improvements in adjuvants have the potential to significantly impact vaccine efficacy, expand vaccination coverage, and contribute to improved public health outcomes.

1.4.2 Advantages of chitosan in adjuvant

Chitosan has attracted attention as a potential adjuvant in vaccine development due to its immunostimulatory properties and biocompatibility. As an adjuvant,

chitosan can:

Improved immune response: Chitosan stimulates the immune system, resulting in a stronger and longer-lasting immune response to vaccine antigens. This can improve the effectiveness of vaccines. Antigen protection and delivery: Chitosan protects antigens from degradation and facilitates antigen uptake by immune cells[43]. This contributes to the efficient delivery of antigens to the immune system and strengthens the body's response to vaccines. Modulating the immune response: The interaction between chitosan and immune cells can modulate the type of immune response elicited by the vaccine. They target specific types of immune responses and, depending on the target of the vaccine, promote stronger cellular or humoral immune responses[43]. Biodegradability and safety: Chitosan is biodegradable and generally considered safe, making it beneficial for use in vaccines. It has low toxicity and is well tolerated by the body, reducing concerns about side effects. Adjuvant combinations: Chitosan can be used in combination with other adjuvants or vaccine delivery systems, potentially increasing its efficacy and reducing the required vaccine dose[44].

Research into chitosan-based adjuvants is ongoing, particularly for their potential to enhance immune responses against advanced pathogens and improve vaccine efficacy in vulnerable populations where standard vaccines may be less effective. Although promising, further research is needed to optimize the adjuvant properties of chitosan, understand its mechanism of action, and ensure its safety and efficacy in various vaccine formulations.

1.4 Presentation of the study

Chitosan has different specifications and varies greatly in its applications. Moreover, there are numerous chitosan derivatives and various performance improvements. Focusing on the particulate form of chitosan, we have investigated its application as a vaccine adjuvant for different routes of administration in mammals and fish and attempted to delve deeper into its mechanism of action in the immunization process.

For the first time, we explore the immune-enhancing effects of chitosan particle-stabilized Pickering emulsion as a vaccine adjuvant. We will explore the mechanism of action of this adjuvant in multiple ways, from antigen presentation to antibody level, and use murine tumor models to validate its adjuvant effect.

As for fish vaccine, our aim was to find methods suitable for large-scale production to prepare chitosan oral vaccine adjuvants. We screen suitable chitosan formulations before introducing the complex components and used a *Vibrio anguillarum* immersion challenge model that simulated a real environment to validate the immunoprotective effect of the chitosan complex adjuvant.

It is hoped that these studies will expand the use of chitosan in vaccine adjuvants and lay the foundation for elucidating its immune-enhancing mechanisms.

Chapter 2 Immune-Boosting Chitosan Formulation Responsive to Acidic Conditions, Transitioning from Particle to Polymer Chain Stabilization

2.1 Abstract

The distinctive attributes of chitosan render it an exceptional candidate for formulating CSPE, an innovative vaccine adjuvant. Its pH sensitivity and biocompatibility make CSPE apt for interacting with biological systems. Additionally, its pliability enables stress deformation upon encountering cell membranes, replicating the actions of natural pathogens and augmenting cellular uptake efficiency.

Within the acidic environment of lysosomes, chitosan molecules within CSPE undergo protonation, heightening their water solubility. This transition from a particle-stabilized to a polymer chain-stabilized state, combined with swelling and proton accumulation, results in lysosomal rupture, facilitating escape of antigens. This process is vital for enhancing cellular immunity through CSPE.

Experimental findings illustrate that CSPE proficiently loads antigens, fosters endocytosis, and enables antigen cross-presentation. Furthermore, it recruits antigen-presenting cells at the injection site, amplifies T-cell activation, and bolsters both humoral and cellular immune responses. These results indicate that CSPE harbors significant potential as a vaccine adjuvant.

In various prophylactic and therapeutic tumor models, such as E.G7-OVA lymphoma and B16-MUC1 melanoma models, CSPE demonstrates notable efficacy in restraining tumor growth and extending the survival of mice. This underscores CSPE's potential as a versatile adjuvant for developing vaccines against diverse diseases.

To summarize, the antigenic lysosomal escape, facilitated by the molecular state transition of chitosan, stands as a pivotal mechanism underlying the immunomodulatory capabilities of CSPE. With its proven capacity to enhance both humoral and cellular immune responses, CSPE emerges as a promising candidate for

vaccine adjuvant formulations.

2.2 Introduction

Pickering emulsions, stabilized through solid particles[45], have garnered significant attention due to their heightened stability in comparison to conventional surfactant-based emulsions. This superior stability has led to their widespread adoption across industries such as food and cosmetics. While biomedical applications of Pickering emulsions have been explored for their stability and high payload capacity[46], their utilization in vaccine adjuvants remains somewhat limited. Notably, in vaccine development, Poly (lactic-co-glycolic acid) (PLGA) particles and aluminum hydroxide microgels have been utilized to stabilize Pickering emulsions[47-49]. These stabilized emulsions have shown superior immune activation compared to commercial Alum adjuvants. Building upon prior research conducted within our group, our investigations have revealed that Pickering emulsions maintain antigen deformability and mobility akin to pathogen-cell interactions to a considerable extent. Additionally, they elicit robust humoral and cellular adaptive responses while exhibiting favorable biosafety profiles. This sets a significant precedent in the exploration of emulsion particle strategies for immune adjuvant development.

The PLGA nanoparticle-stabilized Pickering emulsion adjuvant system has undergone numerous enhancements, particularly surface charge modifications using polyethyleneimine and the encapsulation of polysaccharide immunopotentiators[50-52]. While negatively charged PLGA has found extensive biomedical applications, positively charged materials are better suited for loading antigens with low isoelectric points. In contrast to coating anionic carriers with electropositive substances, employing cationic vehicles streamlines vaccine preparation procedures and facilitates subsequent applications. There are opportunities for further enhancing cellular immunity through adjuvants derived from negatively charged materials. This is because the heightened interaction between positively charged delivery systems and biomembranes enhances cellular uptake and facilitates improved lysosomal escape.[53].

Research on particulate adjuvants has undergone extensive scrutiny due to their ability to stimulate cellular immunity. Investigations have primarily concentrated on parameters such as particle size, charge, composition, and shape[54-56]. Nevertheless, the impact of the molecular state of particles on cellular immunity has received relatively little attention. To address this gap, a set of polymer-toll-like receptor agonist conjugates has been synthesized to investigate the effect of carrier morphology on $CD8^+$ T cell induction. Intriguingly, submicron particles exhibiting superior hydrodynamic properties have shown heightened cytokine production and antigen-specific responses compared to randomly coiled polymers[57]. The limited focus on disparities in intracellular behavior between particles and macromolecular chains can be attributed to the inherent challenges associated with the cellular uptake of coiled polymers. Consequently, research into the impact of intraparticle molecular state on cellular immunity remains sparse in the literature, indicating a promising avenue for future investigations.

Chitosan, being the sole cationic polysaccharide derived from natural sources, offers numerous advantages, including widespread availability, cost-effectiveness, biodegradability, non-toxicity, biocompatibility, fungistatic properties, and potential anti-tumor effects. These characteristics render chitosan highly versatile in various biomedical materials and applications, spanning from bone tissue engineering to the delivery of drugs, proteins, and genes[58-61]. Its broad utilization as an adjuvant in numerous studies extends beyond enhancing mucosal immunity to encompass parenteral routes, including subcutaneous, intraperitoneal, and intramuscular administrations[62, 63]. Notably, chitosan excels in enhancing humoral immune responses, surpassing the efficacy of Alum adjuvants[64, 65]. Furthermore, chitosan exhibits promise in facilitating endosomal escape by increasing the osmotic pressure of lysosomes through the proton sponge effect. This mechanism promotes endosome rupture and subsequent antigen release into cytoplasm[66].

Drawing on the inherent stability at interfaces of Pickering emulsions and the pH-triggered transformation of chitosan particles into molecular chains under acidic conditions[67], we advocate for the creation of chitosan nanoparticle-stabilized

Pickering emulsions (CSPE). This innovation aims to elevate in vivo antigenic escape efficiency significantly. Through the charge bestowed by chitosan, CSPE amplifies the contact area between the emulsion and cell membranes, thereby facilitating antigen uptake by antigen-presenting cells (APCs). Operating as an emulsion stabilized by particles in physiological conditions, CSPE undergoes a transition to an emulsion stabilized by polymer chains within the acidic environment of lysosomes. This shift triggers volume expansion of the emulsion, leading to lysosomal membrane destabilization. Moreover, increased protonation of chitosan within the polymer chain-stabilized emulsion, due to heightened exposure of amino groups, synergizes with the volume expansion effect to hasten and effectively achieve endosomal escape. To validate this conceptual framework, diverse chitosan particle-stabilized Pickering emulsions with varied properties are synthesized to investigate the influence of particle-polymer chain transition and proton accumulation on lysosomal escape and cellular immunity. Furthermore, an exploration of the potential of CSPE as a tumor vaccine adjuvant is initiated.

2.3 Experimental details

2.3.1 Preparation of CSPE

To produce chitosan nanoparticles (CNPs), we employed the salting out method. Initially, chitosan hydrochloride was dissolved in deionized water, and the pH was adjusted to 6.5 using sodium hydroxide. Subsequently, chitosan-stabilized Pickering emulsion (CSPE) was prepared through ultrasonic emulsification using a Sonifier. In this process, chitosan nanoparticles were dispersed in deionized water as the aqueous phase, while squalene (Sigma) served as the oil phase. The aqueous and oil phases were blended in a volume ratio of 9:1 and subjected to sonication for 2 minutes, resulting in the formation of CSPE.

For the formulation methods of other control groups utilized in the study, chitosan microparticles (CMP) had been organized through single-section gel

homogenization. Chitosan hydrochloride turned into solved in deionized water and sodium α, β- glycerophosphate (Sigma) answer turned into delivered drop through drop, curing at 70℃ for 30 min. The gel turned into diluted and dispersed through homogenizer (IKA), observed through sonication to shape uniform microparticles.

Surfactant-stabilized emulsion (SSE) was composed of squalene (10%), Span 85 (Merck, 1%), and Tween 80 (Merck, 1%), and the mixture was emulsified by ultrasound.

Chitosan polymer was prepared by adjusting the pH of chitosan hydrochloride to 5.

CNPs were added to a formaldehyde solution (molar ratio of amino groups and aldehyde groups was 1:1) and cured at 40°C for 1 hour to prepare formaldehyde-crosslinked chitosan particles.

Chitosan nanoparticles cross-linked with sodium tripolyphosphate (TPP) were prepared by ionic gelation. TPP was added to the chitosan solution at pH 5 and stirred for 30 min.

Chitosan polymer-stabilized emulsion (PMSE), formaldehyde cross-linking chitosan nanoparticles-stabilized Pickering emulsion (FCPE), and sodium tripolyphosphate cross-linking chitosan nanoparticles-stabilized Pickering emulsion (TCPE) with the particles mentioned above was also prepared. Treated as CSPE.

MF59-like emulsion (MLE) consisting of squalene (4.3%), Span 85 (0.5%), Tween 80 (0.5%) was produced by us on public revenue.

Commercial aluminum adjuvant (Al hydrogel, Invivogen) was mixed with the antigen according to the manufacturer's instructions, other adjuvants were also mixed with the antigen before use, ensuring uniform chitosan, squalene and antigen content in each recipe.

Different degrees of chitosan deacetylation were prepared and characterized by our laboratory.

2.3.2 Characterization of CSPE

Size, Charge and Morphology

The Nano Zeta Sizer (Malvern) was used to measure the hydrodynamic size and zeta potential of CSPE. For a closer examination of CSPE, Cryo-scanning electron microscopy (Cryo-SEM) was employed. Initially, the emulsion underwent rapid freezing in liquid nitrogen, followed by fracturing of the fixed sample under vacuum at −100 °C. Subsequently, water within it was sublimated under vacuum at -90°C, and then it was coated with gold for observation. Fluorescence micrographs of CSPE and CSPE containing the adsorbed antigen were captured using structured illumination microscopy (SIM). Chitosan was labeled with Cy5, while the antigen was labeled with Cy3.

Deformability

To examine the deformability of CSPE, Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) was utilized. Red blood cell membrane-coated QCM-D chips were employed to mimic a cellular contact surface. Subsequently, CSPE, CNP, and SSE were deposited onto the modified chip, and alterations in frequency and energy distribution were monitored and recorded.

To begin with, a blank chip was equilibrated for 15 min and a baseline was obtained; The RBC membrane suspension was then uniformly applied to the chip using a rotator and equilibrated in the device for 15 minutes. Finally, a 5-fold diluted CSPE emulsion was applied by centrifugation on top of the membrane and the chip was also placed in the device to detect adsorption.

Adsorption was measured as follows: After a blank chip was equilibrated to obtain a baseline, anionic liposomes were applied to the chip and equilibrated for 10 min at 40℃ to obtain a thick film distributed on the chip; Finally, the diluted CSPE emulsion was placed in continuous contact with the chip until equilibrium was reached.

Red blood cell membranes were prepared using the hypotonic method and anionic liposomes (cholesterol hemisuccinate: dipalmitoylphosphatidylcholine = 2: 1) were prepared according to the literature method.

Antigen Loading

In this experiment, two antigens were utilized: ovalbumin (OVA), employed in

most experiments, and MUC1 (SAPDTRPAP, synthesized by GenScript), exclusively used for the B16-MUC1 tumor model.

The vaccine formulation was prepared by combining 100 μL of CSPE with either 10 μg of OVA or 20 μg of MUC1 at 4°C overnight. The antigen adsorption efficacy of CSPE and its control group was assessed by quantifying unbound proteins. OVA was not absorbed in the preparations separated via ultrafiltration, with the pure OVA solution serving as a control to correct for any loss incurred due to the ultrafiltration membrane. The released antigen in the supernatant was analyzed utilizing a MicroBCA kit (Thermo).

1) Drawing of standard curve: take a piece of enzyme plate and add reagents according to the following table.

2) According to the number of samples, prepare the appropriate amount of BCA working solution according to 50 volume of BCA reagent A plus 1 volume of BCA reagent B (50:1), and mix well.

3) Add 200μL of BCA working solution to each well.

4) Place the plate on an oscillator for 30 sec and leave it at 37℃ for 30 minutes, then determine the colorimetry at 562 nm. The standard curve was plotted with protein content (μg) as the horizontal coordinate and absorbance value as the vertical coordinate.

5) Dilute the sample to be tested to a suitable concentration, make the total volume of sample diluent 20 μL, add 200 μL of BCA working solution, mix thoroughly, place at 37 ℃ for 30 minutes, then use the standard curve No. 0 tube as a reference, and then take the colorimetric measurement at 562 nm, and record the absorbance value.

6) According to the absorbance value of the measured sample, the corresponding protein content (μg) can be found on the standard curve, divided by the total volume of the sample dilution (20 μL), multiplied by the number of times the sample was diluted to the actual concentration of the sample (unit: μg/μL).

2.3.3 Cellular Uptake and Intracellular Localization

Cellular Uptake

CSPE and Cy3-labeled antigen were combined and allowed to incubate with Bone Marrow-derived Dendritic Cells (BMDCs) or macrophages for various durations. After treatment with CSPE for 1, 2, and 3 hours, the cells were transferred to glass-bottom cell culture dishes, allowed to adhere for 30 minutes, and fixed with 4% paraformaldehyde for 20 minutes. Phalloidin-Alexa Fluor 488 was used to stain the F-actin of the cells. The uptake of the antigen by Antigen-Presenting Cells (APCs) was observed using structured illumination microscopy (SIM) to capture fluorescent images. Subsequently, BMDCs and macrophages collected after a 12-hour incubation were labeled with fluorescent monoclonal antibodies CD11c-eFluor 450 or CD11b-eFluor 450, respectively. The efficiency of antigen uptake was evaluated by determining the percentage of OVA^+ cells using flow cytometry.

BMDCs were prepared as follows.

1) The hind leg is cut off above the hip joint of the C57BL/6 mouse to expose the femur and tibia, the knee and ankle joints are kept immobile, and as much muscle and tissue as possible is removed.

2) Fill a Petri dish with 70% ethanol and soak clean bones in it for 5-10 seconds to complete external sterilization. Wash all bones and place them in a sterile tube with ice around them.

3) Always using sterile instruments in an ultra-clean bench, the ends of the bone are cut off with scissors as close to the joint as possible. Fill a syringe with pre-cooled RPMI complete medium and insert the syringe needle into the bone to flush out the marrow into the surrounding centrifuge tube on ice. Flush 2-3 times until the bone is completely white. Pipette the bone marrow several times to separate any clumps.

4) Centrifuge the cells in RPMI medium at 1500 rpm for 5 minutes (1-2 times). 5.

5) Resuspend the cell precipitate in RPMI medium and count the viable cells using a hemocytometer and Tapan Blue.

6) Dilute the cells with 10 mL of RPMI + 20 ng/mL GM-CSF and inoculate them into the culture dish at a density of 2×10^6 live cells/dish.

7) Place the dishes in a 37°C incubator with 5% CO_2.

8) Add 10 mL of RPMI + 20 ng/mL GM-CSF on the third day.

9) Remove half of the medium (10 mL) on day six.

10) Briefly centrifuge the removed medium (as DCs are loose walled cells and may be contained in the removed medium) and resuspend the cell precipitate in 10 mL of fresh RPMI + 20 ng/mL GM-CSF and add back to the original dish.

11) Non-adherent and loosely adherent cells in the culture supernatant may be harvested by gentle rinsing with PBS and then combined for subsequent experiments.

Intracellular Localization

Two approaches were employed to observe the intracellular distribution of antigen loaded CSPE. Firstly, cells were labeled with fluorescent dyes, and confocal laser scanning microscopy (CLSM) was utilized. Secondly, cells were fixed at various time points and examined using transmission electron microscopy (TEM). Specifically, BMDCs were exposed to vaccine formulations containing Cy3-OVA for a specified duration. After a 30-minute adhesion period on Petri dishes, both the preparations and non-adherent cells were washed several times with PBS buffer. Lysotracker Green DND-26 (Invitrogen) was then added at a concentration of 50 nM, and the BMDCs were returned to the incubator with 5% CO_2 for 30 minutes. Following additional PBS washes, the co-localization of lysosomes and antigens was observed using CLSM (Nikon).

2.3.4 Antigen Depot

BALB/c mice, 6-8 weeks of age, were randomly assigned to experimental groups (n = 4). Vaccine formulations were prepared by combining the adjuvant with Cy7-labeled OVA and administered subcutaneously near the tail. Fluorescence images of the injection site in anesthetized mice were taken at different time points using an in vivo imaging system. The fluorescence intensity values were also recorded and the ratio to the initial value was calculated, and the relative fluorescence intensity changes were plotted to characterize the antigen release.

2.3.5 Recruitment and Maturation of APCs

C57BL/6 mice, consisting of a sample size of four individuals, were subjected to the administration of vaccine formulations incorporating Cy5-labeled ovalbumin (OVA) following the outlined protocol on the first, third, fifth, and seventh day. Post-administration, the subcutaneous tissue surrounding the injection location along with the inguinal lymph nodes were harvested after euthanizing the mice on the eighth day. Subsequently, the excised subcutaneous tissue was sectioned into 3–4 mm fragments, thoroughly rinsed with Hank's balanced salt solution (HBSS) through multiple washes and subjected to enzymatic digestion facilitated by collagenase (type-II) at 37°C for a duration of 2 hours. Following enzymatic treatment, the digested tissue fragments were mechanically dissociated through a 70-mesh cell sieve to procure a single-cell suspension. These isolated cells were then incubated with fluorescent markers including CD11b-eFluor 450, CD11c-PE, F4/80-FITC, and Ly6C-APC/Cy7 at a temperature of 4°C for a duration of 30 minutes. Notably, the antibodies employed for characterizing each antigen-presenting cell type were as follows: $CD11b^+$ $Ly6C^+$ delineating monocytes, $CD11b^+$ $F4/80^+$ designating macrophages, and $CD11c^+$ representing dendritic cells (DCs). Concurrently, the inguinal lymph nodes were mechanically disaggregated into single-cell suspensions using a sieve and subsequently subjected to staining with fluorescent monoclonal antibodies, specifically CD11c-eFluor 450, CD86-FITC, CD40-PE, and SIINFEKL bound to H-2kb-APC, in addition to MHC Class II (I-A/I-E)-PE/eFluor 610. Following staining, the resulting cell suspensions from both the subcutaneous tissue and lymph nodes underwent thorough analysis using flow cytometry to determine the recruitment and activation status of antigen-presenting cells (APCs) induced by the administered immunoadjuvants.

2.3.6 Antibody, Cytokine and CTL Detection

A total of six C57BL/6 mice between 6 and 8 weeks of age were injected subcutaneously at the back of the neck, each with 100 μL of a different formulation of vaccine, each consisting of 10 μg OVA, twice at two-week intervals. Blood samples

were collected weekly from the retro-ocular plexus of the mice starting on day 14 after the first injection. These blood samples were subsequently centrifuged to obtain serum after a 3-hour incubation period at room temperature. Quantitative assessment of antibody levels for IgG, IgG1, and IgG2a in the serum was conducted using an indirect Enzyme-Linked Immunosorbent Assay (ELISA). OVA was utilized as the coating antigen at a concentration of 20 μg/mL.

Here's a concise protocol for evaluating antigen-specific antibody titers using an indirect ELISA:

1) Antigen Coating: Ovalbumin (OVA) is dissolved in carbonate buffer to attain a concentration of 20 μg/mL. Subsequently, 100 μL of the OVA solution is dispensed into each well of a high-affinity 96-well plate, and left to coat overnight at a temperature of 4°C.

2) Blocking: The plates are washed thrice with Phosphate Buffered Saline with 1% Tween-20 (PBS-T) and then blocked using 0.5% Bovine Serum Albumin (BSA) solution (300 μL/well) at 37 °C for a duration of 60 minutes.

3) Addition of Serum Samples: Following three washes with PBS-T, 2 μl of serum sample, suitably diluted with 0.1% BSA, is added to the first well. Successive dilutions of the serum samples are then made by transferring 1 μl from one well to the next, with each subsequent well containing half the serum concentration of its predecessor. The plates are then incubated at 37 °C for 40 minutes (200 μL/well).

4) Addition of Enzyme-Labeled Secondary Antibodies: Anti-mouse IgG-Horseradish Peroxidase (HRP) antibodies (Abcam) are diluted to a ratio of 1:100,000 with 1% BSA. After four washes with PBS-T, 100 μL of the IgG conjugate solution is added to each well and incubated at 37°C for a duration of 40 minutes.

5) Addition of Substrate: Following six washes, 100 μL of Tetramethylbenzidine (TMB) substrate solution is added to each well.

6) Termination: The enzymatic reaction is arrested after 10 minutes by the addition of 100 μL of 1 mol/L sulfuric acid.

7) Absorbance Reading: The absorbance values are measured at wavelengths of 450 nm and 630 nm using a microplate reader. The antibody titer is determined by

identifying the maximum dilution of the OD_{450}-OD_{630} value that equals or exceeds that of the negative control serum.

Following euthanasia of the mice, spleens were harvested on the thirty-fifth day. Subsequent to this, splenocytes underwent a 60-hour culture period at 37°C within a 5% CO2 incubator. Following culture, these cells were stimulated with either 10 μg/mL of OVA or 2 μg/mL of SIINFEKL. Quantification of Th1/Th2 cytokines in the supernatants was achieved through utilization of the Essential Th1/Th2 Cytokine 6-Plex Mouse ProcartaPlex Panel. Additionally, visualization of cytokine secretion by splenocytes was facilitated via employment of the Mouse IFN-γ or IL-4 Enzyme-Linked Immunosorbent Spot (ELISpot) BASIC kit. Furthermore, splenocytes were subjected to staining with fluorescent monoclonal antibodies including CD8a-PE, CD44-PerCP/Cy5.5, and CD62L-eFluor 450, enhancing the detection capability for memory T cells.

To identify cytotoxic T lymphocytes (CTLs) specifically, surface markers on splenocytes were examined. On the 21st day post-initiation of the described immunization regimen, splenocytes were collected and labeled with CD8a-eFluor 450 and R-PE-tagged H-2Kb (SIINFEKL) pentamers.

To further gauge CTL cytotoxicity, E.G7-OVA lymphoma cells were utilized as target cells. Mitomycin C was applied to halt cell proliferation, followed by its removal after a 1-hour incubation. The E.G7 cells were then washed, suspended in complete culture medium, and adjusted to a concentration of 1.6×10^5 cells/mL. Effector splenocytes were cultured with SIINFEKL and IL-2 for 72 hours, and subsequently combined with the prepared E.G7 target cells at effector-to-target ratios of 5:1, 25:1, and 50:1, respectively. Additionally, 1% Triton X-100 was introduced to the target cells as a positive control. Following a 12-hour incubation, the supernatant was transferred to another 96-well plate, and lactate dehydrogenase activity was evaluated using the lactate dehydrogenase (LDH) Cytotoxicity Detection Kit to assess cytotoxicity.

2.3.7 Anti-Tumor Effect

To explore the potential anti-tumor effects of adjuvant-loaded full-length protein OVA and short peptide MUC1 as representative antigens, murine lymphoma cells E.G7-OVA and melanoma cells B16-MUC1 were employed. E.G7-OVA cells, containing the complete transcript of OVA mRNA and the neomycin (G418) resistance gene, were obtained from American Type Culture Collection (ATCC). B16-MUC1 cells, generated by transfecting the MUC1 gene into B16-F10 cells, were generously provided by Jilin University.

In the prophylactic tumor vaccine study, C57BL/6 mice aged 6-8 weeks (n=8) were vaccinated twice with 100 μL of a vaccine formulation containing 10 μg OVA on day zero and fourteen. Subsequently, on the twenty-first day, the mice received an axillary inoculation of 10^6 E.G7-OVA lymphoma cells.

For therapeutic tumor vaccine studies, mice of the same age and sample size were axillary inoculated with either 5×10^5 E.G7-OVA lymphoma cells or 2×10^5 B16-MUC1 melanoma cells on day zero. Following this, 100 μL of vaccine preparation containing 10 μg OVA or 20 μg MUC1 was subcutaneously injected on the third day and every seven days thereafter for a total of three injections.

Tumor volume was evaluated every other day and calculated using the formula: 1/2 × length × width × width (mm^3). Mice were humanely euthanized upon reaching a tumor volume of 2,000 mm^3. Tumor volume data were recorded for up to sixty days, and survival rates were plotted using GraphPad.

2.4 Results

2.4.1 Preparation, Characterization and Antigen Loading of CSPE

CSPE comprises an oil-in-water emulsion where squalene serves as the oil phase and chitosan nanoparticles act as the stabilizer. Chitosan nanoparticles (CNPs) were synthesized using the salting out method, while the emulsion was generated through ultrasonication (Figure 2.1).

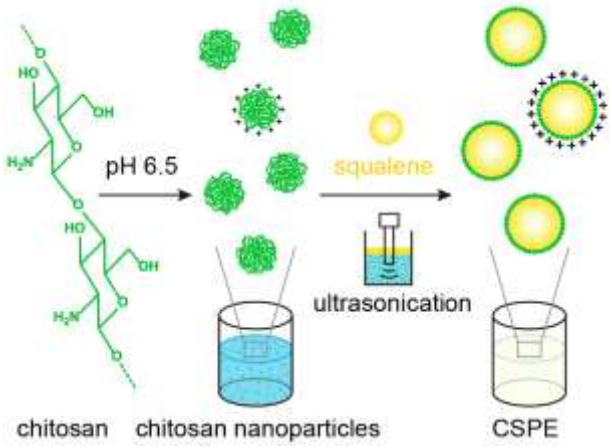


Figure 2.1 Schematic diagram of the preparation process of CSPE

The molecular weight and concentration of chitosan both affected the droplet size of the obtained emulsion. Specifically, Chitosan with a molecular weight ranging from 50 to 150 kDa was utilized to prepare chitosan particles and emulsions at various concentrations. At lower chitosan concentrations, such as 0.1 mg/mL, where fewer chitosan particles were available to stabilize the interface between oil and water, larger emulsion droplets were observed. The size of CSPE droplets then gradually decreased until the chitosan concentration exceeded 2 mg/mL. Notably, at a chitosan concentration of 4 mg/mL, an excess of dispersed particles in the aqueous phase resulted in a minor peak at 100 nm (Figure 2.2), suggesting that there were sufficient particles to stabilize the emulsion.

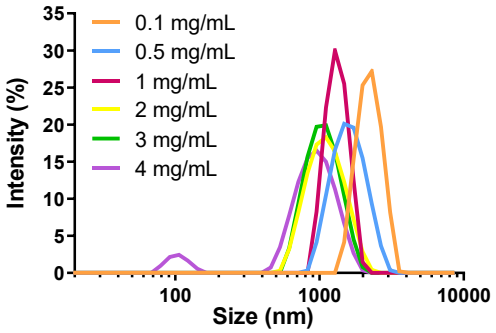


Figure 2.2 Effect of chitosan solution concentration on particle size

Long-term stability assessments revealed that the size of the emulsion remained constant even after two months of storage at 4°C, validating the enduring stability of CSPE across different chitosan concentrations. Further investigations involved storing

emulsions made with chitosan of varying molecular weights at 37°C for fifteen days. It was observed that chitosan of 500 kDa led to excessive adhesion between chitosan particles, resulting in an increased particle size in the emulsion. Conversely, 20 kDa chitosan exhibited degradation during storage, impairing emulsion stability and leading to noticeable changes in droplet size due to particle aggregation (Figure 2.3).

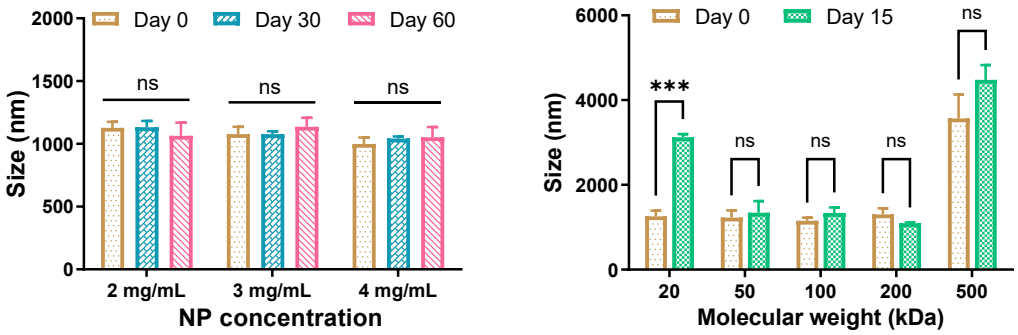


Figure 2.3 Effect of chitosan particle concentration and molecular weight on particle size

In light of these findings, chitosan with a medium molecular weight within the range of 50–200 kDa, at a concentration of 3 mg/mL, was deemed optimal for preparing the CSPE emulsion. Microscopic examination revealed uniform emulsion droplets with visible chitosan particles on their surface (Figure 2.4).

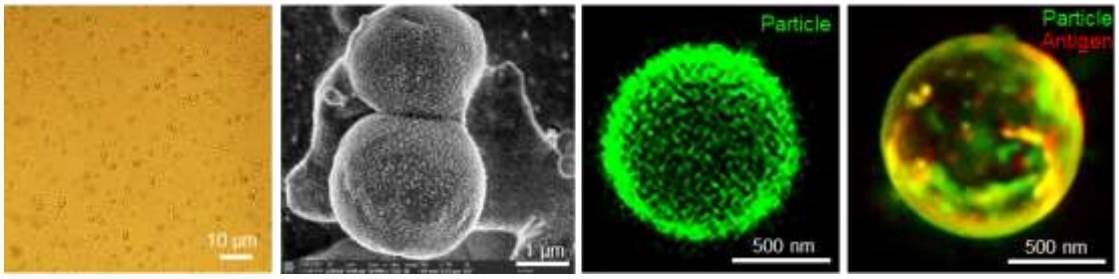


Figure 2.4 Images of CSPE

At the oil-water interface of CSPE, numerous amino-rich, positively charged chitosan particles are positioned, while ovalbumin, being negatively charged at pH 6-7, facilitates the effective adsorption of antigens with a low isoelectric point, like OVA, into the emulsion through electrostatic interaction. Confirmation of this phenomenon was achieved through the reduction in the zeta potential of CNP and CSPE following antigen adsorption (Figure 2.5), along with the substantial alignment

between fluorescence signals of chitosan and OVA (Figure 2.4).

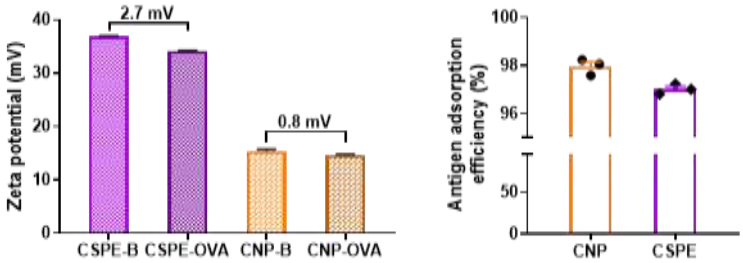


Figure 2.5 Antigen adsorption of CSPE

The schematic depiction of CSPE adsorbing antigen further elucidated the electrostatic adsorption process, highlighting the effective entrapment of antigen within the emulsion matrix (Figure 2.6).

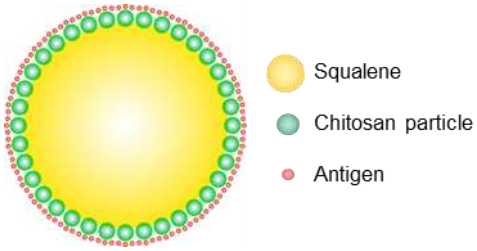


Figure 2.6 Schematic depiction of antigen adsorbed CSPE

2.4.2 Intracellular Uptake and Lysosomal Escape

Cy3-labeled ovalbumin was encapsulated within various adjuvant preparations and subsequently co-incubated with Antigen-Presenting Cells (APCs), including macrophages and BMDCs. Utilizing Structure Illumination Microscopy (SIM), the endocytosis of OVA within CSPE by macrophage and BMDC was visually evident (Figure 2.7).

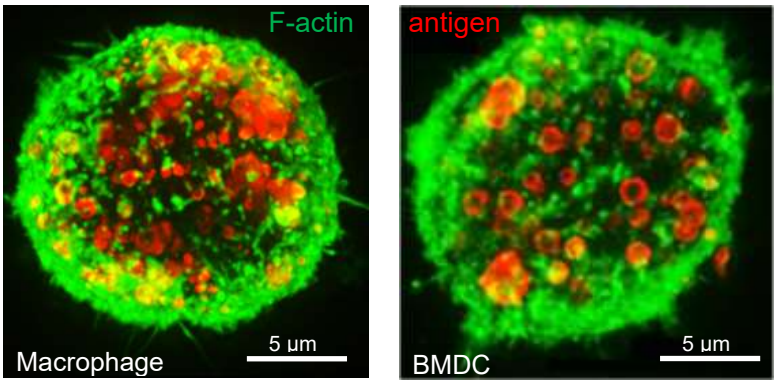


Figure 2.7 SIM images of antigen uptake by APCs

Flow cytometry quantification demonstrated that adjuvants containing chitosan, including CSPE, CMP, and CNP, markedly increased the cellular uptake of antigen by APCs. Particularly, particles at the nanometer scale like CNP demonstrated greater internalization by APCs compared to micro-sized CMP. While antigens within CSPE and CNP were internalized to a comparable degree by BMDCs, those adsorbed in CSPE were remarkably more efficiently endocytosed by macrophages (Figure 2.8). This enhanced efficacy of CSPE in facilitating antigen uptake by APCs can be attributed to its robust surface and deformability as a Pickering emulsion.

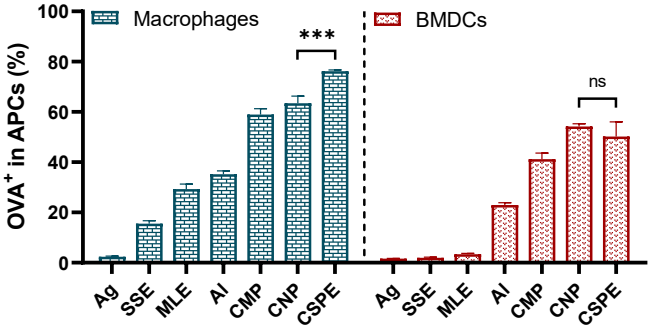


Figure 2.8 Effects of different formulations on antigen uptake by APCs

Quartz Crystal Microbalance with Dissipation (QCM-D) was employed to replicate and measure the interactions between particles or emulsions and cells upon contact. The reduction in oscillatory frequency (Δf, represented by dash lines) demonstrated an augmentation in mass, while the dissipation (ΔD, depicted by solid lines) exhibited a positive correlation with viscoelasticity. Notably, the Δf of CSPE (blue dash line) was significantly decreased, implying its preference for adsorption onto biomembranes compared to SSE or CMP. This phenomenon can be ascribed to the deformation of CSPE upon contact with the cell membrane, consequently augmenting the contact area and intensifying the interaction force. Moreover, The greater ΔD variation of CSPE (blue solid line) indicated its higher flexibility in engaging with cell membranes. 3D reconstruction visualization of particle or emulsion interactions with the cytomembrane further highlighted the excellent

deformability of CSPE. CSPE had better deformability upon contact with cells, thereby mimicking the interactions between natural pathogens and cells in a way (Figure 2.9).

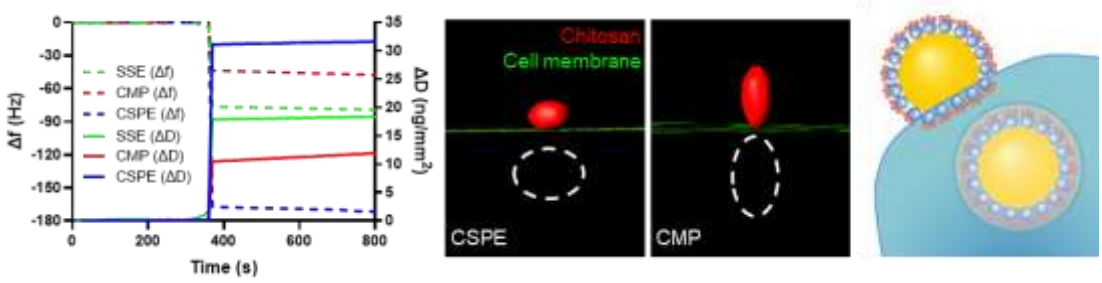


Figure 2.9 Deformability of CSPE

In addition to boosting antigen uptake by APCs, CSPE facilitated the cross-presentation process of exogenous antigens. Notably, 2 hours after CSPE's lysosomal uptake, the lysosomal membrane ruptured, enabling the antigen to escape from the lysosome into the cytoplasm (Figure 2.10). This occurrence may be attributed to the heightened membrane cleavage capacity of CSPE under acidic conditions similar to the pH environment within the lysosome.

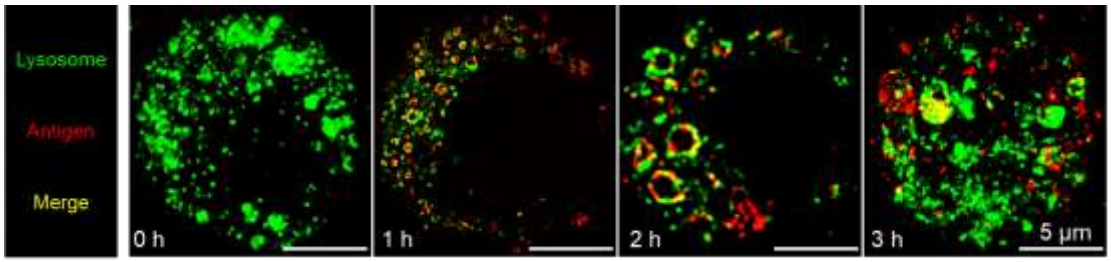


Figure 2.10 Escape of antigen from lysosomes

Furthermore, the comparable zeta potential of CSPE and CNP at approximately pH 5 implied that chitosan particles alone might not be solely accountable for the robust membrane rupture capability of CSPE (Figure 2.11).

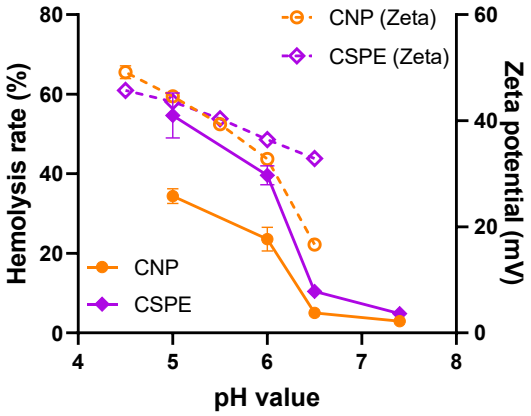


Figure 2.11 CSPE potential and membrane-breaking ability at different pH

Following the same duration, antigen adsorbed CSPE and the lysosomes had a co-localization rate of 20.5%, markedly lower than CNP's 56.4% (Figure 2.12). This observation suggests that the augmentation of lysosomal escape is intricately linked to the inherent properties of CSPE itself, which may not be easily replicated by dispersing chitosan particles in the water phase alone.

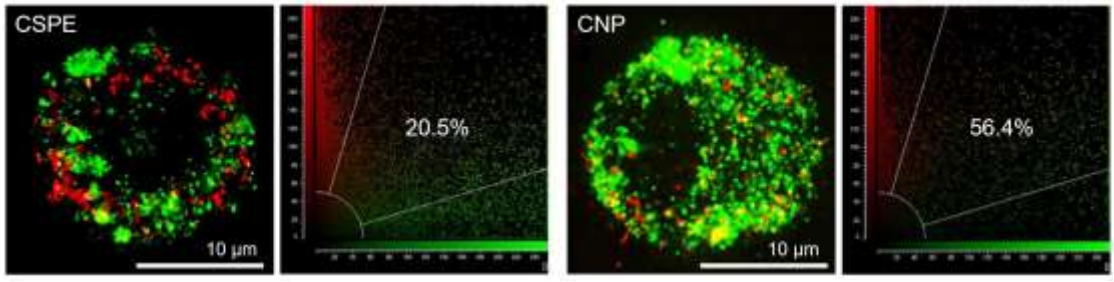


Figure 2.12 Co-localization images of lysosomes and antigens

Several hypotheses have been postulated to explain this phenomenon. Firstly, under acidic conditions, CSPE undergoes a transformation into an emulsion stabilized by polymer chains, leading to swelling. Secondly, a significant portion of particles in CSPE are adsorbed onto the oil-water interface and aggregate within lysosomes, potentially compromising the stability of lysosomal membrane.

Flow cytometry was employed to evaluate the expression of SIINFEKL-specific Major Histocompatibility Complex-I (MHC-I) on Dendritic Cells (DCs) in lymph nodes (Figure 2.13). In this context, 2-CSPE, 3-CSPE, and 4-CSPE denote emulsions stabilized by CNP with chitosan particle concentrations of 2 mg/mL, 3 mg/mL, and 4

mg/mL, respectively. Remarkably, compared to 2-CSPE, particles in 3-CSPE exhibited denser packing at the oil-water interface, leading to increased expression of SIINFEKL-specific MHC-I in DCs following immunization. This highlights the pivotal role of solid particles at the oil-water interface in CSPE.

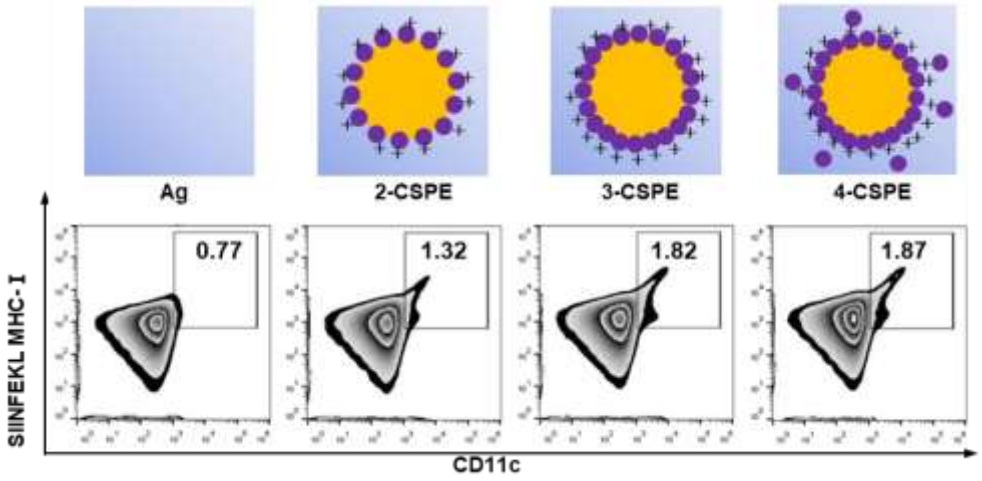


Figure 2.13 Effect of particle amount in CSPE on antigen escape

2.4.3 Mechanisms of CSPE facilitating lysosomal escape

To delve deeper into the function of chitosan particles in facilitating antigen cross-presentation by CSPE, three additional formulations were constructed: chitosan polymer-stabilized emulsion (PMSE), formaldehyde covalently cross-linked chitosan particles-stabilized Pickering emulsion (FCPE), and sodium tripolyphosphate ionically cross-linked chitosan particles-stabilized Pickering emulsion (TCPE). CSPE, FCPE, and TCPE are three emulsions all stabilized by chitosan particles, where the amino group of FCPE is irreversibly crosslinked with the aldehyde group, thus exhibiting resistance to pH fluctuations (Figure 2.14). The antigen loading efficiencies of four kinds of emulsions were all more than 90%, their particle sizes were between 1029–1772 nm (Table 2.1).

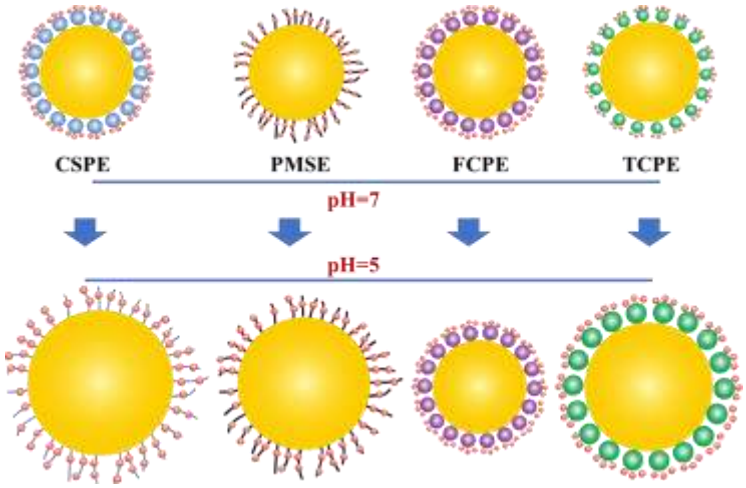


Figure 2.14 Schematic representation of different emulsions

Table 2.1 Characterization of different emulsions

	Antigen loading efficiency (%)	Particle size (nm)
CSPE	93.00 ± 1.50	1106 ± 114
PMSE	92.91 ± 0.99	1555 ± 110
FCPE	90.49 ± 0.99	1029 ± 276
TCPE	93.10 ± 0.91	1772 ± 258

At a pH of 5, formaldehyde-cured CNP particles and tripolyphosphate cross-linked chitosan particles remained unchanged, while CNP underwent a transformation from particles to polymer chains (Figure 2.15).

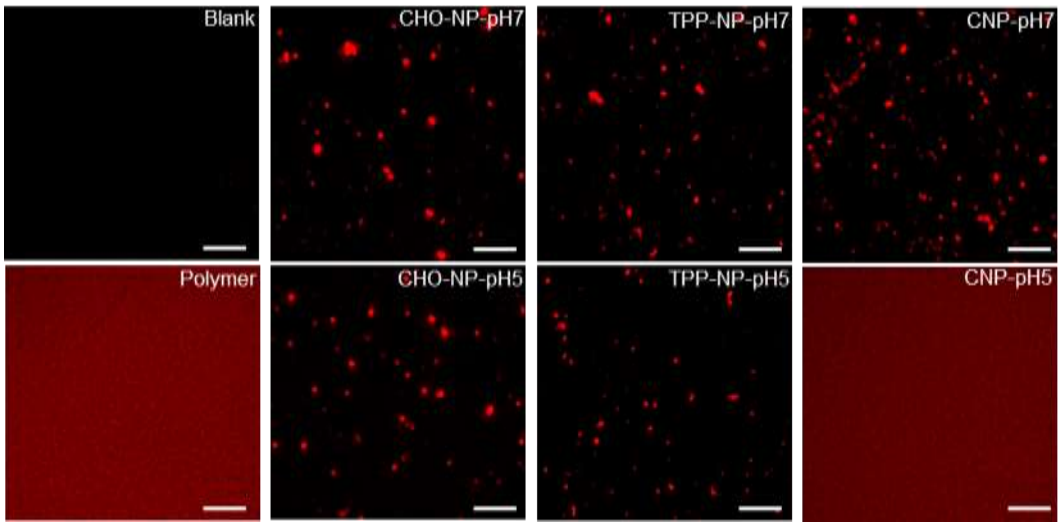


Figure 2.15 Variation of different particles in different pH

At pH 5, the emulsion droplet sizes of CSPE, PMSE, FCPE, and TCPE increased by factors of 4.27, 1.91, 1.02, and 2.37, respectively. In CSPE and PMSE, the chitosan molecules were not cross-linked, resulting in a charge increase to 43–50 mV when the pH was reduced to lysosomal pH 4.5–5. While the ultimate potential of PMSE surpasses that of CSPE, the extent of potential alteration in CSPE (13.2 mV) significantly exceeds that of PMSE (5.3 mV) from endocytosis into endosomes and then into lysosomes (Figure 2.16). The substantial disparity in potential variation in CSPE due to pH shifts could be crucial for the lysosomal escape of the antigen and might result in a more robust cellular immune response.

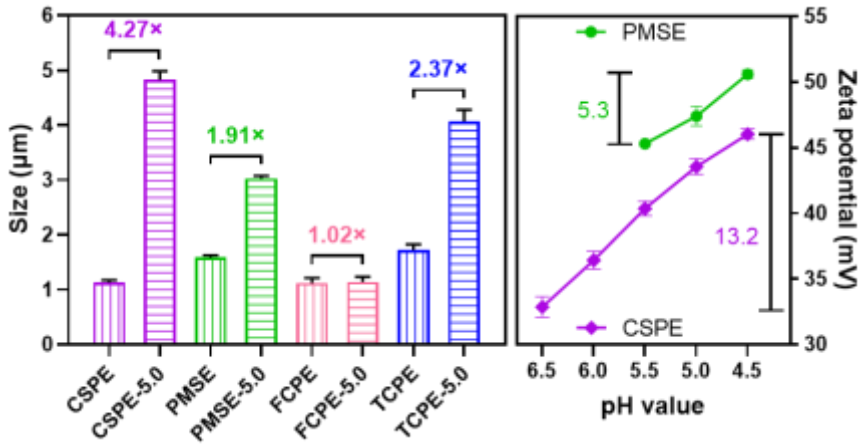


Figure 2.16 Changes in emulsion particle size and potential in different pH

With a decrease in pH, the size of CSPE droplets undergoes a rapid increase, accompanied by alterations in surface morphology. At pH 5, there are no discernible particles visible at the emulsion oil-water interface (Figure 2.17). This observation suggests that chitosan present on the CSPE surface has the capability to shift from particles to polymer chains, thus validating the rationale behind CSPE's earlier observed rapid lysosomal escape. It is hypothesized that the transition of chitosan from a particulate state at pH 7 to a polymer chain state at pH 5, along with the transition of emulsion interface stabilizers from particles to chains, contributes positively to emulsion swelling and plays a pivotal role in lysosomal escape. Subsequent studies focused on comparing the immunization effects of CSPE versus PMSE aim to test this hypothesis.

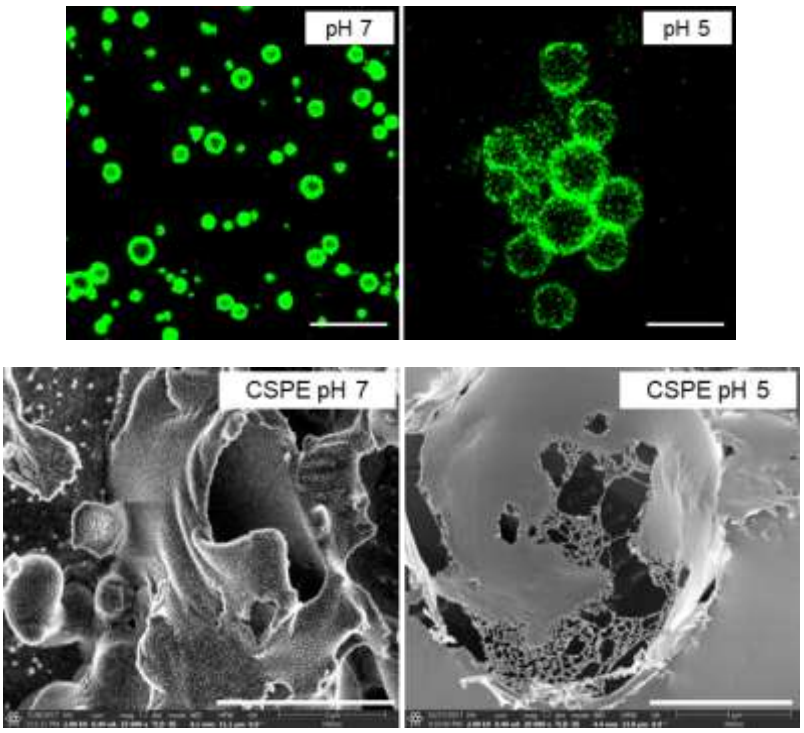


Figure 2.17 Changes in particle size and emulsion surface particle morphology in different pH

During uptake experiments involving various chitosan emulsions by macrophages and BMDCs, CSPE and TCPE exhibited greater antigen uptake efficiency in comparison to PMSE and FCPE (Figure 2.18), indicating a positive correlation between emulsion volume increase and cellular endocytosis.

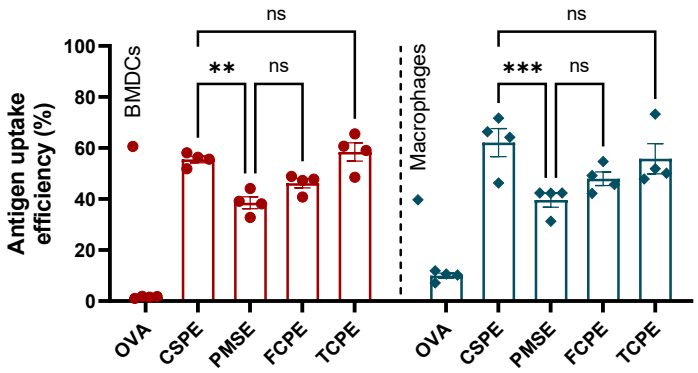


Figure 2.18 Antigen uptake efficiency of different chitosan emulsions

Mice received three immunizations with a vaccine containing OVA mixed with the mentioned chitosan emulsion adjuvant, following which inguinal lymph nodes were harvested. Following immunization with CSPE, there was a significant increase

in the expression of SIINFEKL-specific MHC-I on the surface of macrophages or DCs in lymph nodes (Figure 2.19).

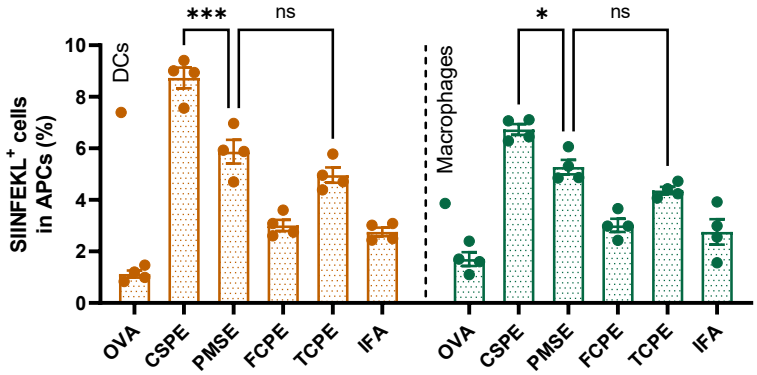


Figure 2.19 Effects of different emulsions on the expression of SIINFEKL-specific MHC-I

Additionally, CSPE exhibited advantages in promoting DC maturation, suggesting its superior ability to facilitate antigenic cross-presentation. Three weeks post-immunization, collected mice splenocytes were stained with SIINFEKL-specific MHC-I pentamer and subsequently analyzed via flow cytometry. The results indicated that the expression of SIINFEKL-specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) was most significantly upregulated in the CSPE group, which was followed by the PMSE group (Figure 2.20).

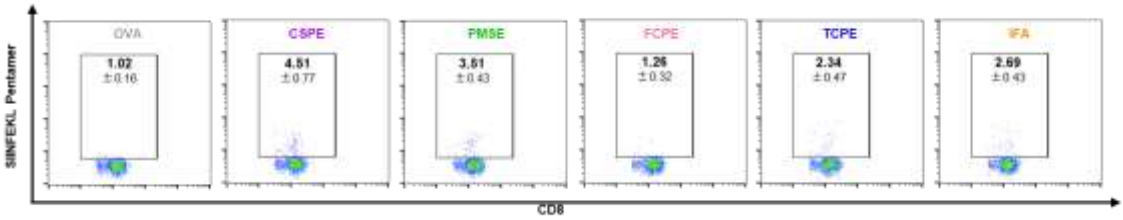


Figure 2.20 Effects of different chitosan emulsions on $CD8^+$ SIINFEKL $^+$ cytotoxic T lymphocytes

While the utilization of chitosan emulsion adjuvants all prompted a Th1-biased immune response, as indicated by an IgG2a/IgG1 ratio >1, CSPE, PMSE, and TCPE elicited varying degrees of IL-4 (a Th2 cytokine) secretion by immune cells following SIINFEKL re-stimulation (Figure 2.21).

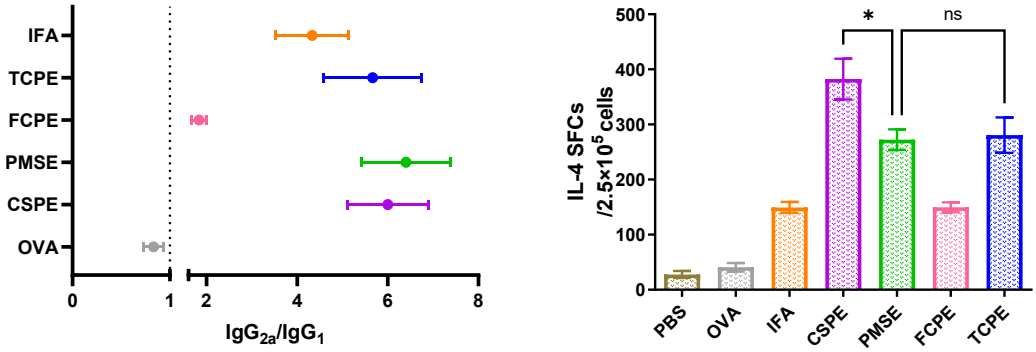


Figure 2.21 Chitosan formulations had effect on IgG_{2a}/IgG_1 ratio of antibodies and IL-4 secretion

CSPE notably enhanced SIINFEKL-specific cytotoxic T-lymphocyte (CTL) responses in comparison to the other groups. This was corroborated through quantitative ELISA analysis of IFN-γ concentration in lymphocyte supernatants and ELISPOT analysis (Figure 2.22).

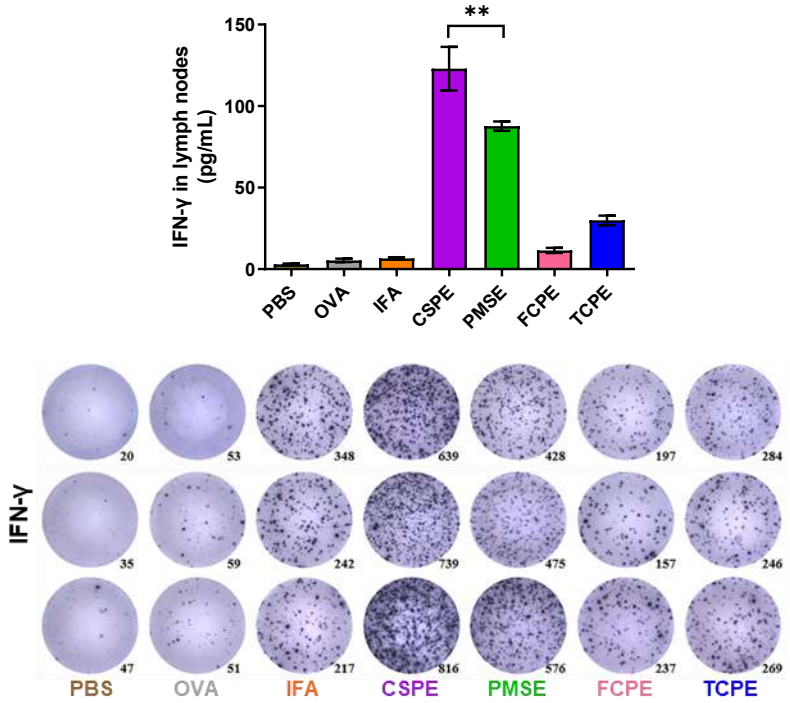


Figure 2.22 Effects of different chitosan formulations on IFN-γ secretion in lymphocyte

Following CSPE and PMSE administration, expression of $CD8^+$ central memory T cells (Tcm, $CD44^+$ $CD62L^+$) was up-regulated (Figure 2.23), and the $CD8^+$ Tcm are

critical for a rapid immune response to reinfection. While direct evidence of morphological transformation of CSPE in lysosomes is currently lacking, results from *in vivo* immunoassays indicate that the transition of chitosan from particle-stabilized emulsions to polymer-chain-stabilized emulsions under acidic conditions, resulting in alterations in zeta potential and emulsion droplet swelling, could be pivotal in conferring the significant advantage of CSPE in promoting antigen lysosomal escape.

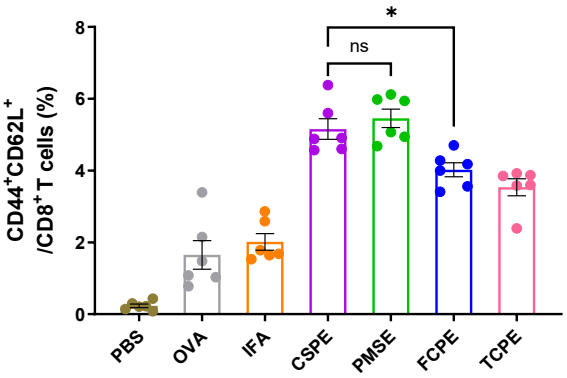


Figure 2.23 Effects of different chitosan formulations on immune memory

2.4.4 Accelerated lysosomal escape by proton accumulation

Chitosan is derived from the deacetylation of chitin (Figure 2.24), leading to an increase in the number of amino groups with the degree of deacetylation. These positively charged chitosan particles can trigger the lysis and rupture of endosomal membranes through the proton sponge effect. While numerous studies have investigated the lysosomal escape rates of biomaterials, exploring variations in types, particle sizes, and surface characteristics, the influence of differences in the degree of protonation on regulating the lysosomal escape process has been rarely explored.

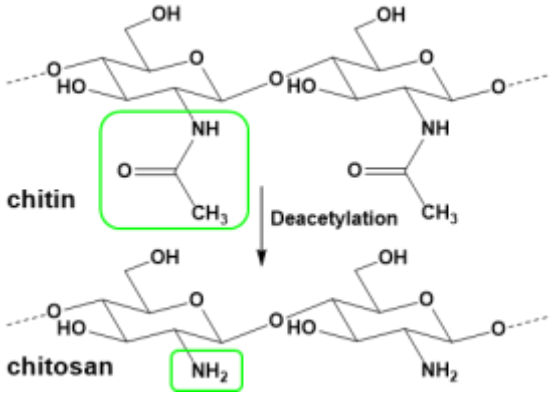


Figure 2.24 Chitin and chitosan

To explore the impact of proton accumulation of CSPE on lysosomal escape, we synthesized three variants of CSPE with varying amino acid contents. These were labeled as 70-CSPE, 80-CSPE, and 90-CSPE, corresponding to CNPs with deacetylation degrees of 70%, 80%, and 90%, respectively (Figure 2.25).

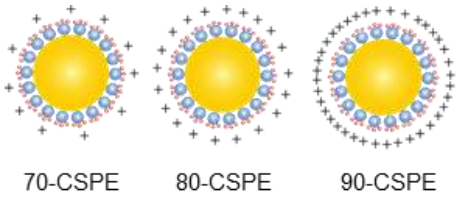


Figure 2.25 CSPE with different degrees of deacetylation

While their particle sizes remained essentially similar and their antigen adsorption efficiencies exceeded 90%, the conversion of CNP into CSPE led to an augmentation in both its potential and its uptake efficiency by BMDCs (Figure 2.26). Remarkably, the uptake rate of 70-CNP was markedly lower compared to that of 90-CNP, primarily influenced by particle size and surface charge, with a higher charge facilitating cellular uptake of particles. Nevertheless, the antigen uptake efficiency of all CSPE did not exhibit significant differences, implying that the deformability of CSPE plays a pivotal role in cellular uptake.

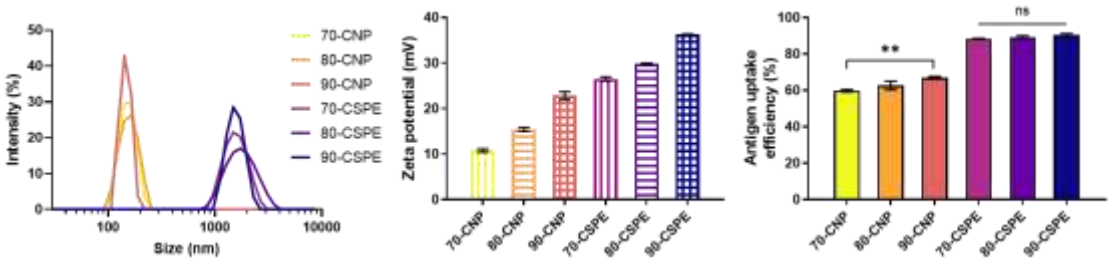


Figure 2.26 Characterization of chitosan formulations with different degrees of deacetylation

Following 12 hours of co-culture with BMDCs, the lysosomal escape rates, calculated as 100% minus the co-localization rate, were 40%, 51%, and 83% for 70-CSPE, 80-CSPE, and 90-CSPE, respectively. These findings strongly suggest that lysosomal escape is significantly influenced by the degree of protonation of CSPE.

Moreover, CSPE with varying of proton numbers resulted in distinct lysosomal escape efficiencies. Specifically, 70-CSPE showed minimal change within the cells from the first hour to seventh hour, with partial emulsion beginning to escape into the plasm only by the 8th hour. The emulsion droplet interface of 80-CSPE began to disrupt at the first hour, with deformation occurring by the 2nd hour. In contrast, 90-CSPE underwent rapid deformation within the first hour, completed deformation by the 2nd hour, and initiated escape attempts. Within 3 hours, large portions of 80-CSPE and 90-CSPE had escaped from the lysosome (Figure 2.27). Most literature suggests lysosomal escape typically occurs around 6 hours, indicating that proton accumulation significantly accelerates lysosomal escape.

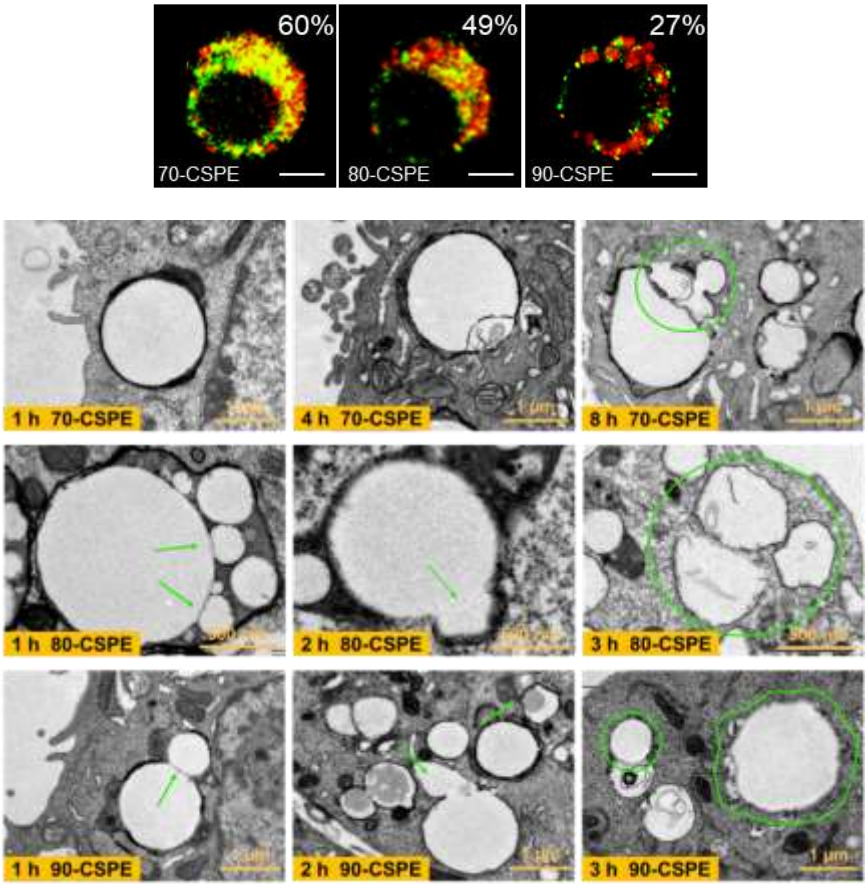


Figure 2.27 Lysosomal escape at different time points

The immunostimulatory impact of CSPE resulting from proton accumulation was further validated using a mouse E.G7-OVA lymphoma model. Splenocytes were collected post-immunization, and effector cells were acquired by SIINFEKL

stimulation. These effector cells were subsequently combined with E.G7-OVA tumor cells in specific proportions.

The Cytotoxic T Lymphocyte (CTL) response elicited by CSPE was stronger than that by CNP, with 90-CSPE exhibiting the highest efficacy. Our study confirms that the lysosomal escape efficiency of CSPE is indeed influenced by the number of protons on its surface, as evidenced by the direct killing of CTL through $CD8^+$ T cell activation via exogenous antigen cross-presentation. Furthermore, we observed a positive correlation between the tumor suppression effect and the number of protons associated with CSPE (Figure 2.28).

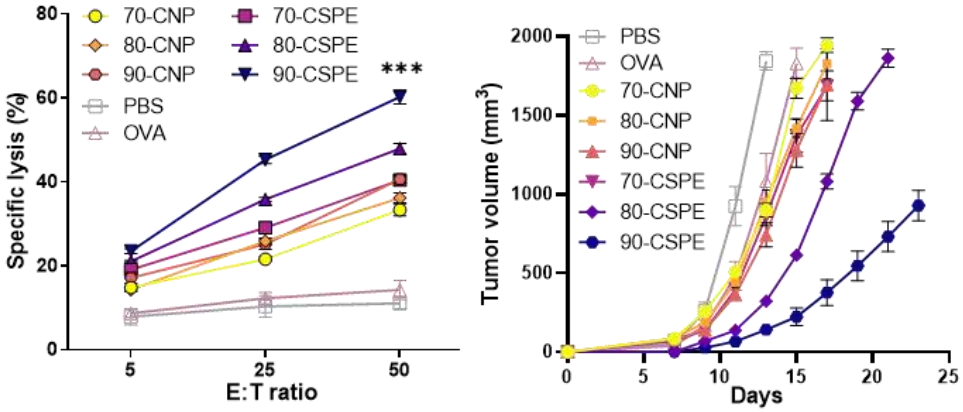


Figure 2.28 Tumor-killing effect of chitosan preparations with different degrees of deacetylation

The trend of differences in the number of cells secreting IL-4 (Th2-type cytokine) among the CNP groups mirrored that of the CSPE group, indicating that proton accumulation did not notably affect the Th2-type immune response. Conversely, while no significant difference was observed in the number of cells secreting IFN-γ (Th1-type cytokine) among the CNP groups, a notable distinction emerged among the CSPE groups. This suggests that proton accumulation significantly bolstered the Th1 immune response (Figure 2.29), further substantiating the role of increased CSPE protons in augmenting cellular immunity in terms of Th2/Th1 immune bias.

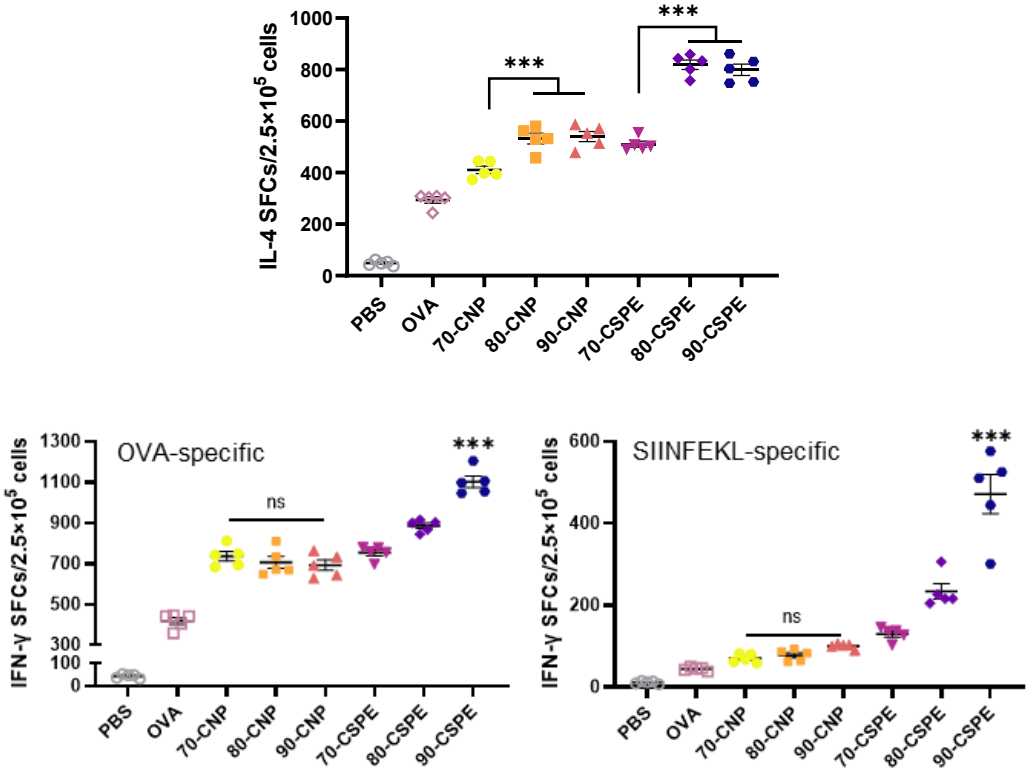


Figure 2.29 IL-4 and IFN-γ secretion of chitosan preparations with different degrees of deacetylation

Following the exploration of protonation levels, we selected chitosan with a deacetylation degree of 91.25% for CSPE preparation, subsequently evaluating its immunological efficacy as an adjuvant. The heightened degree of protonation in CSPE correlated with an augmented antigen lysosomal escape rate, alongside increased parameters related to cellular immunity such as the count of IFN-γ secretory cells and CTL kill rate. These results underscore the involvement of chitosan particle proton accumulation in fostering lysosomal escape and cellular immunity.

2.4.5 Antigen depot and local immune responses

The sustained release of antigen was monitored using an in vivo fluorescence imaging system following subcutaneous injection of vaccine formulations containing fluorescently labeled antigen. After 4 hours, only about 10% of the antigen remained at the injection site in the SSE and MLE (MF59-like emulsion) groups, contrasting

with the antigen retention observed in the pure antigen (Ag) group. The SSE group exhibited a quicker clearance of antigen from the injection site, likely due to simple mixing with the antigen. Conversely, CNP and CMP displayed slightly longer antigen storage times attributed to their cationic nature, which facilitated the adherence and retention of adsorbed antigen at the injection site. Both aluminum adjuvant and CSPE effectively formed antigen reservoirs, retaining approximately 30% and 20% of the antigen at the injection site after 120 hours, respectively (Figure 2.30). The extended presence of gel or emulsion adjuvants at the injection site, coupled with robust electrostatic adsorption between the adjuvant and antigen, contributes to prolonged antigen release and sustained stimulation of the immune system.

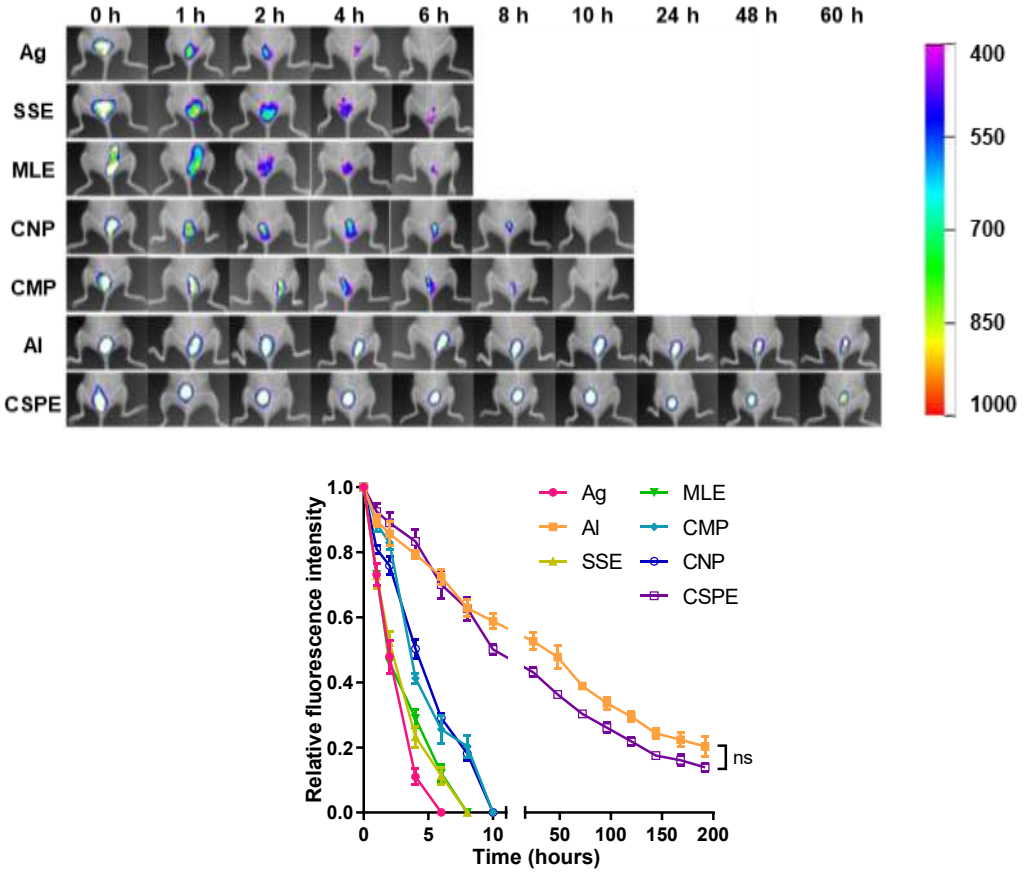


Figure 2.30 *In vivo* fluorescence characterization of CSPE for slow release from antigen depot

Adjuvant retention creates an immunologically activated milieu at the injection site, fostering the recruitment of antigen-presenting cells (APCs) and augmenting antigen uptake by immune cells. Hence, we examined the recruitment of dendritic

cells (DCs), macrophages, and monocytes by adjuvants at the injection site. Representative flow cytometric images depicting the substantial recruitment of APCs by CSPE in comparison to Ag were observed (Figure 2.31).

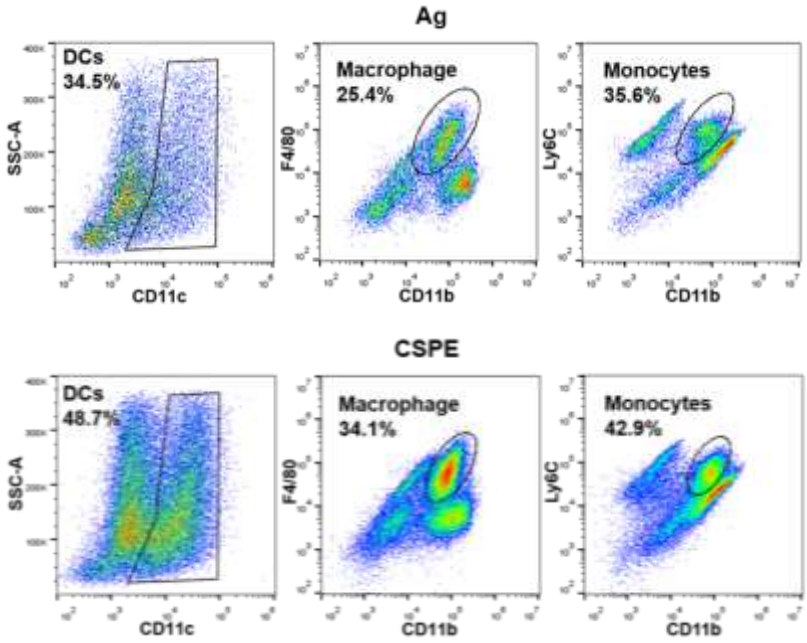


Figure 2.31 CSPE increases recruitment of various APCs

On day 1, CSPE markedly augmented the population of all three APCs capturing OVA at the injection site. Notably, CSPE continued to recruit substantial levels of APCs on day 3 compared to aluminum adjuvant, another notable antigen reservoir; however, by day 7, CSPE recruited fewer APCs than aluminum (Figure 2.32). In contrast to aluminum adjuvants, CSPE promptly attracts APCs that absorb the vaccine formulation, and after approximately one week, it ceases to recruit large numbers of APCs, potentially due to factors such as biodegradation. This characteristic reduces the likelihood of a sustained inflammatory response and enhances biosafety.

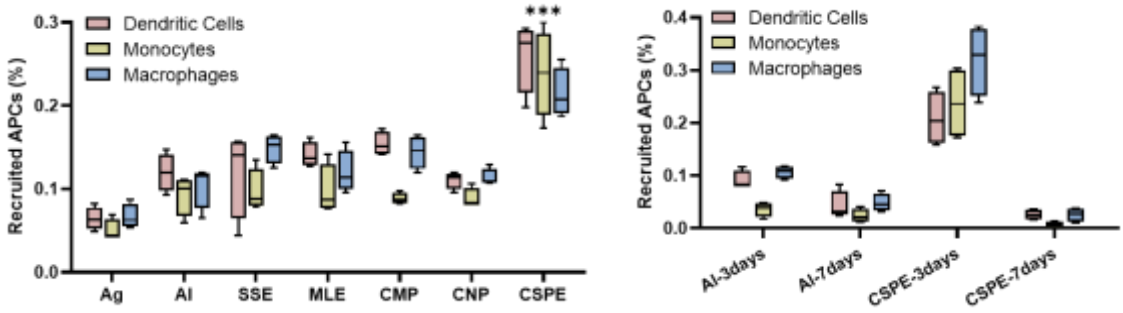
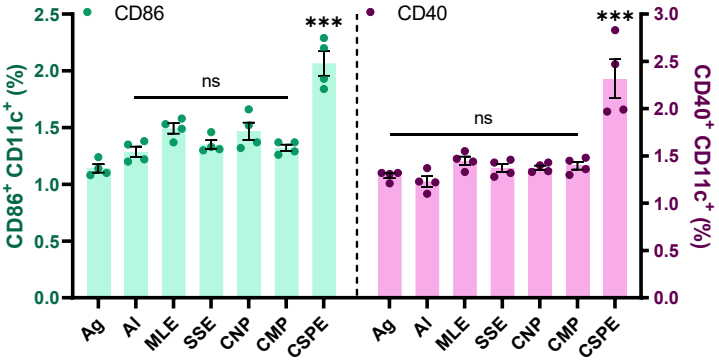


Figure 2.32 Recruitment of APCs by different formulations

Migration of activated DCs from the injection site to the lymph node is pivotal for delivering antigen to specific T cells and effectively activating naïve T cells, a fundamental aspect of the immune response. Assessment of cell activation levels in lymph nodes via flow cytometry revealed that CSPE notably enhanced the expression of co-stimulatory molecules CD86 and CD40, MHC-II, and SIINFEKL-specific MHC-I on the cell surface of DCs in lymph nodes (Figure 2.33), indicative of DC maturation and antigen-presenting proficiency. Consequently, CSPE significantly augmented recruitment of APCs, antigen uptake, activation and maturation of DCs, and antigen cross-presentation.



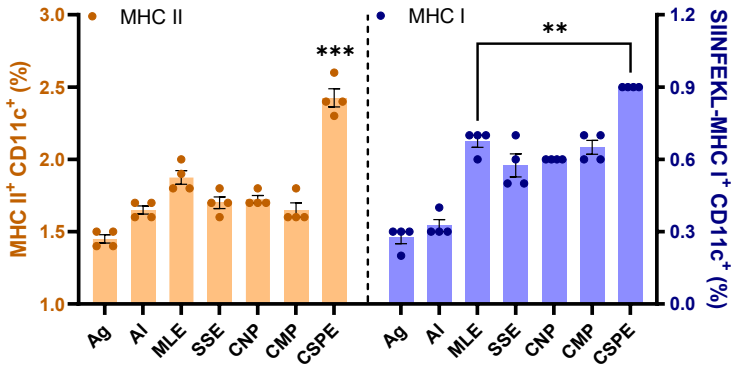


Figure 2.33 CSPE enhances activation of DCs and antigen cross-presentation

2.4.6 Humoral and cellular immune promotion and tumor vaccine effects

In prophylactic vaccination scenarios, the humoral immune response post-immunization plays a crucial role in preventing infection. Following two immunizations, serum levels of antigen-specific antibodies were assessed via ELISA. The CSPE group exhibited the highest antibody levels, distinctly differing from those of the MLE group. Conversely, the Ag group consistently displayed low antibody levels, while those of the adjuvant group peaked at 21 or 28 days before gradually declining (Figure 2.34).

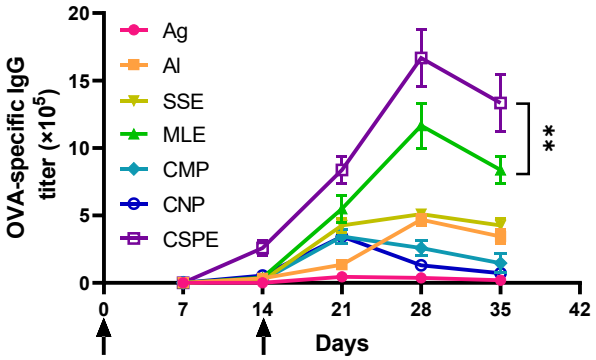


Figure 2.34 Production of specific antibodies in serum

Th1-type cytokines (such as IL-12, TNF-α, and IFN-γ) orchestrate proinflammatory reactions, combating intracellular pathogens, restraining tumor growth, and regulating autoimmune responses. Th2 cytokines (like IL-4, IL-5, and IL-6) orchestrate humoral immune responses against extracellular pathogens. The

balance between Th1 and Th2 responses profoundly impacts tumor vaccine efficacy. This equilibrium can be gauged through the IgG2a/IgG1 ratio and the levels of Th1 and Th2 cytokines. Elevated IgG2a/IgG1 ratios signify a bias toward Th1-type immunity. While CMP and CNP exhibited slightly above 1.0 IgG2a/IgG1 ratios, CSPE notably displayed higher ratios, indicating a robust induction of Th1-type immune responses (Figure 2.35). CSPE robustly induces Th1-type immune responses, primarily via abundant IFN-γ secretion, thereby positioning it as an effective agent in tumor therapy.

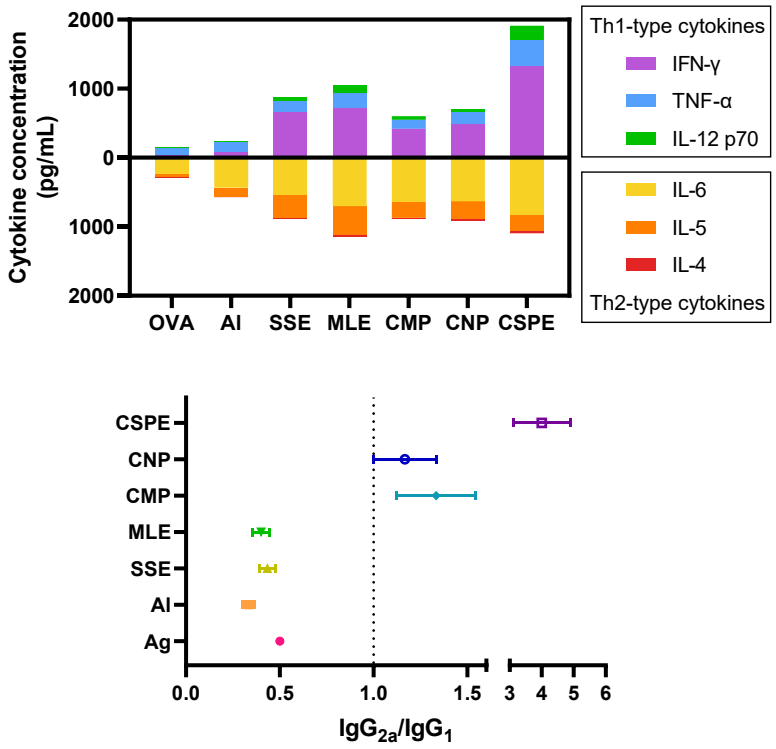


Figure 2.35 CSPE-induced immune bias

Cytotoxic T lymphocytes (CTLs) are the principal effectors in specific cellular immune reactions. Analysis with SIINFEKL-specific MHC-I pentamers revealed a notably higher presence of CSPE-induced antigen-specific CTLs compared to other groups (Figure 2.36).

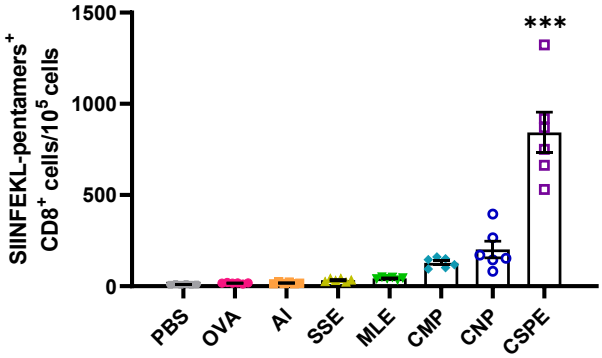


Figure 2.36 CSPE promotes the generation of SIINFEKL-specific CTLs

IFN-γ activation of APCs enhances the generation of antigen-specific $CD8^+$ T cells, a crucial aspect of protective adaptive immunity against pathogens. Following OVA stimulation, the CSPE group exhibited a notably higher count of cells secreting IFN-γ compared to other groups. This indicates that CSPE enhances the cytotoxic capability against virus-infected or tumor cells by boosting the generation of IFN-γ-secreting $CD8^+$ T cells (Figure 2.37). Additionally, $CD8^+$ central memory T cells ($CD44^{hi}$ $CD62L^{hi}$) produced in the CSPE group were important in adaptive immune responses (Figure 2.38).

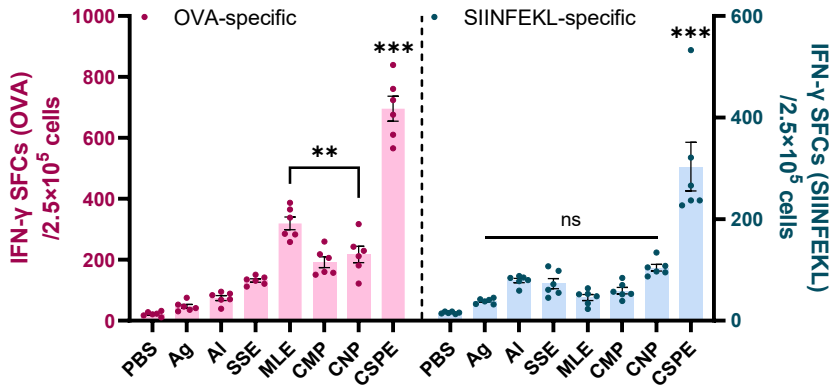


Figure 2.37 CSPE promotes the generation of IFN-γ-secreting $CD8^+$ T cells

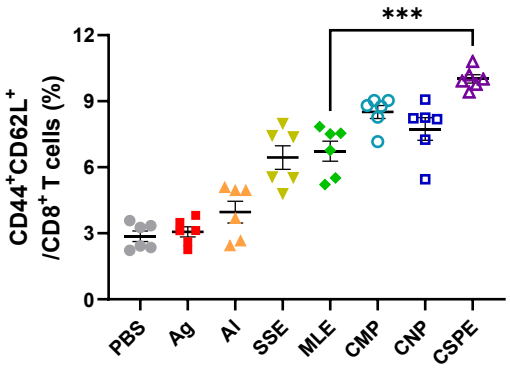


Figure 2.38 CSPE promotes the generation of $CD8^+$ central memory T cells

Based on the promising results from the mouse immunization trials, CSPE demonstrated remarkable enhancement of both humoral and CTL-mediated cellular immunity. Encouraged by these findings, we explored its potential as a preventive and therapeutic tumor vaccine.

In the prophylactic tumor model, mice were inoculated subcutaneously with E.G7 lymphoma cells following two rounds of immunization. Conversely, for the therapeutic lymphoma and melanoma models, mice were initially challenged with E.G7 or B16 cells, respectively, before beginning immunization with vaccine formulations (OVA for E.G7 and MUC-1 for B16). This immunization protocol was repeated three times for each model.

Across all three tumor models, CSPE exhibited significant suppression of tumor growth. Mice treated with CSPE experienced tumor volumes reduced to approximately 15%-40% compared to other groups, leading to substantially prolonged survival times for tumor-bearing mice (Figure 2.39). These outcomes underscore the considerable potential of CSPE as an adjuvant for therapeutic tumor vaccines.

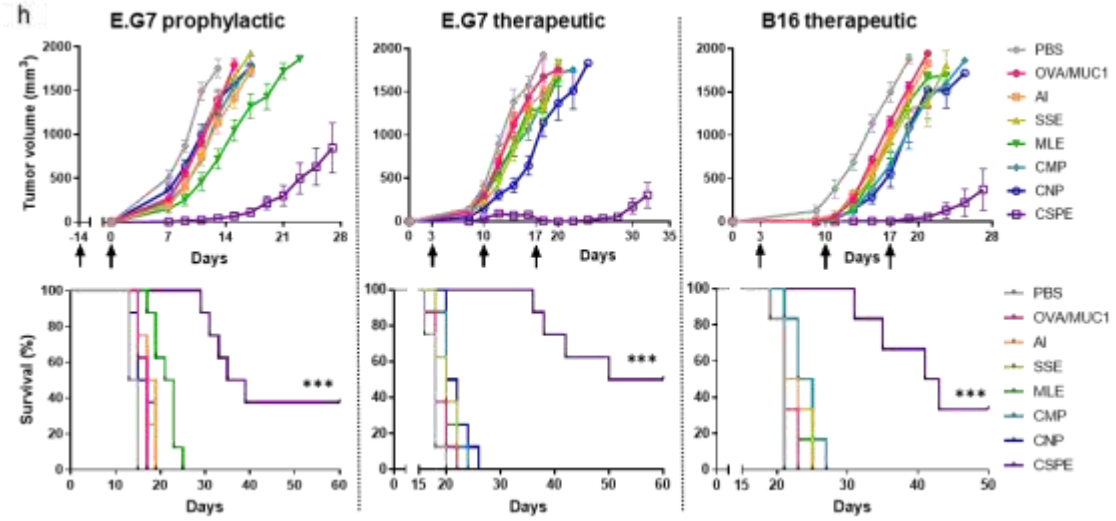


Figure 2.39 CSPE as a tumor vaccine adjuvant to inhibit tumor growth

2.5 Conclusion

In summary, CSPE represents a novel adjuvant stabilized by positively charged chitosan particles, building upon the foundation of PLGA-stabilized Pickering emulsion. Its surfactant-free nature enhances biosafety and provides a suitable environment for simulating pathogen dynamics. Results have demonstrated that CSPE effectively adsorbs antigens and facilitates cellular uptake. A key innovation of CSPE lies in its ability to transition from particles to polymer chain stabilization within the lysosome, leading to rapid lysosomal rupture and consequently higher escape efficiency compared to CNP. When compared to MF59-like emulsion and commercial Al adjuvant, CSPE notably promotes antigen-specific antibody levels, induces a Th1-biased immune response, stimulates the production of IFN-γ-secreting $CD8^+$ T cells, and enhances CTL activity.

Furthermore, through modulation of chitosan deacetylation, emulsions with varying proton content were synthesized, highlighting the possibility of achieving proton accumulation via both particle and amino accumulation. The efficacy of CSPE as an adjuvant was further underscored in select tumor models, where it markedly impeded tumor progression and enhanced mouse survival rates.

In conclusion, CSPE emulsions have been successfully developed, demonstrating efficient and controllable lysosomal escape. Moreover, CSPE shows promising

applications as a therapeutic vaccine adjuvant, highlighting its potential in advancing immunotherapeutic strategies against various diseases, including cancer.

Chapter 3 Application of chitosan and its derivatives as adjuvants for oral vaccines in fish

3.1 Abstract

The significant economic losses incurred by the aquaculture industry due to fish mortality resulting from bacterial and viral infections underscore the urgent need for effective preventive measures. The utilization of fish vaccines and adjuvants presents a promising avenue for safeguarding farmed fish. To streamline the preparation process, optimize adjuvant formulation, and enhance evaluation methodologies, chitosan with a molecular weight of 50 kDa and a concentration of 10 mg/mL was employed. Chitosan particles with uniform particle size were meticulously prepared utilizing the high-pressure homogenization and spray drying technique, tailored for large-scale production. These particles were then loaded with inactivated *Vibrio anguillarum*, subsequently homogenized and emulsified with fish oil to fabricate an oral vaccine. This vaccine was administered to turbot after thorough mixing with feed. To establish an immersion challenge model, the concentration of *Vibrio anguillarum* and immersion duration were meticulously adjusted, resulting in an immersion challenge model with *Vibrio anguillarum* immersed in 10^8 CFU/mL for 12 hours.

In an endeavor to bolster the immunoprotective efficacy of the vaccine, sodium alginate, β-glucan, and other constituents were introduced to formulate a compound adjuvant alongside chitosan and its derivatives. The immunoprotective effects of various compound adjuvants were preliminarily assessed, revealing that the compound formulation comprising chitosan quaternary ammonium salt particles and sodium alginate exhibited heightened expression levels of immune-related cytokines, such as MHC-I. Moreover, turbot survival rates post-challenge experiments were notably augmented. These findings herald a promising development in the realm of oral vaccine adjuvant systems predicated upon chitosan and its derivatives, providing an auspicious avenue for further exploration and refinement.

3.2 Introduction

China, as a prominent player in the global fishery sector, boasts an extensive pond fish farming area exceeding 500,000 hectares and a vast array of fishing nets numbering over 100,000. With the burgeoning development of intensive aquaculture, both the scale of aquaculture operations and stocking densities are poised for steady escalation. Paradoxically, this expansion is accompanied by a concomitant exacerbation of disease-related challenges. The mortality rate among adult fish due to afflictions such as enteritis, gill rot, skin rot, tail rot, and bleeding exceeds 40%, while the survival rate of juveniles in temporary culture languishes at a meager 30%[68]. Historically, disease management has relied heavily on pan-monitoring methodologies entailing the use of chemicals and antibiotics. However, the prolonged and indiscriminate use of antibiotics engenders bacterial resistance, amplifying the risk of drug-resistant strains. Moreover, the pervasive presence of drug residues poses a grave threat to ecological equilibrium[69]. Consequently, there is an urgent imperative to explore novel and efficacious immunoprevention techniques to combat fish diseases. The perturbation of beneficial bacterial populations in aquatic ecosystems and the compromised safety of food fish underscore the pressing need for innovative solutions in this domain[70].

In response to the prevailing challenges, Chinese researchers have undertaken a comprehensive investigation focusing on seawater bass and turbot, two economically significant species. Their research endeavors have revealed Vibrio infection as the primary causative agent of morbidity among bass, a finding with far-reaching implications. Indeed, this disease afflicts not only sea bass but also numerous other saltwater and freshwater fish species, including turbot, salmon, eel, and grouper. Particularly concerning is the rapid proliferation of this pathogen in temperate and subarctic regions, rendering it a formidable threat to farmed fish populations. In fact, Vibrio infection stands as the predominant cause of disease in aquaculture settings across China[71, 72]. Despite fish being categorized as lower vertebrates, they possess relatively intricate immune systems. Studies have elucidated that the kidneys, spleen, thymus, lymphoid tissues within the gastrointestinal tract, as well as the blood and

lymph, serve as pivotal sites for immune responses in fish. Analogous to humans, fish harbor T lymphocytes and B lymphocytes, key lymphocytic populations instrumental in orchestrating immune responses. These findings underscore the physiological parallels between fish and higher vertebrates, highlighting the significance of elucidating immune mechanisms in piscine species for advancing our understanding of immunology and disease management[73, 74].

The identification of pathogens and a deeper understanding of fish immune mechanisms have catalyzed significant advancements in the realm of fish disease prevention and treatment. Leveraging these insights, scientists have devised a novel vaccine through meticulous experimentation and iterative refinement. This vaccine is formulated by isolating the pathogen from afflicted fish and subjecting it to inactivation at elevated temperatures. Upon vaccination of young or adult fish, the body mounts an immune response, generating antibodies targeted against the bacteria. Consequently, when pathogenic bacteria attempt to infiltrate the fish's body via the digestive tract, skin, or mucous membranes, these pre-existing antibodies elicit a robust immune response, thwarting bacterial invasion and averting infections. This innovative vaccine represents a pivotal milestone in mitigating the impact of fish diseases, underscoring the transformative potential of immunoprophylaxis in aquaculture settings[75].

The immune response of fish is intricately influenced by various environmental factors, necessitating a nuanced approach to preventive vaccination. Considerations such as water temperature, seasonal variations, ecological dynamics of the water body, as well as the age and health status of the fish, are paramount in vaccine administration. Three primary methods of vaccination have been delineated to accommodate the diverse needs of aquaculture operations. Firstly, injections are employed for larger fish species possessing higher economic value. This method allows for precise dosing and ensures effective delivery of the vaccine. Additionally, injections can be administered via immersion or by incorporating the vaccine into feed, providing flexibility in application. Secondly, for large-scale vaccination campaigns targeting populations of fish, oral vaccination methods are favored. In this

approach, the vaccine is seamlessly integrated into the fish's diet and administered through feeding. This strategy offers logistical advantages in reaching a broad swath of fish populations efficiently. Compared to conventional antibiotic treatments, fish vaccines confer unparalleled advantages in combating piscine diseases. Notably, they boast a remarkable safety profile, devoid of impurities, and leave no residual traces on fish post-administration. This characteristic not only safeguards the ecological integrity of aquatic environments but also ensures the quality of food fish. Furthermore, the use of fish vaccines mitigates the risk of drug resistance, a pervasive concern associated with prolonged antibiotic use. Thus, fish vaccines represent a pivotal innovation in aquaculture management, heralding a paradigm shift towards sustainable disease control strategies[76].

The adoption of vaccination as a disease prevention strategy has been a longstanding practice in mammals, but its application in fish has progressed more gradually. The earliest documented attempts at fish immunization date back to the late 1930s and early 1940s. In 1938, Sniezko et al. published pioneering research in Polish elucidating immune protection against *Enteroeromonas punctata* in carp (*Cyprinus cario*) [77]. However, the efficacy of this endeavor was hindered by language barriers, impeding widespread dissemination and implementation. Subsequently, in 1942, Duff published the first English-language paper on fish vaccination. His research demonstrated that immersion challenges following prolonged feeding (over 64 days) with bait containing an *Aeromonas salmonicida* vaccine led to reduced mortality in *Salmo clarkia*[78]. A seminal milestone in fisheries vaccine development occurred in 1976 with the registration and manufacturing of the first fisheries vaccine in the United States. This milestone was accompanied by experimental findings showcasing the vaccine's efficacy in preventing intestinal disease in rainbow trout (*Oncorhynchus mykiss*) induced by *Yersinia ruckeri*[79]. In 1986, China achieved a significant breakthrough with the development of the first fisheries vaccine, the inactivated grass carp hemorrhagic disease (GCHD) vaccine[80]. This milestone marked the inception of rapid progress in fisheries vaccine development within China, catalyzing subsequent advancements in the field.

The primary vaccination methods for aquatic vaccines encompass injection immunization, oral immunization, and immersion immunization. Injection immunization stands as the predominant approach, ensuring precise dosing and eliciting robust humoral and cellular immune responses in fish. This method yields varying levels of immune protection, with rates ranging from 50% (via intramuscular injection)[81] to potentially achieving full protection (via intraperitoneal injection)[82]. However, injection immunization presents inherent limitations. It is impractical for fry weighing less than 20 grams, labor-intensive, incurring high labor costs, and induces stress in fish. Moreover, side effects such as growth disorders, localized fibrous peritonitis, granulomas, and darkening at the injection site may occur[83]. Conversely, oral immunization involves delivering vaccines to the gastrointestinal tract of fish through feeding or oral administration, triggering an immune response. This method offers several advantages, including ease of administration, stress avoidance, and suitability for fish of all sizes. Nonetheless, oral immunization does not guarantee precise vaccine dosing, and the vaccine is susceptible to degradation by digestive enzymes in the stomach and foregut, compromising its immunogenicity. Immersion immunization represents the simplest and most direct method of fish immunization. Fish are exposed to a diluted inoculum solution, allowing the antigen to be absorbed through the gills, skin, lateral line, and intestine, thereby stimulating the mucosal immune system. This approach bypasses the need for individual dosing and circumvents issues related to vaccine degradation in the digestive tract[84].

Oral vaccines for fisheries offer compelling advantages, including cost-effectiveness, ease of administration, and suitability for aquatic animals of varying sizes. However, their efficacy is compromised by susceptibility to degradation by gastric acid and digestive enzymes in the gastrointestinal tract, leading to diminished immunogenicity and suboptimal protection rates. Consequently, the central challenge in the development of oral vaccines lies in devising strategies to deliver them safely and efficiently to the target site within the body. This entails ensuring that the vaccines evade degradation and remain intact for an extended duration, thereby facilitating the induction of robust immune responses and augmenting immunity.

Addressing this challenge necessitates innovative approaches to enhance vaccine stability and delivery. Researchers are exploring various encapsulation techniques, such as microencapsulation and nanoparticle-based delivery systems, to shield vaccines from degradation and facilitate their targeted delivery to the intestine. Additionally, advancements in bioadhesive formulations and mucosal adjuvants aim to prolong the residence time of vaccines in the gastrointestinal tract, enhancing their immunogenicity and efficacy. By surmounting the hurdles associated with vaccine degradation and delivery, researchers aim to unlock the full potential of oral vaccines for fisheries, paving the way for improved disease control and enhanced immunity in aquatic species[85].

Currently, two primary categories of vaccine delivery systems have garnered significant research attention: chemical carrier delivery systems and biological carrier delivery systems. Chemical carrier delivery systems encompass both natural and synthetic polymers. Among natural polymers, prominent examples include alginate, chitosan, and liposomes. Alginate, derived from seaweed, and chitosan, sourced from crustacean shells, are biocompatible and biodegradable polymers. Liposomes, vesicles composed of lipid bilayers, offer versatile encapsulation capabilities for vaccine delivery. Synthetic polymers, such as poly (lactic acid) hydroxyglycolic acid (PLGA) and poly (ethylene glycol) polylactic acid (PELA) copolymers[86], afford precise control over composition and properties, facilitating tailored vaccine delivery systems. In contrast, biological carrier delivery systems utilize living organisms as vectors for vaccine delivery. These vectors encompass a diverse array of species, including bacteria, fungi, insects, and plants. These diverse vaccine delivery systems hold immense promise for enhancing vaccine efficacy, enabling targeted delivery, and overcoming challenges associated with oral vaccine administration in aquatic species. Continued research and innovation in this field are pivotal for advancing the development of effective oral vaccines for fisheries and aquaculture.

Vibrio anguillarum poses a significant threat to various fish species, leading to hemorrhagic septicemia and substantial economic losses in mariculture[87]. Turbot (*Scophthalmus maximus*), a prized species in aquaculture, is particularly susceptible

to *Vibrio anguillarum* infection[88]. Vaccination represents a cornerstone in the arsenal of protective measures for farmed fish, with an attenuated *Vibrio anguillarum* vaccine having secured veterinary medicine certifications[89]. While some polyvalent vaccines offer protection against multiple strains of pathogens, adjuvant systems play a crucial role in enhancing the efficacy of attenuated vaccines. In aquaculture settings, oral and immersion vaccines are favored for their efficiency in time and labor[90]. Oral vaccines can be seamlessly incorporated into fish feed, facilitating precise dosing and observation, thus cementing their status as indispensable tools in fish vaccination programs[91].

Chitosan, a prominent cationic polysaccharide derived from natural chitin, boasts an array of favorable attributes, including abundance, affordability, biodegradability, non-toxicity, biocompatibility, and notable antifungal and antitumor properties. These qualities have catalyzed its widespread adoption across diverse biomedical applications and fields, ranging from tissue engineering to various biological delivery systems[92]. Chitosan has been used as an adjuvant in large number of studies. Notably, chitosan's positive charge enables the formation of mucosal adhesion bonds with negatively charged groups on epithelial cell surfaces[93]. This mucosal adhesion, coupled with its intestinal immunomodulatory functions, renders chitosan an ideal candidate for the development of oral vaccine delivery systems[94]. Moreover, the amino and hydroxyl groups of chitosan lend themselves readily to chemical modification, yielding numerous derivatives that afford diverse formulation types capable of influencing bioactivity[95]. This versatility underscores chitosan's pivotal role in advancing the frontier of oral vaccine delivery and underscores its potential as a multifaceted tool in biomedical research and application.

The evaluation of vaccine efficacy necessitates the use of appropriate challenge animal models. While the injection challenge model offers high reproducibility and manipulability, the fish immersion model provides a more realistic simulation of infection within a natural water environment, thereby better reflecting the true protective effect of the vaccine[96]. Nonetheless, the immersion challenge model presents numerous manipulative variables, posing challenges in directly applying

established parameters. As such, there is a critical need to develop immersion challenge models tailored to specific fish species, strains, and culture environments. By customizing these models to suit the unique characteristics of the target species and their rearing conditions, researchers can enhance the relevance and reliability of vaccine efficacy assessments in real-world aquaculture settings. This tailored approach not only facilitates more accurate evaluations of vaccine performance but also fosters advancements in disease control strategies for the aquaculture industry.

The primary objective of this research endeavor is to pioneer the development of an oral fish vaccine optimized for mass production and practical implementation within aquaculture contexts. This investigative study commences with an exhaustive screening process aimed at identifying optimal formulations of chitosan and its derivatives. Through meticulous examination, we seek to elucidate the key factors influencing chitosan formulations and to identify formulation methodologies conducive to large-scale production. Subsequently, we intend to assess the efficacy of the selected chitosan formulations utilizing an immersion-challenged fish model. This model will serve as a pivotal tool for evaluating the immunoprotective properties of chitosan when employed as an adjuvant. Additionally, we endeavor to incorporate various complementary ingredients suitable for oral delivery systems to fabricate compound adjuvants in conjunction with chitosan. Through systematic experimentation, we aim to discern synergistic compound combinations that exhibit enhanced adjuvant effects, thus establishing a robust foundation for subsequent optimization endeavors and in-depth investigations into the mechanisms underlying immune enhancement. In essence, this scholarly pursuit represents a concerted effort towards advancing the frontier of aquatic vaccine development, with the overarching goal of enhancing disease resistance and promoting the sustainable growth of aquaculture industries.

3.3 Experimental details

The methodologies outlined in this protocol have received official approval from the Institute of Process Engineering, Chinese Academy of Sciences. Furthermore, all

animal experimentation procedures strictly adhere to the Regulations for the Care and Use of Laboratory Animals, as well as the Guideline for Ethical Review of Animal Research (China, GB/T35892-2018). These stringent ethical and regulatory frameworks ensure the humane treatment of animals involved in our study, underscoring our unwavering commitment to upholding the highest standards of research integrity and animal welfare.

3.3.1 Fish, strain and attenuation

Fish

The experimental turbots, purchased from Yantai Oriental Ocean Science and Technology Co., Ltd. in Yantai City, Shandong Province, possessed an average body weight of 80 g and body length of 12 cm. Prior to experimentation, these turbots were temporarily accommodated in a cement pool measuring 7 m × 7 m × 1 m. Their dietary regimen comprised three daily feedings, administered at a rate ranging from 1.2% to 1.5% of the fish's body weight. To maintain optimal aquatic conditions, water renewal occurred following each feeding cycle, with approximately two-thirds of the pool volume replaced. The culture water temperature was meticulously regulated within the range of 10–20℃ to ensure favorable environmental conditions for the turbots.

Preceding the commencement of experiments, a subset of 3–5 fish was randomly selected for bacterial isolation procedures. Subsequent examination of the liver, spleen, and kidney tissues confirmed the absence of detectable bacterial presence, thereby validating the suitability of these fish for subsequent experimental procedures.

Strain

Vibrio anguillarum MVM425 was a gift from East China University of Science and Technology (Shanghai, China). It was incubated in Luria-Bertani medium adjusted with 20 g/L sucrose, 1 g/L NH_4Cl, 25 g/L NaCl and 0.4 mmol/L Fe^{3+} at 28 ℃ with a shaking rate of 150 r/min. The micronutrient concentrate contains: H_3BO_3 0.2g/L, $ZnSO_4 \cdot 7H_2O$ 0.5g/L, $MnSO_4$ 0.8g/L, $CoCl_2 \cdot 6H_2O$ 0.4g/L, $CaCl_2$ 0.5g/L, $CuSO_4 \cdot 5H_2O$ 0.5g/L, $FeSO_4 \cdot 7H_2O$ 0.4g/L, $AlCl_3 \cdot 6H_2O$ 0.2g/L,

$NiCl_2 \cdot 6H_2O$ 0.2g/L.

Attenuation

Pick 3~5 single colonies from TSA plate after activation and inoculate them into 5 ml Tryptic Soy Broth (TSB) tubes, incubate at 28℃, 150 r/min shaking bed overnight to get the seed solution, and then transfer the seed solution to 200 ml TSB (containing 3% glucose) at 1% inoculum and incubate at 28℃, 150 r/min shaking bed. The 24 h culture product was collected, and 37% formalin solution was added to the culture product to a final formaldehyde concentration of 0.2% (V/V) and inactivated at 28 ℃, 150 r/min. The inactivated bacteria at 12 h and 24 h were planted into Tryptic Soy Agar (TSA, Solarbio T8650) plates and Tryptone Soy Broth (TSB, Solarbio LA0110) tubes, respectively. After no viable bacteria were detected, complete inactivation was determined. The inactivated bacterial solution was centrifuged at 6000 r/min for 10 min at 4℃, the bacteria were collected, washed twice with PBS buffer (0.2 mol/L, pH=7.6), and then the bacteria were suspended with PBS buffer containing 0.1% formaldehyde, and the inactivated *Vibrio anguillarum* MVM425 was obtained as the antigen of the vaccine, which was stored at 4℃ for future use.

3.3.2 Preparation of chitosan particles and antigen loading

Chitosan of different molecular weights and 80%–90% deacetylation were purchased from Xi'an Tianbao Bio-Tech., Ltd. (Xi'an, China). Sodium alginate and β-glucan were purchased from PuriPharm Co., Ltd. (Huzhou, China).

N-[(2-Hydroxy-3-trimethylammonio) propyl] chitosan chloride (HTCC) was synthesized with chitosan and (2,3-Epoxypropyl) trimethylammonium chloride (ETA) by our laboratory. Briefly, adding isopropanol 15 mL and distilled water 45 mL to 4.83 g chitosan and 0.06 mol ETA (the mol ratio of ETA to amino groups of chitosan was 4:1) in a water bath at 85 ℃ under magnetic stirring for 5 h. After the reaction was completed, ice acetone was added to make the product precipitate from the solution, and the product is allowed to settle and then pump-filtered. The product was washed with a mixed solution of acetone and ethanol (4:1, v/v) to obtain chitosan

quaternary ammonium salt (HTCC). The product was dried at 60 ℃ for 6 h in an infrared electric heating drying oven and then prepared for use.

Chitosan nanoparticles (CSNP) were prepared by high pressure homogenization. Chitosan with a molecular weight of 50 kDa was dissolved with acetic acid, and then the pH value of the solution was adjusted to around 6.8 by adding NaOH dropwise. This was followed by pre-homogenization (3000 rpm, 10 min) and the resulting product was then subjected to high-pressure homogenization (10,000 psi).

N-[(2-Hydroxy-3-trimethylammonio) propyl] chitosan chloride nanoparticles (HTCCNP) were prepared by adding α, β-glycerophosphate (α, β-GP, Sigma) to HTCC drop by drop while stirring at 4 ℃ for 10 min and followed by homogenization. Finally, the product was spray dried. The inlet temperature was 110°C, the outlet temperature was 70°C, and the pressure was 0.2 MPa.

The antigen was loaded by gently mixing of positively charged CSNP or HTCCNP and negatively charged attenuated *Vibrio anguillarum* MVM425 for several hours.

3.3.3 Characterization of chitosan particles

The hydrodynamic size and zeta potential of CSNP were measured by Nano Zeta Sizer (Malvern). To describe the size and zeta potential of CSNP, dilute 10 μL of CSNP in 1 mL of deionized water and transfer the diluent solution to a DTS0012 cell. Turn on the computer and dynamic light scattering (DLS) analyzer, then insert the cell with sample into the DLS system. To begin the operation, launch the Zeta Size Software and generate a new measurement file. Then, select parameters item by item under the entry for particle size. Last, begin the determination procedure to acquire the particle size distribution. To determine the Zeta potential, transfer the diluted sample to a DTS1070 cell, change the parameters of zeta potential option, then start the measurement.

Surface morphology of particles was observed by scanning electron microscope (SEM, JEOL). To observe the morphology of CSNP, 0.1 mL of CSNP aqueous dispersion was diluted 10 times and evenly applied to a 5 cm × 5 cm aluminum foil,

and the water was allowed to evaporate naturally overnight in a well-ventilated fume hood. A small portion of the aluminum foil with the air-dried sample, approximately 5 mm × 5 mm, was cut and fixed to the sample stage with conductive tape. Spray the sample surface with gold at 10 mA for 120 seconds. The surface morphology of the samples was then observed using a scanning electron microscope (SEM).

3.3.4 Preparation and characterization of compound formulations

In order to achieve better oral delivery, we added some other components to the chitosan adjuvant, such as sodium alginate, β-glucan, etc. Sodium alginate and β-glucan are both negatively charged so that they could be electrostatic adsorbed by cationic chitosan. β-glucan was dissolved in deionized water and adjusted to a concentration of 20 mg/mL; sodium alginate was dissolved in deionized water to a concentration of 10 mg/mL. Spray-dried CSNP or HTCCNP was coated with the β-glucan and sodium alginate solution for 2 h and spray dried at the end.

The success of plating is mainly judged by the change of particle potentials. After coating, the particles sample was dispersed in water and diluted to 1 mg/mL, and 1 mL of diluted sample was added into the DTS1070 cell for the Zeta potential measurement by Nano Zeta Sizer (Malvern). A decrease in the potential of the coated particles compared to the uncoated particles indicates successful coating.

3.3.5 Oral immunization

Newly imported turbots were immediately randomly sampled for health checks. Confirmed healthy turbot could be used for subsequent experiments after one week of domestication. It was administered orally at a feeding rate of 2% (m/m) of body weight for 5 days, followed by 5 days interval and another 5 days for a total oral administration of 10 days. The vaccine dosage at 5% (v/m) mixing ratio resulted in an attenuated *Vibrio anguillarum* dose of 1.35×10^9 CFU/fish.

3.3.6 RT-PCR analysis

28 days after the date of the first immunization, 5 turbot in each group were randomly selected and dissected to obtain intestines. After tissue grinding, total RNA

was extracted using TRIzol (Thermo) and reverse transcribed into cDNA using TaKaRa Reverse Transcription Kit, and then mRNA expression levels of immune-related cytokines were detected by RT-PCR.

Total RNA was extracted as follows. Take 50 mg of turbot intestine, kidney and spleen tissues for homogenization, add 600 μL of TRIZOL lysate, mix upside down, then add 200 μL of chloroform, shake and mix for 5 sec on a mixer, centrifuge at 12000 rpm for 15 min at 4°C. Pipette 500 μL of the supernatant, add 400 μL of isopropanol, and leave it at -20°C for 30 min. centrifuge at 12000 rpm for 15 min. pour off the supernatant; then add 600 μL of 75% ethanol to wash the precipitate, centrifuge at 12000 rpm for 5 min and gently pour off the supernatant. Centrifuge at 12000 rpm for 15 min and pour off the supernatant; then add 600 μL of 75% ethanol, wash the precipitate, centrifuge at 12000 rpm for 5 min and gently pour off the supernatant. The RNA precipitate was dried at room temperature for 3 min, then 20 μL of water was added and mixed gently to dissolve the RNA on the wall of the tube. centrifugation was performed at 2,000 rpm for 5 sec and stored on ice. RNA samples were processed to remove genomic DNA contamination using the Turbo DNA-free Kit (Ambion, USA). The RNA OD_{260}/OD_{280} of turbot samples was determined to be 2.0 by a nucleic acid analyzer, and the RNA concentration was adjusted to 100 ng/μL.

1.0 μg of total RNA from each tissue was reverse transcribed to cDNA using Superscript First Strand Synthesis System (Invitrogen, USA). reaction system: 10 μL fluorescent dye, 0.2 μmol/L primer (forward/reverse), 1.0 μL cDNA template (10 ng/μL), ddH_2O was made up to 20 μL. The reaction program: 95 ℃, 10 min; 95 ℃, 10 s, 60 ℃, 30 s, 72 ℃, 25 s for 40 cycles. Melting curve analysis was performed at the end of 40 cycles to determine the specificity of PCR products. Three replicate experiments were set up for all samples. In the gene expression analysis, β-actin was used as an internal reference gene, and the fold change in the expression level relative to that in the control was calculated according to the $2^{-\Delta\Delta CT}$ method.

3.3.7 *Vibrio anguillarum* challenge

The growth curve was plotted by measuring the optimal density (OD) value at

600 nm of the bacterial solution at different time points using a spectrophotometer. 28 days after first immunization, *Vibrio anguillarum* challenge was performed by injection or immersion. *Vibrio anguillarum* intraperitoneal challenge dose is 5×10^6 CFU/fish, the immersion dose varied from 10^7–10^8 CFU/fish. For the immersion challenge, *Vibrio anguillarum* was collected by centrifugation, quantified by OD value, adjusted to a certain concentration and mixed with artificial seawater, and the immunized turbot was transferred to culture tanks containing *Vibrio anguillarum*, and after a period of time, it was transferred back to the normal rearing environment.

Relative percent survival (RPS) was calculated for both experiments using the following formula: RPS = [1 - (the percent of mortality of vaccinated individuals/the percent mortality of controls)] × 100.

3.4 Results

3.4.1 Preparation and characterization of chitosan particles

To identify a suitable adjuvant for scaling up fish oral vaccines, chitosan's molecular weight and concentration underwent meticulous screening. Initially, chitosan was solubilized to a final concentration of 30 mg/mL. As the molecular weight increased, the dissolution time of chitosan prolonged, accompanied by an escalation in solution viscosity. Notably, at a molecular weight of 600 kDa, the chitosan solution exhibited markedly diminished fluidity (Figure 3.1).

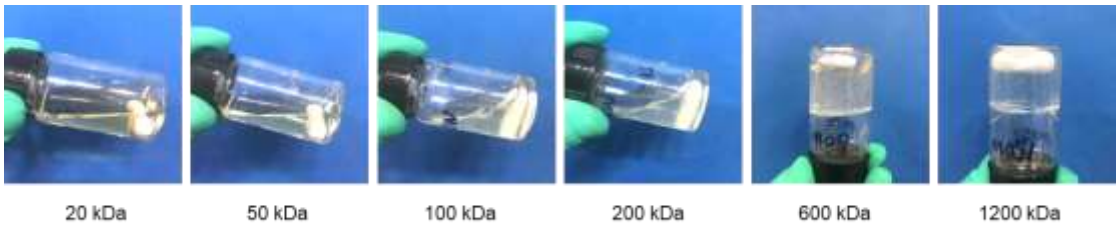


Figure 3.1 Chitosan solutions of different molecular weights

Subsequently, chitosan particles following spray drying were subjected to scanning electron microscopy (SEM) analysis. Lower molecular weight chitosan particles demonstrated reduced strength and increased folding, while higher molecular

weight counterparts exhibited heightened inter-particle adhesion (Figure 3.2).

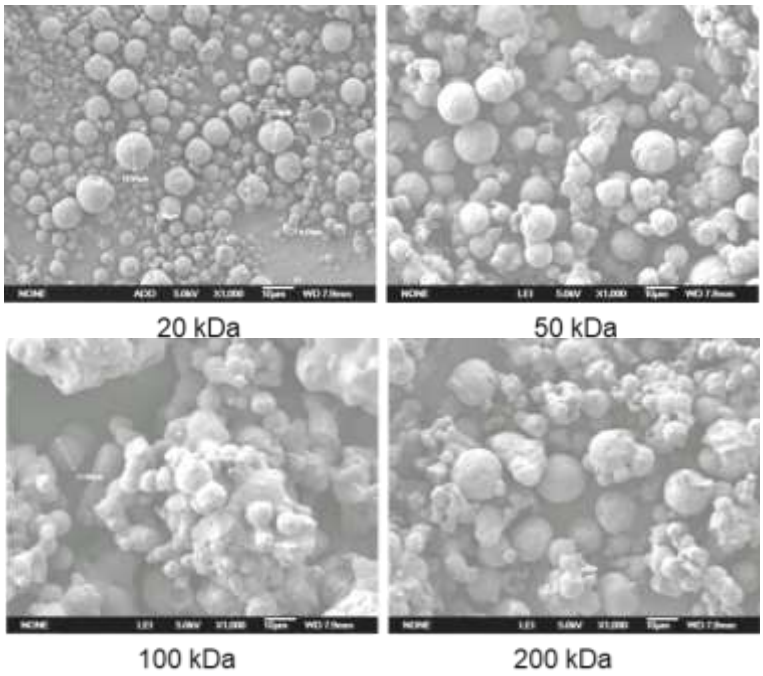


Figure 3.2 SEM images of particles prepared from chitosan of different molecular weights

Moreover, particle wrinkling gradually improved with escalating chitosan concentration, as evidenced by investigations utilizing chitosan particles prepared with a 50 kDa molecular weight (Figure 3.3).

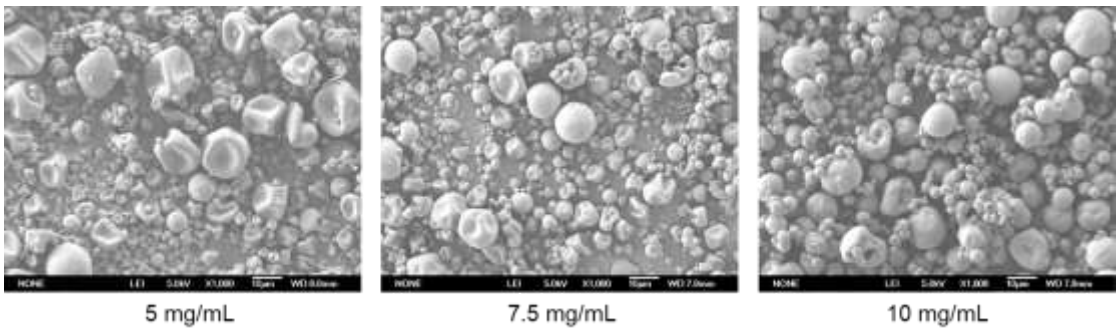


Figure 3.3 SEM images of particles prepared from chitosan of concentrations

Following comprehensive analyses, chitosan possessing a molecular weight of 50 kDa, coupled with a concentration of 10 mg/mL, was deemed optimal for subsequent experiments owing to its lower viscosity and desirable characteristics. Subsequently, N-[(2-hydroxy-3-trimethylammonio) propyl] chitosan chloride nanoparticles (HTCCNP) underwent a series of processing steps, including high pressure

homogenization and spray drying. The resultant HTCCNP exhibited an average size of 220.5 ± 1.2 nm, with a polydispersity index (PDI) of 0.177 ± 0.015, indicative of uniform particle size and favorable dispersity (Figure 3.4).

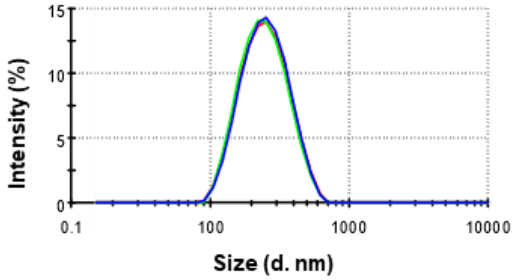


Figure 3.4 Size distribution of HTCCNP

Subsequent experimentation involved the co-mixing of CSNP or HTCCNP with attenuated *Vibrio anguillarum* MVM425. Notably, CSNP possessed a potential of 17.5 ± 0.7 mV, whereas the bacterium exhibited a potential of -5.18 ± 1.12 mV. The electrostatic adsorption facilitated the loading of antigens into CSNP, resulting in a potential of 13.6 ± 0.9 mV post co-mixing; the adsorption of antigens onto HTCCNP leads to a reduction in the electric potential from point 22.9 ± 3.4 mV to 19.8 ± 1.4 mV (Figure 3.5), thus affirming the successful encapsulation of antigens within chitosan particles.

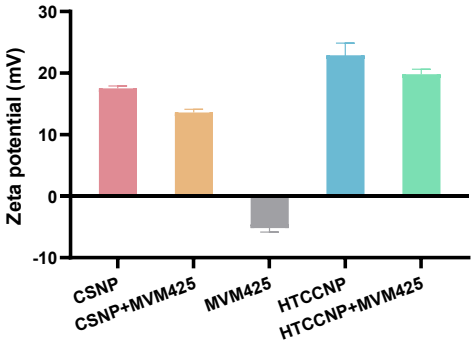


Figure 3.5 Potential changes in CSNP and HTCCNP after adsorption of *Vibrio anguillarum*

SEM images obtained post-spray drying of chitosan particles blended with antigens showcased homogeneous mixing and excellent homogeneity in the final product (Figure 3.6).

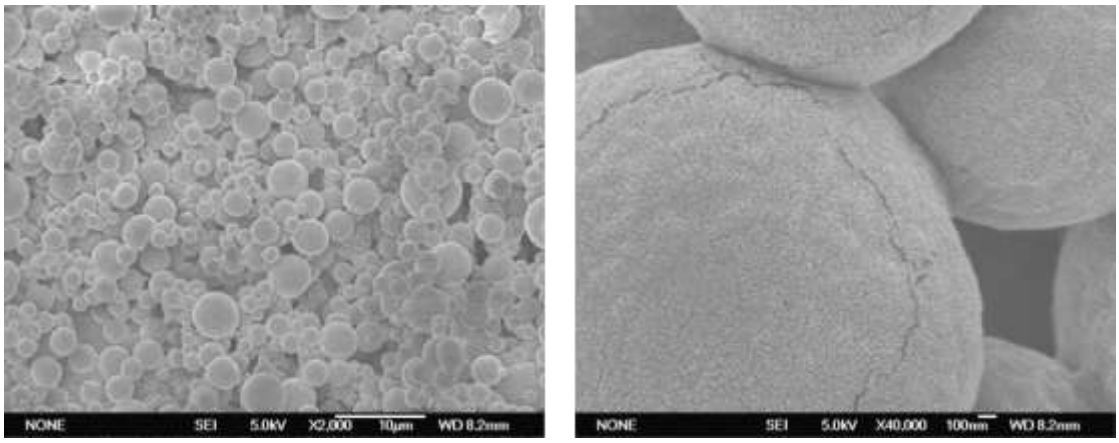


Figure 3.6 SEM images of HTCCNP after adsorption of *Vibrio anguillarum*

3.4.2 Preparation of oral vaccine and validation of the chitosan effect

To enhance the uptake of the oral vaccine, we formulated CSNP emulsions through homogenization, with chitosan particles adsorbed with attenuated *Vibrio anguillarum* MVM425 serving as the aqueous phase, and fish oil as the oil phase (Figure 3.7).

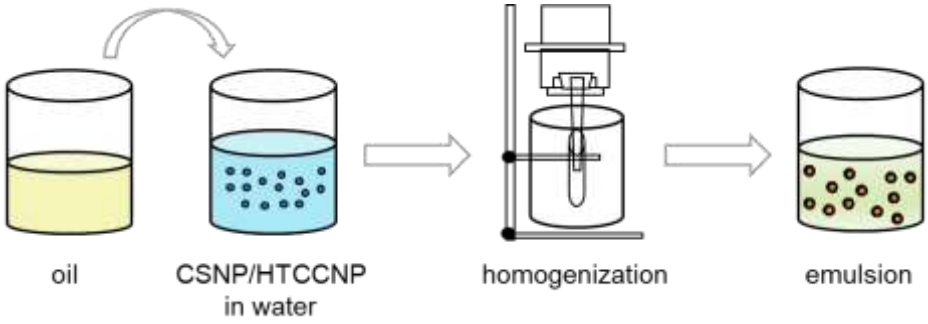


Figure 3.7 Vaccine preparation process

The resulting emulsion exhibited a milky white appearance and demonstrated excellent homogeneity when examined under an optical microscope (Figure 3.8). Prior to administration, the oral vaccine was gently stirred and thoroughly mixed with the fish feed. Remarkably, aside from a noticeable increase in humidity, no significant alterations were observed in the mixed feed (Figure 3.9).

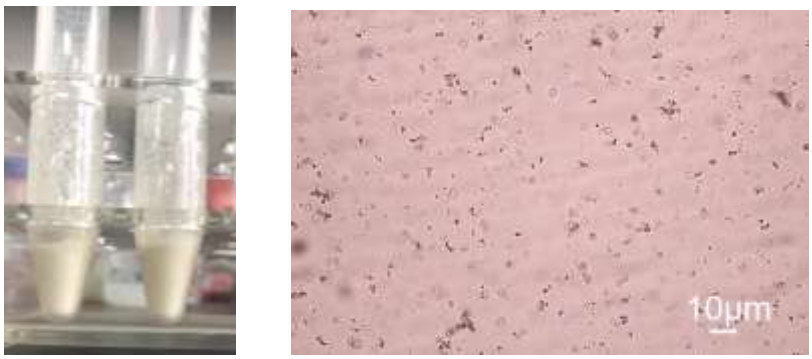


Figure 3.8 Appearance and microscopic images of the vaccine

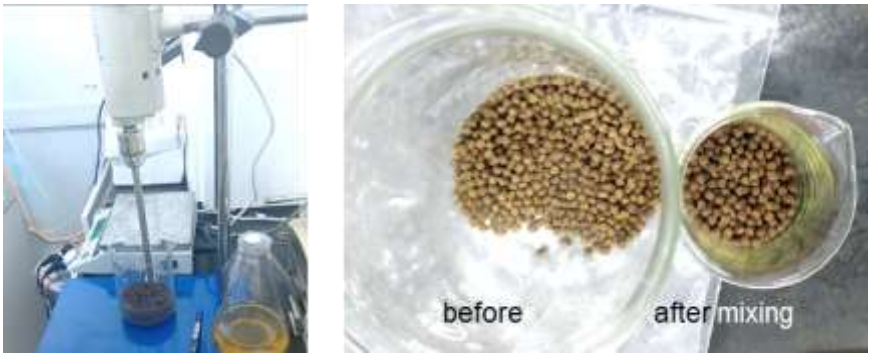


Figure 3.9 Mixing of vaccine and feed

An initial oral immunization pre-test was conducted to evaluate the efficacy of the chitosan adjuvant. Utilizing a well-refined injection challenge fish model, 160 turbots were randomly allocated into 4 groups (n=40), receiving a 5-day administration followed by a 5-day cessation period, and subsequent secondary immunization for 5 days (Figure 3.10), with an antigen dose of 1.35×10^9 CFU per fish. The experimental groups comprised the chitosan particle group (MVM425+CSNP), the CS solution group (MVM425+CS), and the antigen emulsion group (MVM425+fish oil). Intraperitoneal challenge with *Vibrio anguillarum* MVM425 was conducted 28 days post-initial administration, followed by a 10-day observation period recording turbot mortality.

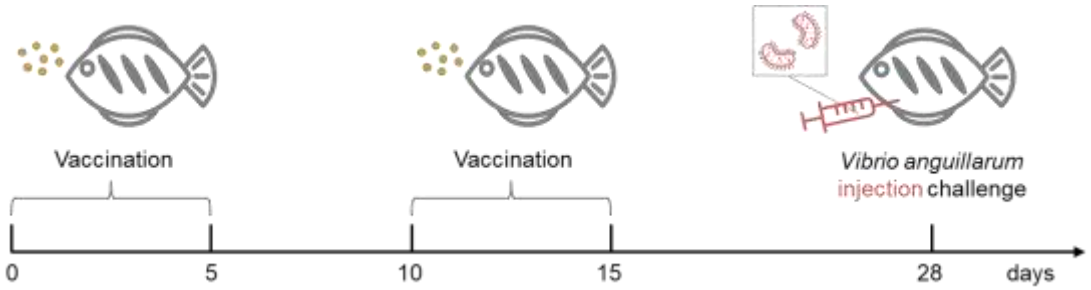


Figure 3.10 Immunization Procedure for Pre-experimentation

During the observation period, the cumulative mortality rate of the control group reached 91.7%, validating the efficacy of the challenge experiment. Comparative analysis revealed that attenuated bacteria as antigens induced modest protective responses. The cumulative mortality trend observed in the MVM425+CS and MVM425+CSNP groups exhibited similarities, with slightly lower mortality rates recorded in the CSNP particle group compared to the CS solution group. Notably, the relative percent survival (RPS) of both groups exceeded 40% (Figure 3.11). These findings preliminarily suggest that irrespective of its formulation, chitosan serves as an effective immune booster. Further investigation is warranted to ascertain whether the particle form of chitosan confers enhanced and more stable immune-boosting effects.

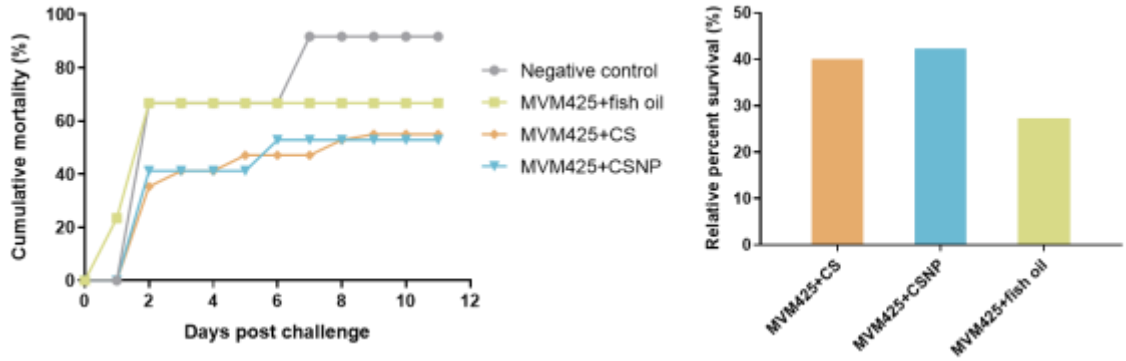


Figure 3.11 Protective effect of chitosan oral vaccine

3.4.3 Exploration of immersion challenge model

To establish a suitable immersion challenge modeling method, our focus

primarily centered on determining the concentration of *Vibrio anguillarum* and the immersion duration. Due to limitations in laboratory culture conditions, the daily production of *Vibrio anguillarum* MVM425 was constrained. Following the exclusion of bacterial media and pH-related effects on turbots, we primarily investigated the influence of immersion duration on challenge modeling.

Initially, we delineated the growth curves of two volumes of MVM425 in the laboratory culture environment. *Vibrio anguillarum* MVM425 exhibited logarithmic growth at 7–8 hours with heightened activity, presenting an opportune time point for expansion of cultivation or fish challenge post-collection. Notably, the volume of culture exerted minimal influence on growth patterns, with higher culture volumes not necessarily correlating with enhanced bacterial growth (Figure 3.12).

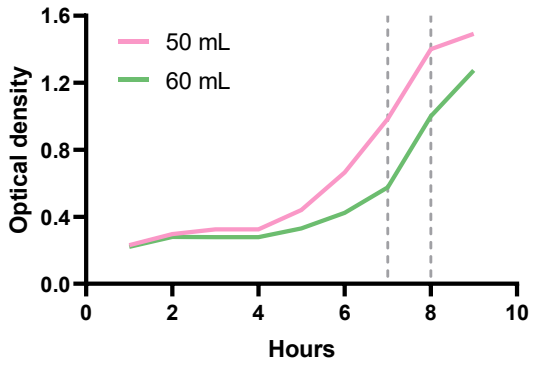


Figure 3.12 Growth curves of *Vibrio anguillarum* MVM425

Our first attempt involved immersing turbots in a high concentration of *Vibrio anguillarum* MVM425 (approximately 10^8 CFU/mL) during the logarithmic growth stage for a brief period, followed by transfer to a normal rearing environment. Interestingly, all turbots immersed for 2.5 hours survived after one week, whereas those immersed for 4.5 hours experienced mortality on the first day, with a subsequent survival rate drop to 20% after 6 days (Figure 3.13).

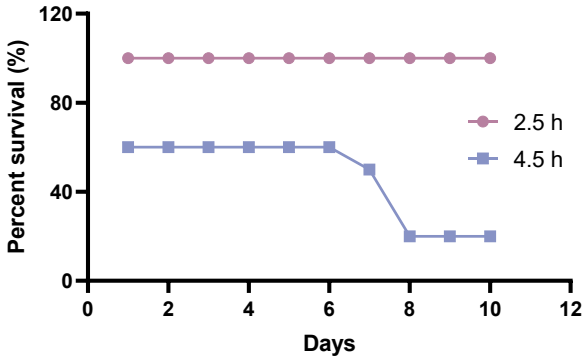


Figure 3.13 Survival of high concentration (10^8 CFU/mL) immersion challenge model

Subsequently, we explored a medium-concentration immersion (approximately 5×10^7 CFU/mL). In this scenario, the 4.5-hour group exhibited mortality onset after 6 days, eventually stabilizing at a high survival rate of 70%, while the 7-day group experienced mortality between day 4 and day 6 in all instances (Figure 3.14).

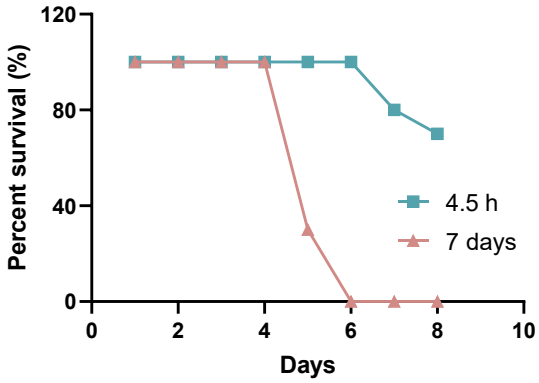


Figure 3.14 Survival of medium concentration (5×10^7 CFU/mL) immersion challenge model

Drawing upon the insights gained from the aforementioned immersion challenges, we experimented with both high concentration (approximately 10^8 CFU/mL) immersion for a short duration and low concentration (approximately 10^7 CFU/mL) immersion for an extended duration. The 7-day group displayed mortality initiation on the fifth day, with final mortality stabilizing at 40%, whereas the 12-hour group witnessed 80% mortality on the second day, culminating in a final lethality rate of 90% (Figure 3.15). Ultimately, the high concentration immersion for 12 hours met the requisite criteria of 60% to 90% lethality for the challenge model, with a moderate

modeling duration, thereby warranting the adoption of this immersion challenge model.

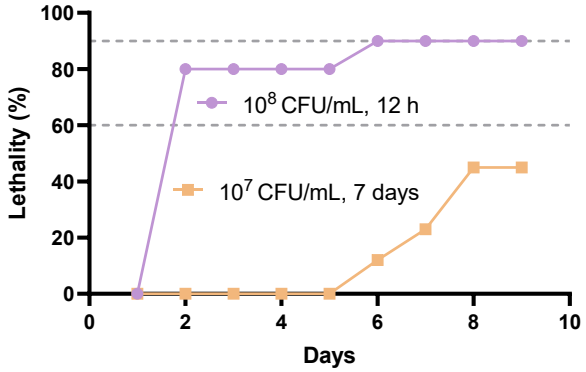


Figure 3.15 Comparison of the lethality rates between the high concentration short-time immersion and low concentration long-time immersion challenge models

Vibrio anguillarum infection elicits internal hemorrhaging in fish and is highly lethal. Under normal conditions, turbot typically exhibit a blackish-gray body surface adorned with evenly distributed spots, coupled with healthy internal organs. Conversely, turbot afflicted with *Vibrio anguillarum* infection display distinct alterations in appearance and internal physiology. Specifically, the body surface of infected turbot may exhibit partial fading to white, indicative of compromised health. Moreover, the internal organs, particularly the intestines, assume a reddish hue, consistent with the manifestation of hemorrhagic septicemia attributed to *Vibrio anguillarum* infection (Figure 3.16).

Figure 3.16 Comparative photographs of the skin and viscera of normal and *Vibrio anguillarum*-infected turbot

3.4.4 Investigation of the efficacy of chitosan-complexed adjuvant

In the pursuit of practical application for complex fish vaccines, reliance solely on chitosan as a vaccine adjuvant may present challenges. To address this limitation and enhance adjuvant efficacy while improving palatability, additional components were incorporated into the formulation. Alongside chitosan, N-[(2-Hydroxy-3-trimethylammonio) propyl] chitosan chloride (HTCC), a chitosan derivative boasting quaternary ammonium groups, was selected for adjuvant preparation due to its heightened antimicrobial activity and water solubility.

The immunization protocol for the oral vaccine entailed feeding turbot a mixture of vaccine-infused feed for 5 consecutive days, followed by a transition to normal feed for 5 days. Subsequently, the second immunization phase involved continued administration of the vaccine mixture for an additional 5 days, totaling 10 days of oral vaccine administration. Four weeks post-initial vaccine administration, a *Vibrio anguillarum* MVM425 immersion challenge was executed following the optimized method described in section 3.4.3 (Figure 3.17).

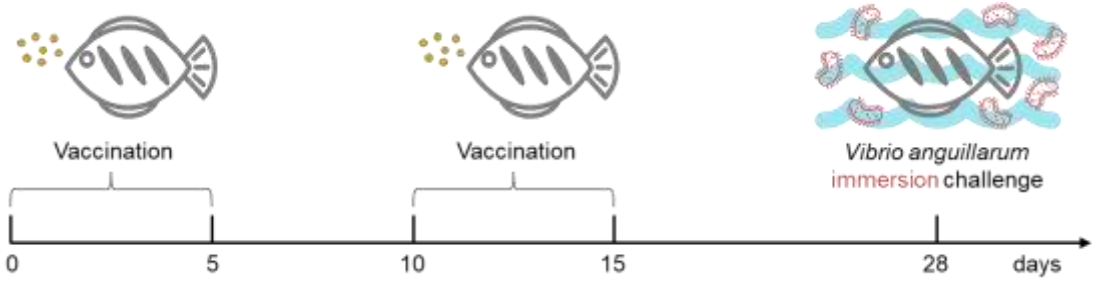


Figure 3.17 Comparative photographs of the skin and viscera of normal and *Vibrio*

On day 28, turbots were randomly selected from each group prior to the *Vibrio anguillarum* challenge, sacrificed, and intestinal RNA was extracted to detect the expression of immune-related factors via fluorescence quantitative PCR. Post-*Vibrio anguillarum* immersion challenge, survival of the remaining turbots in each group was observed and recorded.

The overall expression of cytokines in the HTCCNP group surpassed that of the regular chitosan adjuvant group, potentially attributed to HTCC's higher positive charge and water solubility, facilitating better antigen loading compared to chitosan

alone. Additionally, the addition of dextran, which directly impacts intestinal cells, likely contributed to this observation. The HTCCNP group supplemented with sodium alginate and calcium exhibited elevated expression of antigen presentation-related factors MHC-1 and MHC-2, particularly MHC-1, along with heightened expression of TNF-α and IL-12 (Figure 3.18). This suggests that the combination of HTCC and sodium alginate facilitates antigen cross-presentation, activates cytotoxic T cell-dominated immune processes, and promotes macrophage and NK cell proliferation and cytotoxicity. However, variations in cytokine expression among individuals necessitate further optimization of experimental methods, including increased sample size and other approaches.

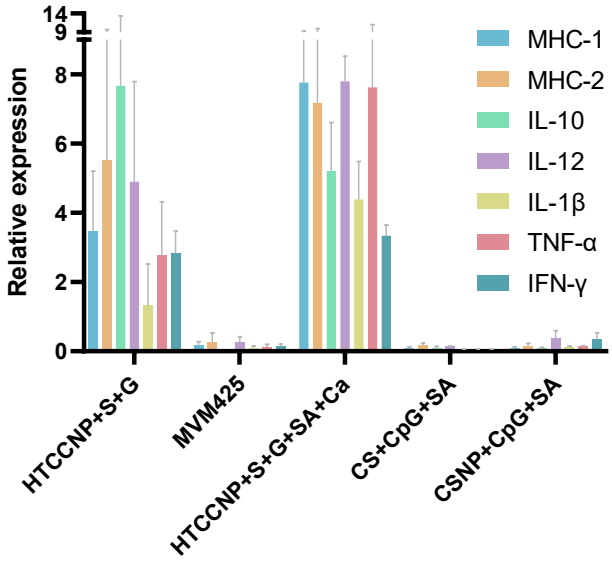


Figure 3.18 Relative expression of cytokines after two weeks of immunization

Over the 12 days following the *Vibrio anguillarum* MVM425 immersion challenge, all turbots in the negative control group succumbed to mortality the subsequent day, indicating potential over-application of strains in this challenge. Notably, the chitosan solution (CS) group exhibited lower immunoprotective efficacy compared to other groups. Interestingly, the HTCCNP+sucrose+glucan group demonstrated low mortality in the initial days, yet final protection was comparable to that of the CSNP group. The HTCC group supplemented with sodium alginate exhibited the most favorable outcomes, with approximately 30% of turbots ultimately

surviving the *Vibrio anguillarum* challenge (Figure 3.19). This underscores the potential superiority of the SA and HTCC combination for oral vaccines within the chitosan adjuvant system.

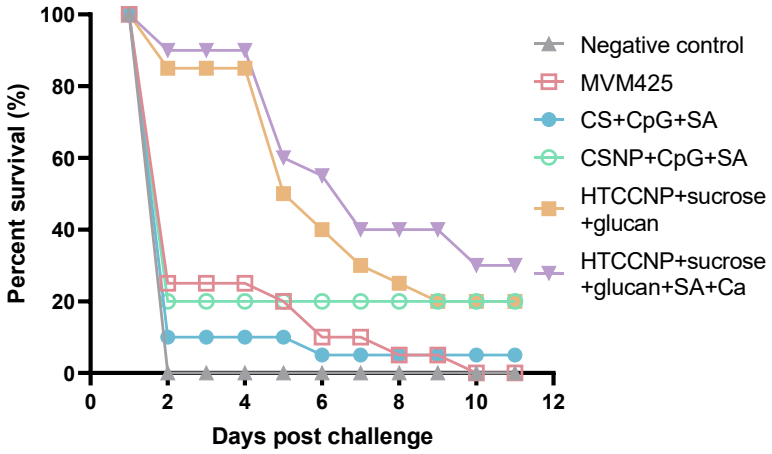


Figure 3.19 Survival of turbot after *Vibrio anguillarum* immersion challenge

3.5 Conclusion

In the aquaculture industry, oral vaccines represent a convenient method for immunizing fish against pathogenic threats, thereby mitigating economic losses. Incorporating adjuvants into these vaccines reduces antigen requirements while enhancing immunoprotection. In our study, geared towards large-scale vaccine production, we meticulously optimized each step, from preparation processes to adjuvant formulation, to ensure suitability for industrial production.

Initially, chitosan particles with optimal molecular weights were screened based on particle morphology and workability. Preparation methods such as high-pressure homogenization and spray drying were adopted for easy scale-up, resulting in chitosan particles characterized by uniform sizes, robust mechanical strengths, minimal batch-to-batch variations, and efficient antigen loading. These chitosan particles were then emulsified with fish oil and incorporated into fish feed for administration to turbot. To simulate real aquaculture conditions, we explored a condition-adapted immersion challenge model, starting from *Vibrio anguillarum* concentration and immersion time.

Subsequently, to enhance adjuvant potency and palatability, we introduced other

components suitable for oral delivery to be combined with chitosan and its derivatives, forming a complex adjuvant. Evaluation via the immersion challenge model revealed that the combination of chitosan derivatives, HTCC, and SA effectively promoted the expression of cytokines such as MHC-I and IL-12, activating turbot adaptive immune responses and improving survival rates.

Despite the anticipated immunostimulatory effect, the addition of CpG ODN did not yield the expected results, possibly due to oligonucleotide degradation hindering intestinal delivery. HTCC, a chitosan derivative, demonstrated superior immunoprotection, likely attributed to its higher positive charge and water solubility, facilitating intestinal delivery. Additionally, β-glucan, an immune enhancer, may play a role, although further verification is required. Sodium alginate coating of chitosan particles was found to mitigate vaccine degradation in the stomach, improving intestinal delivery efficiency. These compounds are commonly used in oral delivery to prolong drug or antigen action.

Our study lays the foundation for large-scale production of fish oral vaccines based on chitosan and its derivatives, establishing an immersion challenge evaluation system, and demonstrating the effectiveness of HTCC and SA complex adjuvants. Future endeavors will involve investigating additional compound adjuvants, exploring the effects of different component ratios, optimizing industrial vaccine production processes, and developing efficient universal fish vaccine adjuvants suitable for multivalent vaccines against various pathogens.

Chapter 4 Concluding remarks

In this thesis, we have explored the development and application of vaccines and adjuvants, focusing on the pivotal role of adjuvants in enhancing immune responses and overcoming challenges in their development. Chapter 1 provides an overview of vaccine and adjuvant evolution, emphasizing the significance of adjuvants and highlighting chitosan's potential in biomedicine, particularly as a promising adjuvant candidate for vaccines.

In Chapter 2, we introduced a novel formulation termed CSPE (Chitosan Stabilized Pickering Emulsion), a surfactant-free emulsion with enhanced biological safety and pathogen dynamics simulation capabilities. Our findings demonstrate CSPE's efficacy in antigen adsorption and cellular uptake, with lysosomal disruption facilitating efficient escape. Immunological studies reveal CSPE's ability to significantly elevate antibody levels, induce Th1-biased immune responses, and promote the production of IFN-γ-secreting CD8+ T cells and CTL cells, surpassing traditional adjuvants like MF59 and aluminum. Furthermore, by modulating chitosan's degree of deacetylation, we constructed emulsions with varying amino group content, underscoring the importance of both particles and amino groups in proton accumulation. In tumor models, CSPE exhibits promising adjuvant effects, inhibiting tumor growth and enhancing mouse survival, whether for prevention or treatment purposes.

Chapter 3 details the development of an oral fish vaccine adjuvant conducive to scale-up production. We meticulously screened chitosan for optimal molecular weights and formulated concentrations suitable for large-scale preparation, employing high-pressure homogenization and spray-drying to yield chitosan adjuvant particles with excellent stability and homogeneity. Following preliminary validation of its efficacy as an oral vaccine adjuvant, we established a *Vibrio anguillarum* immersion challenge model tailored to our needs. Subsequently, we introduced additional ingredients to synergize with chitosan, forming compound adjuvants. Preliminary

comparisons of various compound preparations as oral fish vaccine adjuvants underscored their immunoprotective effects.

Collectively, this thesis contributes to advancing the field of vaccine adjuvant development, showcasing the potential of chitosan-based formulations and emphasizing their efficacy in enhancing immune responses, both in traditional vaccination and oral vaccine settings.

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