



DEVELOPMENT OF CHITOSAN-BASED CHITOSAN NANOPARTICLES FOR
INTRANASAL VACCINATION AND DISSOLVING MICRONEEDLES FOR
INTRADERMAL VACCINATION OF JAPANESE ENCEPHALITIS



By
MR. Ekachai DUMKLIANG

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL TECHNOLOGY
(INTERNATIONAL PROGRAM)

Department of PHARMACEUTICAL TECHNOLOGY
Graduate School, Silpakorn University
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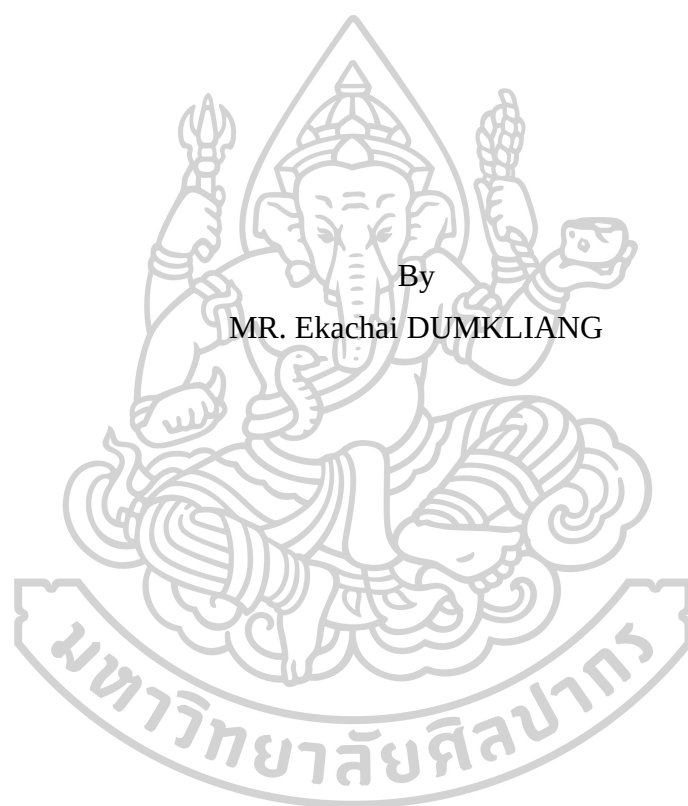
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MR. EKACHAI DUMKLIANG : DEVELOPMENT OF CHITOSAN-BASED CHITOSAN NANOPARTICLES FOR INTRANASAL VACCINATION AND DISSOLVING MICRONEEDLES FOR INTRADERMAL VACCINATION OF JAPANESE ENCEPHALITIS THESIS ADVISOR : PROFESSOR PRANEET OPANASOPIT, Ph.D.

In this study, chitosan-based nanoparticles (CS-NPs) for intranasal (IN) immunization and chitosan-based MNs patch (CS-MNs patch) for intradermal (ID) immunization, non-invasive alternative approaches for Japanese encephalitis (JE) vaccination were developed to improve the immune responses of the live attenuated Japanese encephalitis-chimeric virus vaccine (JE-CV), a commercial JE vaccine, compared with traditional subcutaneous (SC) immunization. This work, therefore, demonstrates the formulations, characterization, and efficacies of using JE-CV-loaded CS-NPs for IN immunization and JE-CV-loaded sCS-MNs patches for ID immunization. For IN immunization study, positive charges CS-NPs at the mass ratio of vaccine antigen: NPs of 6:1 with an incubation time of 1 h at 4°C were successfully developed with high loading efficiency (LE) >85% and loading capacity (LC) $>1.2 \times 10^4$ PFU/mg. FITC-BSA-loaded NPs exhibited superior mucoadhesive and uptake properties on *ex vivo* nasal swine mucosa, compared to antigen alone without NPs. Mice immunized with JE-CV at 1×10^4 PFU (full dose vaccine) via IN immunization stimulated systemic and mucosal immune responses in mice, while SC immunization only stimulated systemic immune responses but lack of mucosal immune responses. IN immunization elicited PRNT₅₀ at seroprotection level (PRNT₅₀ titers ≥ 10 , as recommended by WHO) similar to SC immunization with JE-CV solution. In the case of IFN- γ , a major cell-mediated immune response for preventing flavivirus amplification, the results revealed that IN immunization of JE-CV in mice with CS-NPs produced more IFN- γ than that of the JE-CV solution. Moreover, IN immunization did not induce adverse events when observed body weight change in mice. In the case of ID immunization, the blended polymer of NaHCO₃:CS:polyvinyl alcohol (PVA) (0.25:1:4) was used for the CS-MNs patch fabrication and successfully developed with an elegant appearance, able to resist sufficient compression forces with good ability for skin insertion. In addition, JE-CV was loaded into a CS-MNs patch to obtain the optimal mass ratio of 1:4. The JE-CV-loaded CS-MNs patch was then successfully prepared using a cellulose membrane as an MN base to achieve a separating CS-based MNs patch (sCS-MNs patch). The morphology, mechanical strength, and penetration properties of the JE-CV loaded sCS-MNs patch were evaluated and showed desirable results with the detachable property. The percentages of LE and LC of the JE-CV-loaded sCS-MNs patch were approximately 79% and 1.2×10^4 PFU/g, respectively. For safety, after applying MNs, the skin appeared the microholes and naturally resealed and completely recovered at 48 h without skin infection after the MNs patches removal. ID immunization in mice with a half-dose of JE-CV-loaded sCS-MNs patch (5×10^3 PFU/mouse) effectively elicited PRNT₅₀ and cell-mediated immune responses (CMIR) similar to full dose vaccination via SC. Therefore, immunization with JE-CV-loaded CS-NPs via IN and JE-CV-loaded sCS-

MNs patch via ID could be promising approaches for effective immunization.



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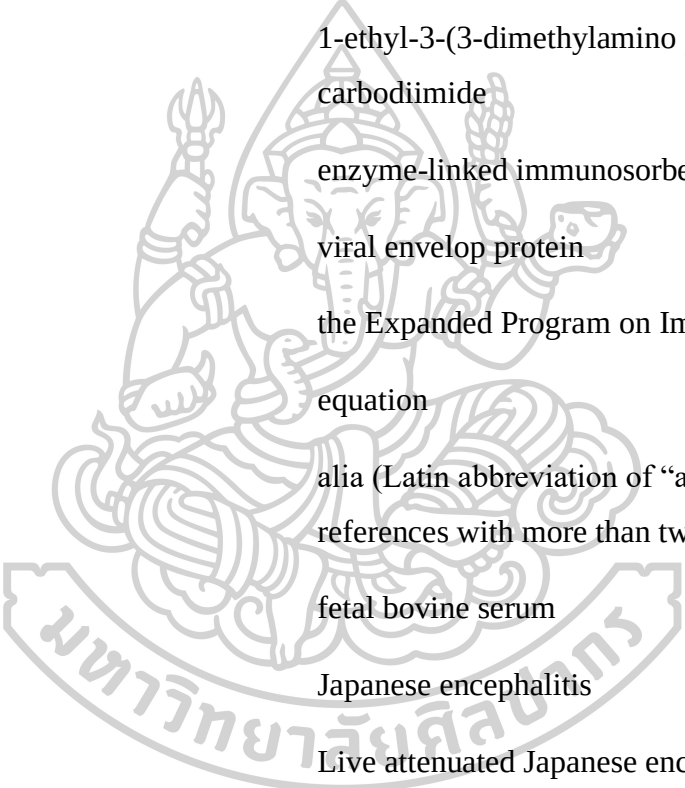
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LIST OF ABBREVIATIONS



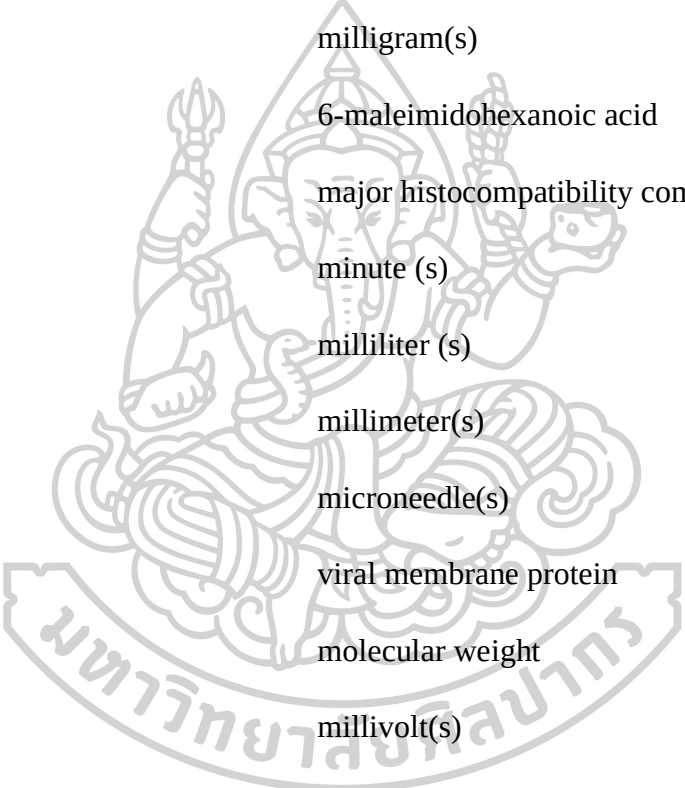
ACK lysing buffer	ammonium chloride potassium lysing buffer
AEs	adverse events
ANOVA	analysis of variance
anti-JEV	antibody against Japanese encephalitis virus
APCs	antigen presenting cells
BSA	bovine serum albumin
Cl	calcium chloride
CLSM	confocal laser scanning microscope
CNS	central nervous system
CM	chitosan maleimide
cm ²	square centimeter(s)
cm ³	cubic centimeter(s)
CMC	carboxymethyl cellulose
CMIR	Cell-mediated immune response
CM-NPs	chitosan maleimide nanoparticles
CS	chitosan
CS-NPs	chitosan nanoparticles
CS-MNs	chitosan-microneedles
CS-based MNs patch	chitosan-microneedles patch
CO ₂	carbon dioxide
C protein	viral capsid protein



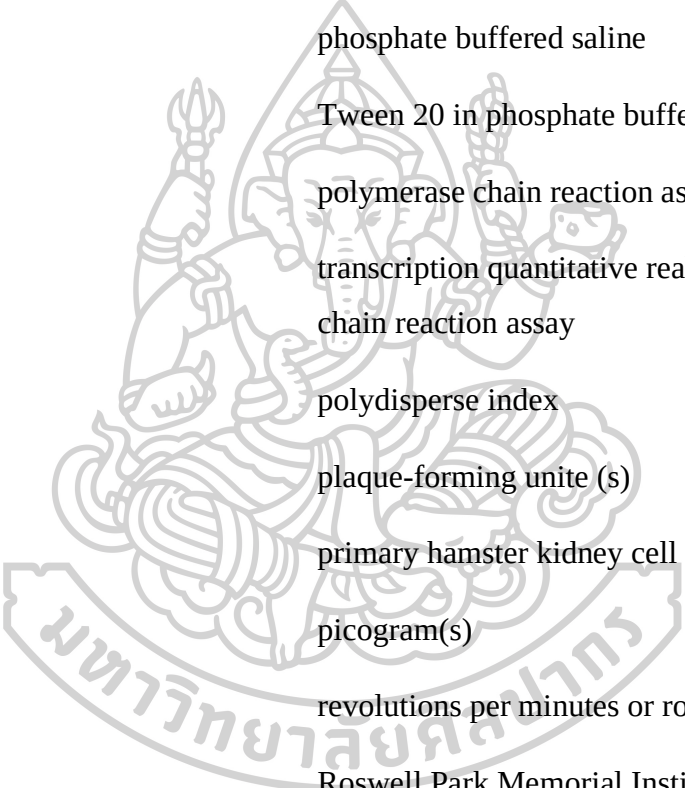
CTL	cytolytic T lymphocyte (s)
Da	Dalton (s)
DE	delivery efficiency
DI water	deionized water
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
EDAC	1-ethyl-3-(3-dimethylamino propyl) carbodiimide
ELISA	enzyme-linked immunosorbent assay
E protein	viral envelop protein
EPI	the Expanded Program on Immunization
Eq.	equation
et al.	alia (Latin abbreviation of “and others”; For references with more than two authors)
FBS	fetal bovine serum
JE	Japanese encephalitis
JE-CV	Live attenuated Japanese encephalitis – chimeric virus vaccine
JE-CV-loaded sCS-MNs patch	Live attenuated Japanese encephalitis – chimeric virus vaccine loaded separating chitosan-based microneedles patch
JEV	Japanese encephalitis virus
FI	fluorescence intensity
FITC-BSA	fluorescein conjugated bovine serum albumin



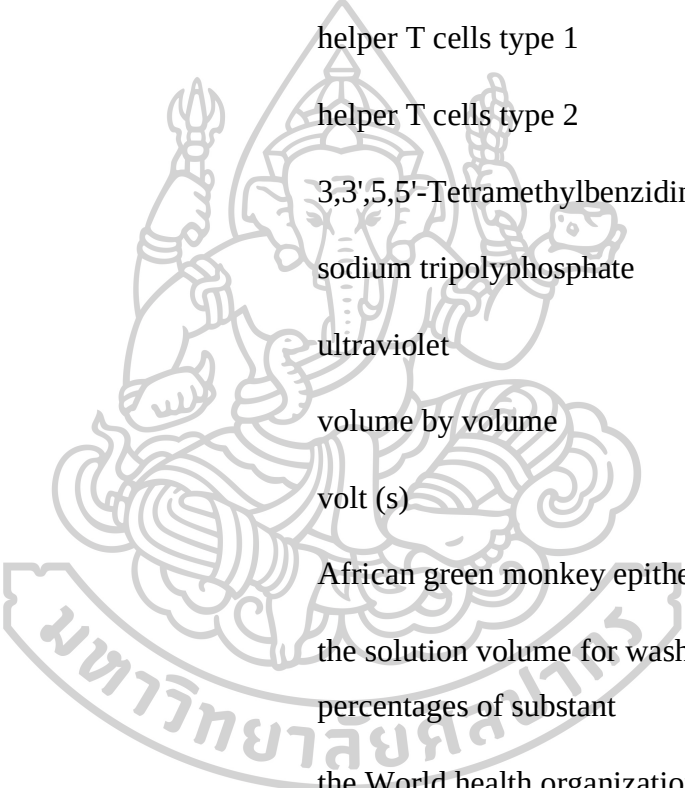
FTIR	Fourier transform infrared spectroscopy
h	hour(s)
H ₂ SO ₄	sulfuric acid
HCl	hydrochloric acid
HIR	Humoral immune response
HRP	horseradish peroxidase
ICH	international council on harmonisation
ID	intradermal
i.e.	id est (Latin abbreviation); that is
IFN- γ	interferon-gamma
IgA	immunoglobulin A
IgG	immunoglobulin G
IL-4	interleukin-4
IM	intramuscular
IN	intranasal
J	steady-state flux
JE	Japanese encephalitis
JE-CV	Japanese encephalitis chimeric virus vaccine
JEV	Japanese encephalitis vaccine
KCl	potassium chloride
kb	kilobase (s)
kDa	kilodalton (s)
kg	kilogram (s)
L	liter (s)



LC	loading capacity
LE	loading efficiency
log P	log partition-coefficient
LLC-MK2 cell	Rhesus monkey kidney epithelial cell
mBar(s)	millibar(s)
M-cell(s)	microfold cell(s)
mg	milligram(s)
MHA	6-maleimidohexanoic acid
MHC	major histocompatibility complex
min	minute (s)
mL	milliliter (s)
mm	millimeter(s)
MN	microneedle(s)
M protein	viral membrane protein
MW	molecular weight
mV	millivolt(s)
N	newton
NaCl	sodium chloride
NALT	nasal-associated lymphoid tissue
NaHCO ₃	sodium bicarbonate
NaOH	sodium hydroxide
ng	nanogram(s)
NHS	N-hydroxysulfosuccinimide

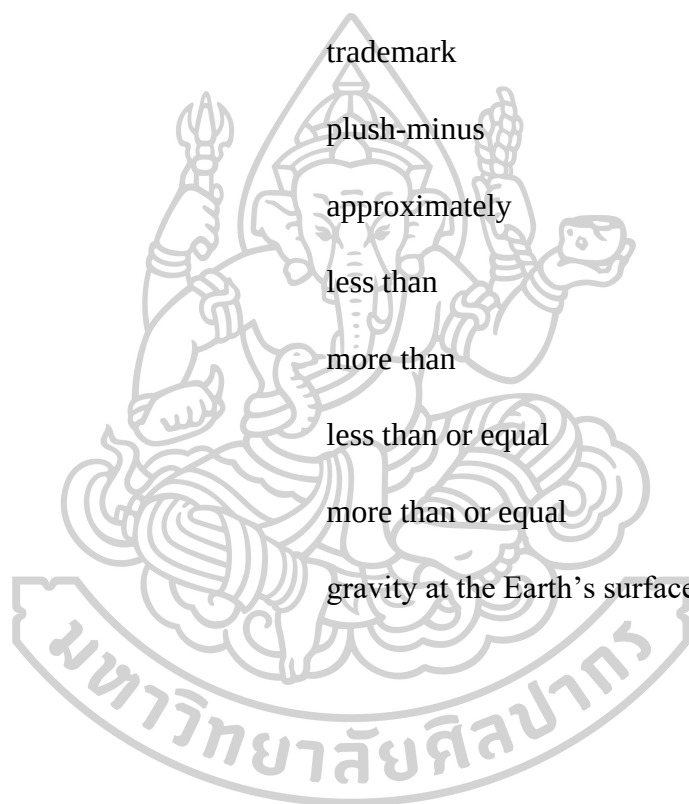


nm	nanometer(s)
NMR	nuclear magnetic resonance spectroscopy
NP(s)	nanoparticle (s)
NT	neutralization titer (s)
OD	optical density
OVA	ovalbumin
PBS	phosphate buffered saline
PBS-T	Tween 20 in phosphate buffered saline
PCR	polymerase chain reaction assay
RT-qPCR	transcription quantitative real-time polymerase chain reaction assay
PDI	polydisperse index
PFU	plaque-forming unite (s)
PHK cell	primary hamster kidney cell
pg	picogram(s)
rpm	revolutions per minutes or rounds per min
RPMI-1640	Roswell Park Memorial Institute-1640 medium
PRNT ₅₀	50% of plaque reduction neutralization titer(s)
PVA	poly (vinyl alcohol)
PVP	polyvinylpyrrolide
RNA	ribonucleic acid
RT-qPCR	reverse transcription-quantitative real-time polymerase chain reaction
s	second



SC	subcutaneous
sCS-MN(s)	separating chitosan-microneedle(s)
SD	standard deviation
SEM	scanning electron microscope
SNF	simulated nasal fluid
TEM	transmission electron microscope
Th ₁	helper T cells type 1
Th ₂	helper T cells type 2
TMB	3,3',5,5'-Tetramethylbenzidine
TPP	sodium tripolyphosphate
UV	ultraviolet
v/v	volume by volume
V	volt (s)
Vero cell	African green monkey epithelial cell
WO ₅₀	the solution volume for washing out 50 percentages of substant
WHO	the World health organization
w/w	weight by weight
3D	Three-dimensional
µg	microgram(s)
µL	microliter(s)
µm	micrometer(s)
µmol	micromole(s)
µs	microsecond

%	percentage (s)
γ	gamma
λ	lambda
°	degree angle
°C	degree Celsius
®	registered trademark
TM	trademark
$\pm$	plus-minus
$\sim$	approximately
$<$	less than
$>$	more than
$\leq$	less than or equal
$\geq$	more than or equal
$\times g$	gravity at the Earth's surface



CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the research problem

Japanese encephalitis virus (JEV) is a cause of Japanese encephalitis (JE), a common viral encephalitis virus throughout the country in Asia-Pacific regions. Due to no specific treatment, successful strategic preventions with human immunization are essential. According to the Expanded Program on Immunization recommended by the World Health Organization (WHO), all children in many endemic countries undergo JE immunization, including Thailand [1, 2].

Several types of JE vaccines have been used to control JE in an endemic area and protect the visitor from the non-endemic site, such as inactivated vaccines from mouse brain or cell cultures and live attenuated vaccines JEV or chimeric virus. Live attenuated Japanese encephalitis chimeric virus vaccine (JE-CV) is a new type of JE vaccine. In many clinical studies, it showed the highest efficiency and safety compared to others. After the parenteral administration via subcutaneous (SC) or intramuscular (IM) injection, the JE vaccines successfully improve the humoral immune responses (HIR) via IgG as a serum antibody. WHO suggests that neutralizing antibody function of IgG at 50% plaque reduction neutralization titer (PRNT_{50}) ≥ 10 titers is a suitable protection level against JEV. In contrast, a cytokine as a cell-mediated immune response (CMIR) is unclear the protective relationship [1]. The typical limitations of parenteral immunization, such as high costs, low compliance, and harmful infection risk, have also been reported [3, 4]. Consequently, novel vaccine delivery technologies and administration routes were developed to improve patient compliance. It should be non-invasive and overcome the disadvantages of parenteral immunization as described. Using microneedles (MNs) patches technique by intradermal (ID) immunization and nanoparticles (NPs) via intranasal (IN) immunization are non-invasive with self-administration. They were studied both in preclinical and clinical studies. Several studies explain their efficient

protection as well as conventional parenteral vaccines [5, 6]. Therefore, their delivery platforms are interested in JE immunization.

For IN immunization, the inductive sites are the nasal-associated lymphoid tissue (NALT), which contains several immunological cells such as antigen-presenting cells (APCs), T- and B- lymphocytes. NALT is covered with microfold cells (M-cells) antigen sampling that prefers to take up NPs rather than microparticles or solutions. It transports the antigen from the nasal lumen to the underlying mucosa and leads to a mucosal immune response via secretory IgA (IgA) as HIR in the oronasal site. Moreover, APCs' function causes a systemic immune response via HIR and CMIR, such as IL-4 and IFN- γ cytokines. However, the significant limitation of IN immunization is poor absorption due to rapid mucociliary clearance (half-life 15-20 min). Therefore, the critical issue for effective IN immunization is the antigen-retention time at the inductive site, which enables its uptake by M-cells and the interaction with the lymphatic system [3, 7].

Chitosan-based NPs (CS-based NPs), the NPs which are prepared from chitosan (CS) and its derivatives, are among the most attractive for IN delivery systems because of their safety, biocompatibility, mucoadhesion, and M-cell uptake enhancement. They show mucoadhesion property due to electrostatic interactions between the amine group's positive charge in the CS and the negative charge of sialic acid (N-acetylneuraminic acids) in mucin on the mucosa tissue. However, the major drawback of CS (pKa $\sim$ 6.5) is insoluble at physiological pH. Therefore, CS derivatives such as trimethyl chitosan and glycol chitosan have been synthesized to improve their solubility, enhance their mucoadhesive properties, and increase the adjuvant effect [8]. Chitosan maleimide (CM), a new derivative of CS, can react with the thiol group of mucin via a Michael reaction. It shows strong mucoadhesion via the covalent bond and does not depend on pH [9]. However, there is no report of using CM for IN delivery system; this leads to our curiosity resulting in this study.

Intradermal (ID) vaccination is a method to increase the effectiveness of vaccines because the skin contains a high concentration of APCs, such as dermal dendritic cells and Langerhans cells, in the viable epidermis and dermis layer, known to participate in vaccine-induced immune responses. However, the stratum corneum (the dead epidermis layer) as a superior skin layer makes it a significant barrier for antigenic

compounds (usually high molecular weight >500 Da with hydrophilic property) to penetrate the viable epidermis and dermis layer for immunization [6]. A conventional ID immunization for tuberculosis vaccine and rabies vaccine, the Mantoux technique, is pierced through the stratum corneum and directly inserted into the dermis as an inductive site. However, it is a difficult technique to inject vaccines precisely into the ID tissue and requires trained health professionals to ensure safety and efficacy [10]. To overcome these limitations, the use of MNs as an ID vaccination has been developed as an alternative approach to traditional ID immunization. MNs device comprises micron-sized needles with a typical 25 to 1,000 μm length that are long enough to penetrate the upper dermis but do not penetrate through the deeper dermis layer where plenty of nerve ending is located. The MNs are beneficial because they are minimally invasive, pain-free, reduce the risk of infections, and are easy to use. In addition, it can induce strong immune responses at both systemic and mucosal sites with painless in the case they comprise an antigen [11].

MNs are classified into five types based on patterns of the delivery system, including hollow, solid, coated, dissolving, and hydrogel-forming MNs [12]. Hollow MNs contain a hollow bore similar to hypodermic needles; however, the size of hollow MN is much smaller than that of hypodermic needles. Hollow MN permits the pressure-driven flow of a liquid formulation through microconduits created by the needles. For solid MNs, needles create tiny holes in the skin before vaccine application. High vaccine wastage and variability efficiency of solid MNs lead to developing the coated MNs. The vaccine is mixed with a polymer and coated on solid MNs. Coated MN might reduce the administration step compared to solid MNs; however, the amount of drug coated on the needles is limited. Dissolving MNs are fabricated from soluble/biodegradable material in which mixed with the vaccine. Once the application, the needles are dissolved/degraded for vaccine release when in contact with skin interstitial fluid. It increases vaccine amount with efficient accuracy when compared with solid and coated MNs. Moreover, it does not create needle waste that prevents contamination to others and reduces the cost of biohazard sharp waste management, unlike previous MNs types. Hydrogel-forming MNs, new type MNs, are fabricated from hydrogel-forming polymer. After insertion into the skin, interstitial fluid diffuses through MNs to the backing layer of the vaccine, then swells and allows

vaccine diffusion into the skin. However, hydrogel-forming MNs have low delivery efficiency for macromolecule delivery [13]. Among the various types of MNs, dissolving MNs have been widely utilized for the transdermal delivery of various drugs due to their high biocompatibility and functionality. Micromolding technique is typically used to produce dissolving MNs because of its simplicity and low cost. Dissolving MNs can be fabricated from biodegradable or biocompatible polymers, such as CS, polyvinyl alcohol (PVA), and methylvinylether-co-maleicanhydride (Gantrez[®], Ashland corporation, UK) [12, 14]. CS-based MNs have many advantages, such as protecting antigen degradation, facilitating antigen uptake, and adjuvant properties. However, CS required increasing in a network polymer density to improve the mechanical strength of MNs. For example, CS concentration should be increased to 10%w/v or combined with PVA before molding [15, 16]. Another limitation of CS-MNs is that entirely CS-MNs patch is slowly dissolved in the skin at least four days after application, leading to uncomfortable wearing [15]. To decrease wearing time, increasing the dissolution rate with porous forming in a degradable polymer, such as adding NaHCO₃ in the fabricated polymer, would be a benefit [17]. Additionally, separating MNs patch was developed by separating CS needles tip and baseplate of MNs. Once skin insertion, CS baseplate detaches and separates from the tip of the needles. CS needles were then embedded in the skin and released antigens for immunization [18]. In the case of MNs for JE vaccine, Dean et al. (2005) reported that using hollow MNs for JE-CV immunization in monkeys induced PRNT₅₀ 7 times more than that of SC injection [19].

This study aims to develop CS-based NPs for IN vaccination and separating CS-based MNs (sCS-MNs) patch for ID vaccination to improve the immune responses of the JE-CV vaccine, the highest efficiency type of JE vaccine, compared with the conventional SC. For this purpose, CS-NPs and CM-NPs loading with JE-CV were prepared and physically characterized in particle size, zeta potential, mucoadhesive, and model antigen uptake properties. Moreover, the systemic immune response, including HIR (IgG and PRNT₅₀), CMIR (IL-4 and IFN- γ), and IgA as a mucosal immune response from nasal washing and saliva of mice, was also evaluated for the efficacy of the formulations. In the case of sCS-MNs patch, a ready-to-use patch by detaching the baseplate layer of separating MNs, the combination of

CS:PVA:NaHCO₃ was used to fabricate the patch. The patches were characterized for morphology, mechanical strength, skin insertion, and *in vitro* skin penetration. Optimal MNs patch is then loaded with JE-CV for ID immunization. The efficiency in stimulating immunization and safety of the sCS-MNs patch were also evaluated. The immunization study was compared to conventional SC immunization.

1.2 Aims and objectives

1. To develop JE-CV-loaded CS-based NPs (CS-NPs and CM-NPs) for IN immunization.
2. To develop a JE-CV-loaded sCS-MNs patch for ID immunization.
3. To evaluate the efficacy and safety of JE-CV-loaded CS-based NPs and JE-CV-loaded sCS-MNs patch compared to commercially available SC vaccines in mice model.

1.3 Hypothesis

1. Formulations of CS and CM NPs can be prepared with low PDI using the ionic gelation method. JE-CV can be successfully incorporated into NPs and shows good physical characteristics.
2. sCS-MNs patch successfully fabricated by micromolding techniques with desirable characteristics.
3. JE-CV-loaded CS-based NPs for IN immunization and JE-CV-loaded sCS-MNs patch for ID immunization can be prepared as alternative to commercially available SC vaccination.

Chapter 2

Literature review

2.1 Japanese encephalitis (JE)

2.1.1 Virology and epidemiology

Japanese encephalitis virus (JEV) is a cause of Japanese encephalitis (JE) as the major viral encephalitis disease in Asia-Pacific regions. The first JEV was isolated and identified in Japan, giving us the Nakayama strain from a brain of a fetal case in 1935 [20]. JEV is included in the *Flavivirus* genus of the *Flaviviridae* family as an enveloped virus, a positive-sense single-stranded RNA genome, as well as Dengue, West Nile, Yellow fever, and Zika virus are included in *Flavivirus*. Nowadays, several kinds of JEV have been reported and classified into 5 genotypes based on the RNA sequence for the viral envelope protein-encoding. All genotypes spread out in the endemic area, but genotype III is the most prevalent found, especially the Beijing strain [1, 21].

JEV is a small virus with a size of about 50 nm in diameter (Figure 1A). It contains three protein structures with different functions (Figure 1B), including (1) membrane protein (M) covers the whole virus and protects it from an unfavorable environment. (2) envelop protein (E) integral membrane protein involved in several aspects of the virus' life cycle, which forms the outermost layer of the virus. It is used to attach a cell host for entry of infection. Therefore, E protein is the most potent antigen and has the potential to elicit the highest immune response of JEV, which is important for vaccine development. (3) capsid protein (C) covers a core structure of JEV that contains genomic RNA. The sequence RNA of JEV (Figure 1C) is about 11kb and is separated into two parts based on translation protein synthesis, such as protein-structural genes that translate to E, M, and C protein structures and non-structural genes which do not translate to the protein structures but it associates for the translation protein synthesis and RNA replication in infected cells [22, 23].

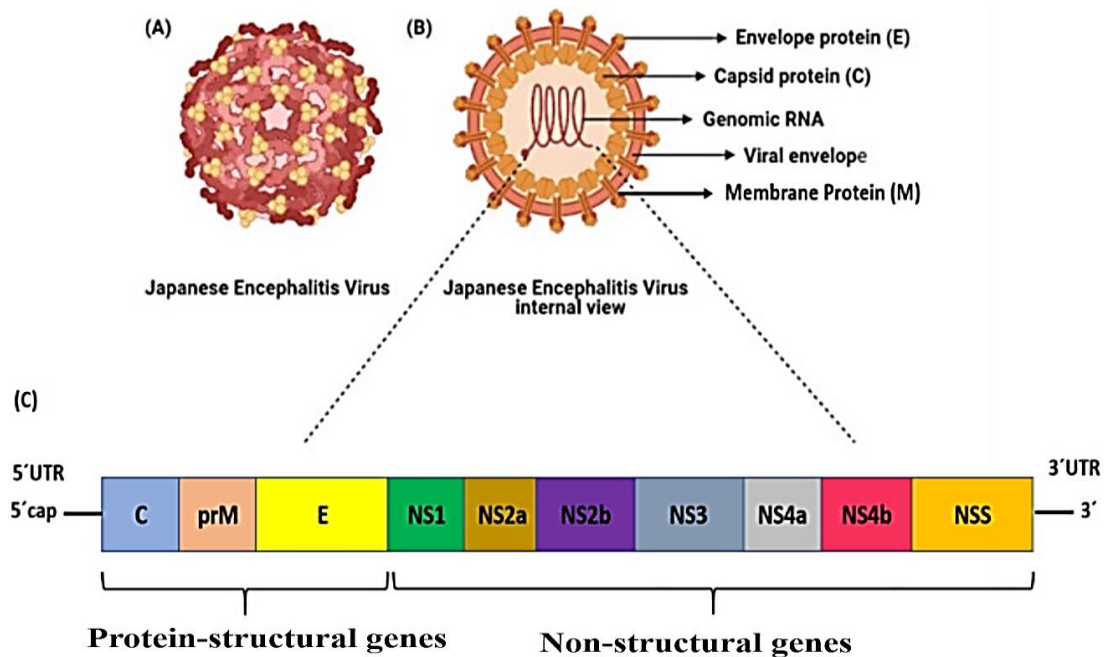


Figure 1 The structure of JEV: overview structure (A), internal view structure (B), and genomic structure (C) [24].

JEV is a mosquito-borne virus transmitted in an enzootic cycle between *Culex* mosquitoes as a viral vector and amplifying/natural host, as shown in Figure 2. The *Culex* mosquito genus is an important vector for JEV, especially *Culex tritaeniorhynchus*, a night-biting mosquito, the primary mosquito vector of JEV in Asia, especially in the tropical-temperate regions. Pigs and birds are the main amplifying host in an enzootic cycle because JEV can amplify with a high viremia titer capable of transmitting JEV to the mosquito. Then, JEV infects the salivary gland of the mosquito. When infected mosquito bites naïve animals (not infected animals), JEV in the saliva is transmitted to the skin of the bitten animal. If the bitten animal is amplifying host, JEV is amplified and transmitted via mosquito biting in an enzootic cycle as a natural cycle. However, if the infected mosquito transmits JEV to humans or other animals that cannot produce high viral titer of the virus in the bloodstream, JEV does not sufficiently transmit to other animals via mosquito. This is an accidental cycle; humans are called accidental dead-end hosts. The JEV transition cycle indicates that pigs, birds, and *Culex* mosquitoes are very important as amplifying hosts for JEV spreading [2, 25]. Interestingly, both wild and domestic pigs are amplifying hosts;

however, the predominantly domestic pig is a major amplifying host for JEV transition to human. Domestic pigs are the primary concern because they produce high viremia titer with long-term presenting, and pig farm in Asia is also close to human habitat, increasing the JE incident in humans [26]. In addition, JE can directly infect without the mosquito vector, between the infected pig to the naïve pig. The saliva of an infected pig contains a sufficient JEV titer that can infect the other pigs via the oronasal route, and then, they become the best JEV reservoirs [27]. For birds, wild ardeid waterbirds, such as egrets and herons, are another prominent amplifying host because it lives near rice fields or water reservoirs that are abounded with mosquitoes for JEV transition. Additionally, ardeid birds are seasonal migratory birds that potentially enhance the JEV infection and contribute to the transmission of the virus to other areas as a destination region, especially topical-temperate regions, where the rainy season is plentiful in meal and habitat, causing seasonal JE outbreaks [26].

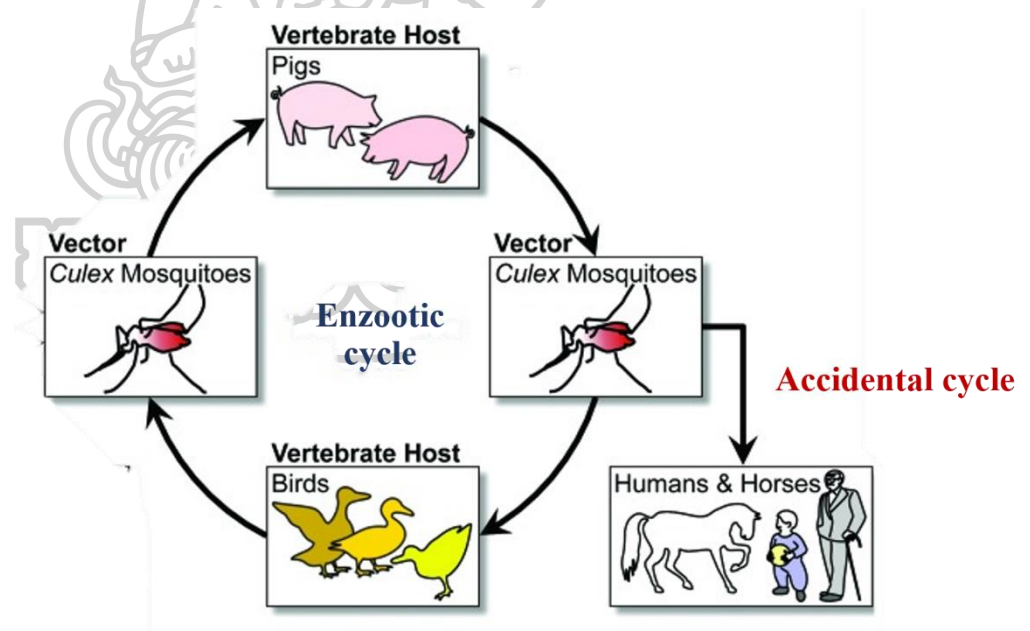


Figure 2 Transmission cycle of JEV [25].

Asia-Pacific region, the endemic area of JE spreading, has residencies of about 60% of the global population. In 2019, the World Health Organization (WHO) reported that the annual JE case is about 68,000 cases with 13,600-20,400 deaths (~

30% mortality rate) around the world, and half of the survivors show permanent of serious neurologic congenial sequela. JE case is found at any age, especially in children (0-15 years) in the endemic area with the highest incidence and higher mortality rate with more neurological complications than other ages. Furthermore, all traveler-visited JE endemic areas can occur due to the absence of protective immunity [20, 25]. Therefore, JE is indicated to be a serious health problem, and JE immunization for all children in endemic countries and the traveler is recommended by WHO [28, 29]. Thailand included the JE vaccine in the Expanded Program on Immunization (EPI) and has given it to all Thai new-born since the 1990s [30].

In the case of epidemiology, JEV is not a viral infection disease for person to person via direct contact. Besides natural infection, JEV can infect person to person through organ transplantation and blood transfusion. Several preclinical information in monkeys, mice, and pigs reported that JEV could infect via the oronasal route. The mucosal infection depends on viral virulence, the amount of the virus, and the health status of the host. These factors are essential and widely investigated in the JEV laboratory. According to the recommendation of The Centers for Disease Control and Prevention of The United States of America, the JEV study is restricted up to biosafety level III, which protects against mucosal and inhalational exposures, different from attenuated JEV study that allowed using biosafety level II. Moreover, JE vaccination prior to starting the experiments is recommended for lab workers [2, 25].

2.1.2 Pathogenesis and clinical management

JE infection starts after the infected mosquito biting, as shown in Figure 3. The saliva of the infected mosquito, JEV reservoirs, is inoculated into the dermis layer of the skin during the biting period. The virus infects and replicates cells surrounding the inoculation site, such as fibroblasts, endothelial cells, monocytes, and antigen-presenting cells (APCs, dendritic cells and Langerhans cells). Then replicated virus goes to local lymph nodes containing B lymphocytes and several types of T lymphocytes [22, 23]. In this step, APCs present JE antigen to both types of helper T lymphocytes (Th; Th₁ and Th₂) for stimulation of cell-mediated immune responses (CMIR). Stimulated Th₁ secretes IFN- γ cytokine that directly induces apoptosis of

infected cells or stimulates cytotoxic T lymphocytes (CTLs) for the highest IFN- γ production. Moreover, CTLs facilitate the activation of macrophages and several immune cells to destroy the infected cells via phagocytosis and apoptosis induction [20, 31]. For B lymphocytes, stimulated Th₂ secretes IL-4 cytokine to activate B lymphocytes, producing IgG as an antibody, humoral immune response (HIR), against JEV. IgG, called neutralizing antibody, can bind with the E protein of JEV and neutralizes it to protect cells from JEV infection [23]. IFN- γ is a major cytokine of Th₁ for CMIR against the infected cells as an endogenous pathogen. IL-4 is a major cytokine of Th₂ for HIR stimulation and neutralizing antibody, exogenous protection from JEV. Th₁ and Th₂ are balanced by the negative feedback between IFN- γ and IL-4 mechanism [32]. A local lymph node is an organ that stimulates and then releases the immune responses to eliminate JEV. In the case it cannot eliminate JEV, the virus will enter the bloodstream and stimulate systemic immune responses from lymphatic organs, especially the spleen, the largest lymphatic organ. In severe cases, the systemic immune responses are insufficient to eliminate the JEV; JEV penetrates the CNS across the blood-brain barrier, resulting in brain inflammation, called encephalitis. The clinical data of survivors show that IFN- γ is increased in an early phase of JEV infection, IL-4 and IgG are dominantly increased leading to increase in neutralizing antibody level after JEV spreading into the bloodstream, followed by recovery of IFN- γ level. The immune responses pattern as described indicated the HIR and CMIR that affects the JE protection [23, 33].

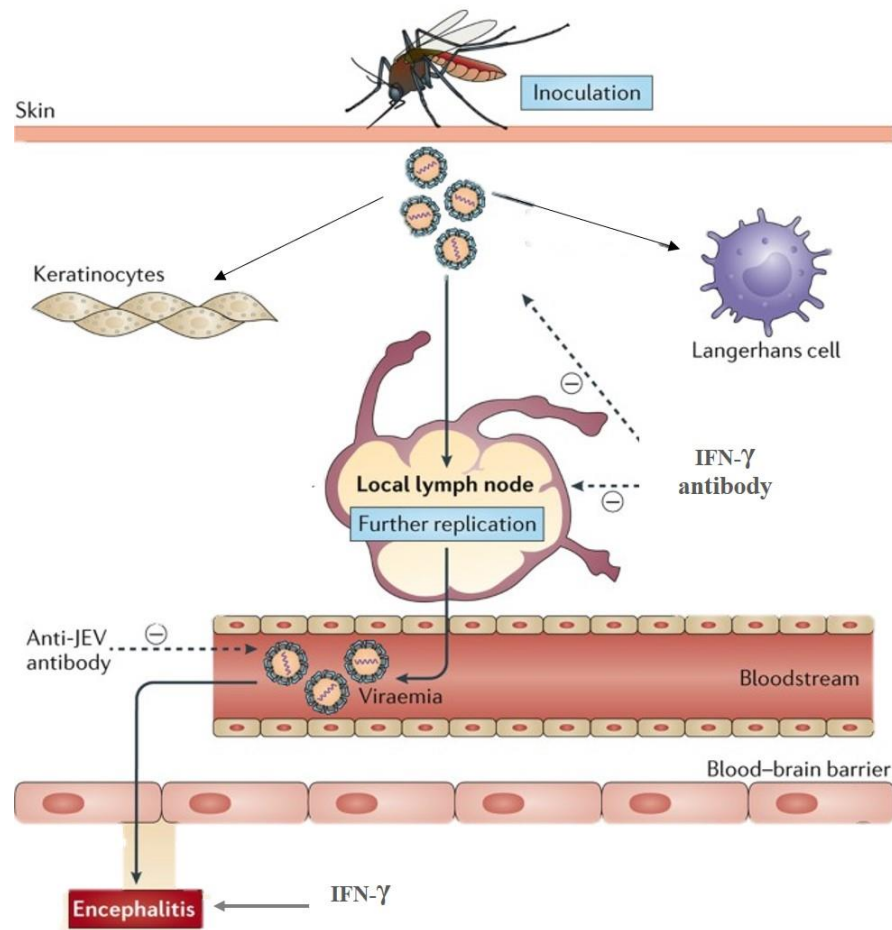


Figure 3 Pathogenesis of JE in humans [23].

The initial symptom after the infected mosquito biting is fever or asymptomatic. Only 0.1-1% of infected person has developed encephalitis because many patients can eliminate the JE virus as a self-limiting illness without the involvement of the CNS. Among patients who develop clinical symptoms, the incubation period is believed to be about 5–15 days after the mosquito biting. Initial symptoms are usually nonspecific, including fever, headache, vomiting, and diarrhea. Then, neurologic deficits, mental status changes, and seizures are present. Moreover, an important sign indicated that JE involvement in the brain is a parkinsonian syndrome; the complex clinical signs of mask-like facies, tremors, cogwheel rigidity, and choreoathetoid movements (episodes of irregular jerking or shaking movements) are specificity. The poor outcome is dead from brain hypoxia and aspiration

pneumonia complications. For diagnosis, plague assay, ELISA, and RT-qPCR are generally used in the clinical field [2, 34]. JE therapy still has no specific treatment and no specific antiviral drug. Palliative care for JE clinical magnifications in each patient is a recommended therapy. Therefore, prevention is only one strategy for JE management. Based on the transmitting cycle, JE prevention can be divided into four methods; 1) mosquito control with chemical or biological substances, the method is unsuitable in wild areas or clinical fields 2) avoiding mosquito biting by applying mosquito repellent, staying in mosquito net accommodation or changed activity for avoiding mosquito, an unsuitable for long-term prevention and disturb life-activity 3) pig immunization, as successful method but high cost for immunization because life-period of pigs is short and short breeding period for getting the new generation is required tremendously of vaccines, 4) human immunization, a suitable method for long-term prevention with cost-effectiveness is recommended by WHO for JE prevention [1, 25]. Based on the neutralizing antibody property to protect JEV infection, the plaque reduction neutralization test is a qualitative method for the evaluation of neutralizing antibody levels in serum. The level of PRNT₅₀ ≥ 10 in human and animal models is indicated complete JE protection, called "seroprotection", which is recommended by WHO. For IFN- γ level, it is unclear about the protective levels; however, long-term persistent IFN- γ levels found in vaccinated persons are related to JE survival. Therefore, neutralizing antibodies and IFN- γ levels are the primary requirement for JE immunization [1, 35].

2.1.3 JE immunization

Based on antigen characteristics and preparation process, JE vaccines are classified into four types; 1) mouse brain-derived, inactivated JE vaccine, 2) cell culture-derived, live attenuated JE vaccine, 3) cell culture-derived, inactivated JE vaccine, and 4) live attenuated chimeric vaccine [1].

2.1.3.1 Mouse brain-derived, inactivated JE vaccine

Mouse brain-derived inactivated JE vaccine is the first licensed JE vaccine developed in Japan in 1954 and has been used internationally for a long time. It was produced using the Nakayama strain in mouse brains, then inactivated virus with formalin before collecting inactivated JEV as a vaccine antigen. However, it had

several disadvantages, including manufacturing variability, serious adverse events (AEs), etc. Especially, neurological AE might relate to the residual of basic myelin protein from mouse brain host. In Korea, inactivated mouse brain-derived vaccine showed severe neurological AE leading to two fatal cases in 1994. Therefore, the cell culture-derived JE vaccines were developed to overcome the limitations of mouse brain-derived, inactivated JE vaccines and achieve non-neurovirulent from mouse brain related to safety, high productibility with reproducibility, and low cost of manufacturing. Therefore, the WHO replaced cell culture-derived JE vaccines with mouse brain-derived inactivated JE vaccines for recommended JE vaccines. The mouse brain-derived was discontinued in 2006 [36].

2.1.3.2 Cell culture-derived, live attenuated JE vaccine

It is the second type of JE vaccine developed in China and approved in 1998. This vaccine was prepared by multiple passages of JEV SA14 stain, as wild-type on PHK-cell culture to obtain live attenuated JEV and was called "SA14-12-2 strain", exhibiting mid-virulence virus in healthy people persons and is highly immunogenic with safety. Therefore, cell culture-derived, live attenuated JE vaccine is SC injected for two doses/regiment. Difference from inactivated mouse brain-derived JE vaccine that is SC or IM injected for 4-5 doses/regiment, as explained in Table 1. Theoretically, live attenuated JEV might be recovery virulence in people with immunocompromised (people who reduced ability to fight infections and other diseases), such as AIDS, cancer, diabetes, malnutrition, certain genetic disorders patients, pregnant women, and lactating women, leading to contraindication [1].

2.1.3.3 Cell culture-derived, inactivated JE vaccine

Several vaccine developers produced this type of vaccine with various kinds of JEV and cell cultures. Briefly, JEV was passaged and cell-cultured, then killed with chemical reagents for obtained inactivated JEV as a vaccine antigen. Regarding efficacy, the cell culture-derived-inactivated JE vaccine has lower immunogenic efficiency than the live attenuated cell culture-derived JE vaccine. Therefore, it requires an adjuvant for enhancing immune stimulation. Cell culture-derived inactivated JE vaccine is SC or IM injected for 3-5 doses/regimen depending on the developer, as explained in Table 1. Beneficially, the cell culture-derived inactivated

JE vaccine has no virulence; therefore, it can be used in immunocompromised patients [1, 37].

2.1.3.4 Live attenuated chimeric vaccine

JE-CV was first licensed in 2010 and was created with genetic-engineering technology. Briefly, live attenuated YFV 17D strain as a safe virus was used as a vector virus. The prM and E genes of the JEV SA14-12-2 strain were replaced with the prM and E genes of the vector virus. The replaced vector virus was then transferred into Vero cells for replication and amplification. The obtained chimeric virus shows M and E protein of JEV on the surface of the live attenuated chimeric virus. It is a new type of JE vaccine with the highest efficiency and safety in clinical use. However, it is not recommended for immunocompromised patients. The JE-CV (10^4 - $10^{5.8}$ PFU/dose) is SC or IM injected at 2 doses/regiment as the JE-CV immunization program [1, 36], as explained in Table 1.

The first preclinical JE-CV study in mice by Guirakhoo et al. (1990) reported that the single dose SC injection of JE-CV at less 10^4 PFU/mouse in female C57BL/6 age 3-4 weeks stimulated seroprotection ($\text{PRNT}_{50} \geq 10$ titers) after immunization on 21 days and exhibited 100% survival against JEV challenging after immunization on 28 days [38, 39]. JE-CV 10^3 PFU/mouse stimulated PRNT_{50} about 8 titers while exhibiting 12.5% against JEV challenging [40], according to $\text{PRNT}_{50} \geq 10$ titers as a seroprotection level in human and mice, and $\text{PRNT}_{50} < 10$ titers as partial protection [1]. Therefore, in this study, the schedule of JE-CV immunization in mice was selected based on the study of Guirakhoo and $\text{PRNT}_{50} \geq 10$ titers recommend by the WHO were used as criteria for seroprotection

Table 1 Licensed JE vaccine: type, regimen, and efficiency with safety [1, 29, 36, 37].

Types of vaccine	JEV strain/ Substitute	Manufacturer/ trade name	Regimens	Seroprotection (%)¹	AEs (%)²
Mouse brain-derived, inactivated	Nakayama strain/ mouse brain	Biken, Japan/ JE-VAX	• SC or IM injection for 5 doses • Start on days 0, 7, 28 at 12-24 months of age; booster after 12-14 months, then every 3-5 years	≥ 80%	~ 35 % total AEs, 1% of neurologic AEs, and 2 fetal case reports Discontinue in 2006
Cell culture-derived, inactivated JE vaccine	SA-14-14-2/ Vero cell culture	Example: Novartis, Austria/ IXIARO™	• SC injection for 3 doses • Start on days 0, 28 as early as 2 months of age; booster after 1 year	≥ 85 %	Common AEs; fever and local reactions (redness, swelling, and pain at injected site) were pooled AEs about 12.49% without serious AEs
	Beijing/ Vero cell culture	Example: Biken, Japan/ JEBIK-V	• SC or IM injection for 4-5 doses • Start on days 0, 7, 28 at 12-24 months of age; booster after 12-14		

Types of vaccine	JEV strain/ Substitute	Manufacturer/ trade name	Regimens	Seroprotection (%) ¹	AEs (%) ²
			months, then every 3-5 years		
Cell culture-derived, live attenuated JE vaccine	SA-14-14-2/ PHK cell culture	Chengdu Institute of Biological Products, China/ CD.JEVAX™	• SC injection for 2 doses • Primary vaccination at ≥ 8 months of age, booster after 12-24 months	99.15%	13.12% - 37.5% of common AEs, without serious AEs
Live attenuated chimeric vaccine	Chimeric YFV 17D containing JEV antigen/ Vero cell culture	Saofi-Pasteur, Australia/ ChimeriVax-JE™ and Imojev® GPO-MBP, Thailand/ Imojev®, Thaijev®	• SC or IM injection for 2 doses • Primary vaccination at ≥ 9 months of age, booster after 12-24 months	100%	12.41% - 39.0% of common AEs, without serious AEs

¹ the percentage of obtained seroprotection cases/total cases

² the percentage of obtained AEs/ total cases

2.2 Vaccine administration

Vaccination is a prevention method against infectious diseases and has been recommended as the EPI for children by WHO since 1977, depending on the diseases spreading in each region. Vaccination is the most significant cost-effective method for decreasing mortality and morbidity according to the prevention estimated 2.5 million fetal cases/year [21]. Vaccines stimulate the body's immune system to develop immune responses and thereby recognize and pre-fight against pathogens. The route of administration is one factor for prevention success. Vaccines have conventionally been injected through hypodermic needles with SC and IM routes since the 1850s, which exhibited the successful vaccination and disease control method. However, vaccine injection has the limitations, such as needle phobia (anxiety associated with needles), pain from invasive technical leading to poor compliance, biohazardous sharps waste of needle after injection that requires a specific method for destroying and preventing the contamination, required healthcare provider, etc. Therefore, non-invasive immunization is developed to overcome the disadvantages of the conventional immunization method [22, 23]. Oral and IN, non-invasive mucosal immunizations, are mostly investigated, especially IN shows the superior benefits such as avoiding antigen destruction from the highly acidic condition and digestive enzymes in the gastrointestinal tract. FluMist/FluenzTM (MedImmune, USA) and NasovacTM (Serum Institute of India, India) are IN vaccine licenses for live attenuated influenza vaccine [24, 25]. ID immunization, a minimally invasive immunization, is an interesting alternative to conventional immunization. ID vaccination, called the Mantoux technique, uses hypodermic needles with 25G-27G needles and shallow injection into the skin dermis layer with a 5°-15° angle from the skin surface. Ideally, the needle penetrates deep into the skin, about 25 μ m -2,000 μ m from the skin surface, and minimally pain stimulation (mild pain). In the case of incorrect vaccination technique, too deep injection, the needle delivers the vaccine into SC or IM layer leading to ineffective vaccination and stimulating pain; however, if too shallow vaccination, a vaccine may leak out, resulting in insufficient dosages delivered. Therefore, this technique is difficult to control and requires special training and regular practice by healthcare workers. Moreover, WHO suggests ID immunization as the alternative administration for tuberculosis and rabies vaccines

because it can reduce antigen amount compared to conventional immunization, reducing the cost of immunization [10, 41].

2.2.1 CS-based NPs for IN immunization

2.2.1.1 Nasal anatomy and immune response

The nasal cavity is the first part of the respiratory system where air enters the lung. The total surface of the nasal cavity is 160 cm², divided into two nostrils with a nasal septum. The nasal mucosa of each nostril is divided with structure and function into three regions: vestibule and atrium region, respiratory region, and olfactory region. Posteriorly, the nasal passage is connected to the nasopharyngeal region, as shown in Figure 4 [42].

The vestibule and atrium region, the anterior part of the nostril, contains stratified squamous with non-ciliated, keratinized epithelial cells that are tightly sealed by intercellular junctions (named as the 'tight junctions') leading impermeable of vaccine and other biomacromolecules. Moreover, the vestibule part has numerous hairs for filtering air particles before entry into the respiratory region. Therefore, this part is not interested in vaccine delivery [7].

The respiratory region, the largest surface area of the nasal cavity, contains three parts of turbinate (inferior-, middle- and superior turbinate). Turbinate is constituted of ciliated epithelial cells and goblet cells. The goblet cells create mucus covering the nasal surface with about 10-15 thicknesses. The mucus comprises 95% water, 2.5–3% mucin, and 2% electrolytes, proteins, lipids, enzymes, and antibodies. Nasal mucus is slightly negative charges with a physiological pH value between 5.5–6.5 [7]. If the drug or inhaled substance deposits on the nasal mucosa, it is attached to mucus and moved with cilia movement from a nasal mucosa to an esophagus and stomach for substance destruction. The cooperation of mucus and cilia of epithelial cells is called mucociliary clearance. It is the main issue for IN delivery because of rapid mucociliary clearance with a half-life to clearance of about 15-20 min, not long enough for vaccine absorption. Therefore, mucoadhesive delivery systems are developed against mucociliary clearance and prolonging vaccine retention on the nasal mucosa, leading to enhanced vaccine absorption. The underneath epithelial layer has high blood circulation for air warming before entering the nasopharyngeal region.

The drug or active substances that pass the mucus and epithelial layer exhibit systemic delivery via IN administration. However, a tight junction of ciliated epithelial cells can open and close for hydrophilic substance transportation via the junction among epithelial cells called paracellular transport. The junction size is less than 10 Å. The other transport pathway is called transcellular transport, and the substance is directly transported via epithelial cells. The small size (< 1 kDa) with hydrophilic substances is general transport via transcellular epithelial cells. In the case of IN vaccination, a biomacromolecule has poor absorption through nasal epithelium via both transcellular transport and paracellular transport, with lower than 1% bioavailability. Therefore, most vaccines are eliminated via mucociliary clearance [3, 42].

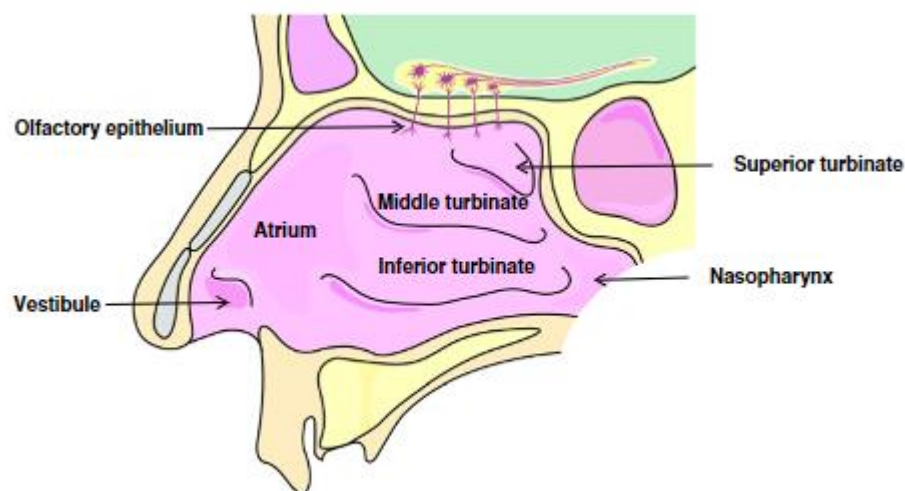


Figure 4 Nasal anatomy [42].

Fortunately, some part of the respiratory region consists of a lymphoid follicle called NALT, including T lymphocytes, B lymphocytes, and APCs, as shown in Figure 5. It is covered with microfold cells (M-cells) as thin, non-epithelial cells, showing high antigen uptake ability compared to other epithelial cells. Therefore, M-cells are called "antigen sampling cells" and prefer to absorb particulate antigen in nano-sized more than micron-size and antigen solution, respectively. Consequently, NALT is the target of IN vaccine delivery system. Mucoadhesive nanoparticles are an ideal delivery system for IN immunization [43]. M-cells attach the particulate antigen, then uptake and transported to submucosa for APCs uptake, then antigen is presented

to B-lymphocytes for secreting IgA at the inductive site as mucosal immune responses. Moreover, APCs at NALT can migrate via the common lymphatic pathway and present antigens to lymphoid node/lymphoid organ sites for HIR and CMIR stimulations, leading to systemic immune response and mucosal immune responses. However, antigen trafficking from IN immunization to the spleen is restricted, resulting in strong mucosal immune responses with low systemic immune responses [44].

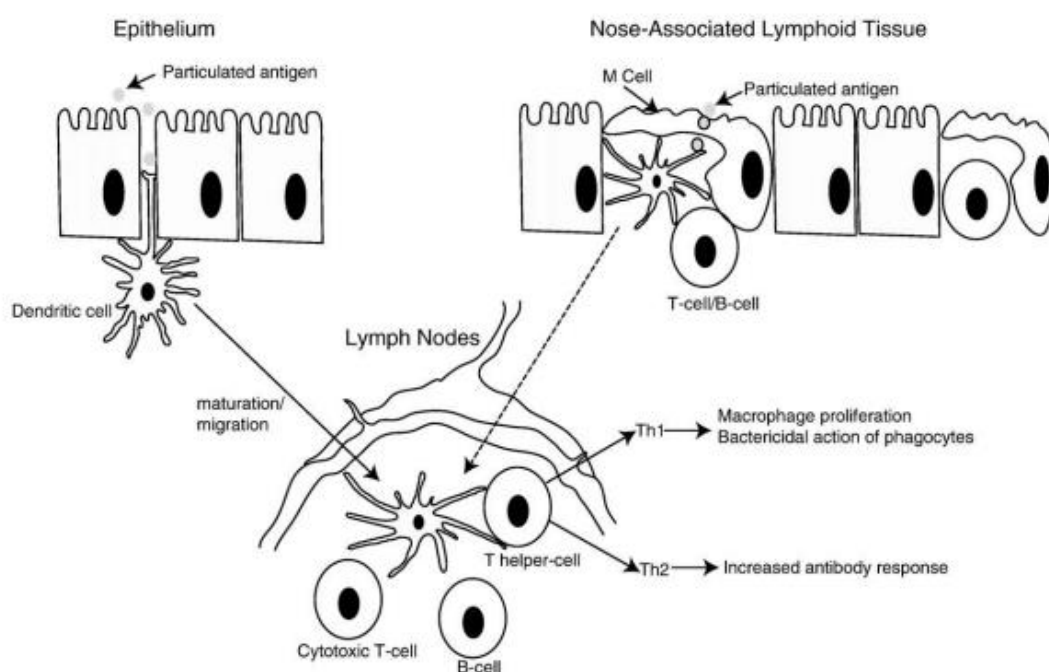


Figure 5 The structure of NALT and immune stimulation [44].

The third part of the nasal mucosa is the olfactory region that locates upper to the respiratory region, which is a small surface area, approximately 3 -5 % of the total surface nasal mucosa (Figure 4). The olfactory epithelium is cilia-epithelial cells with tightly tight junctions; therefore, the small molecules (< 1 kDa) with high lipophilicity are preferred absorption through this nasal mucosa region. Therefore, it is unsuitable for vaccine delivery; however, it is interested in developing CNS drug delivery due to bypassing the blood-brain barrier via olfactory nerves. In addition, because this region has olfactory receptors in the mucosa for attaching odor molecules, the axons of olfactory receptors connect to CNS for smell responses [42].

2.2.1.2 CS-based NPs

2.2.1.2.1 CS properties

CS is a polysaccharide mainly composed of D-glucosamine and N-acetyl-D-glucosamine units randomly β -(1-4)-linked (Figure 6A). It is the partially deacetylated more than 65% deacetylation of chitin. Chitin is a natural exoskeleton of crustaceans, insects, and other organisms; however, mainly acquired from marine crustaceans such as crabs and shrimp [45]. The large volume of chitin is generated from the seafood industry and is simple to transform into CS, obtaining the low price of CS and available mass for commercial use [46]. CS has been approved for pharmaceutical excipient by the Food and Drug Administration of The United States of America. It has adjuvant effects and other excellent properties such as biocompatibility, biodegradability, non-toxicity, extended-release, and protein-stabilizing properties. Composing of D-glucosamine creates CS with a pKa value of 6.5. At pH lower than 6.5, amine (NH_2) of D-glucosamine is protonated to positive charges anime (NH_3^+) that can dissolve in acid conditions. On the other hand, at physiological condition pH 7.4, positive charges anime are deprotonated and cannot dissolve, which is a significant drawback of CS for parenteral use [47]. Usually, CS formulations are developed for mucosal administration because it exhibits mucoadhesive property and is soluble in an acid condition of mucosae such as nasal mucosa and vaginal mucosa [43]. For nasal mucosa administration (pH 5.5-6.5), CS exhibits mucoadhesive properties due to two mechanisms: (1) Electrostatic interaction between negative charges of sialic acid in mucus and positive charge anime (NH_3^+) groups of CS and (2) Hydrogen bonding interaction between hydroxyl groups (OH^-) of mucin as a glycoproteins and positive charge anime (NH_3^+) groups of CS. Effective of both mechanisms depends on a suitable pH environment and generally shows weak interaction. Therefore, modifying CS with the specific covalent bond-forming functional groups, such as CS-cysteine and CM as the CS conjugated with maleimide functional groups, are developed for enhancing mucoadhesive properties (Figure 6B). CM exhibits stronger mucoadhesive properties than CS- cysteine, and CS (native) because maleimide can react to the thiols of mucin via a Michael-type addition reaction that is strong covalent bonding and does not dependent on pH conditions.

CM exhibits excellent mucoadhesive properties with safety in *in vitro* study, IN applications have also been reported [9, 48].

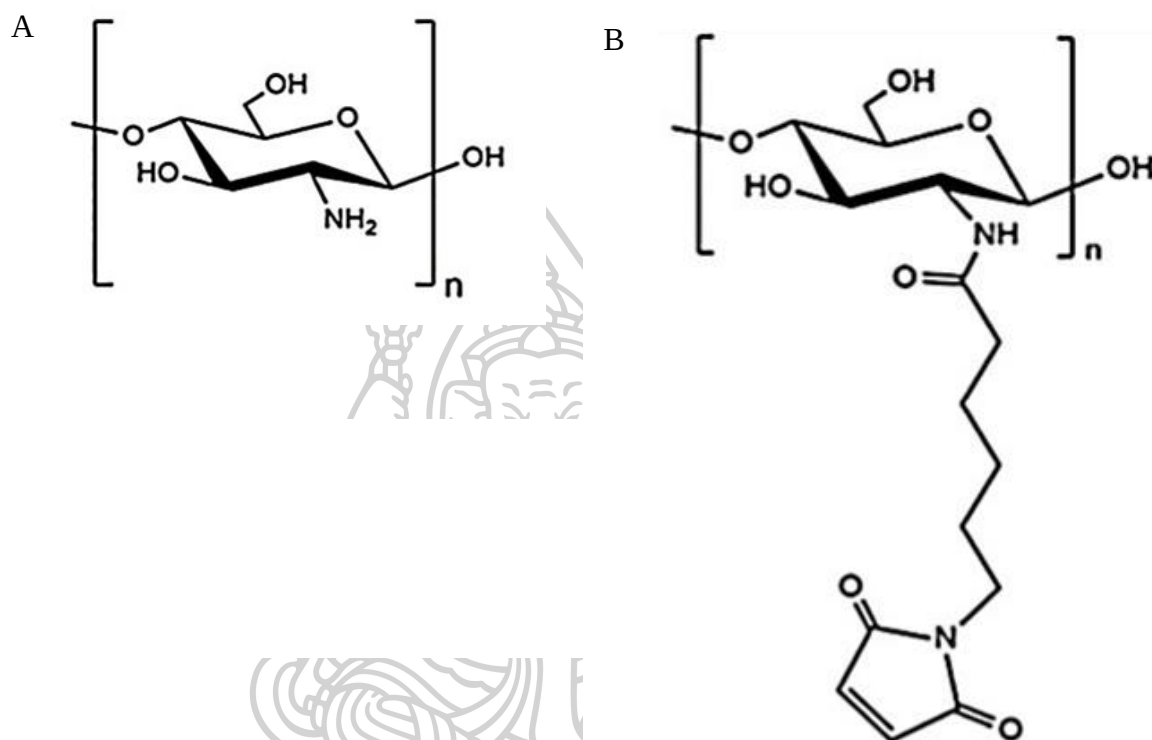


Figure 6 Structures of CS (A) and CM (B) [9].

2.2.1.2.2 Preparation and characterizations of CS-based NPs

NPs is a colloidal system of nano-sized particles as a dispersed phase dispersing in a continuous phase, but it does not combine to form a true solution. The highest ratio of surface area to particle volume creates new raw material properties. For example, large surface areas enhance cellular attraction for particle uptake, prevent antigen damage from enzymatic or extreme conditions, increase bioavailability, prolong antigen half-life, etc. Generally, the smaller particle size has antigen delivery efficiency higher than larger-sized particles and raw material [49]. NPs dispersed in a continuous phase with the balance between the attraction force of Van der Waals forces and electrostatic repulsion forces for a similar surface charge.

Therefore, the absolute value of surface charge affects the stability of NPs for suspending in a continuous phase and prevention aggregation as instability of NPs, size changing, and sedimentation of NPs [50]. PDI is a measurement of the size distribution of NPs, ranging from 0.0 to 1.0. The PDI of NPs system ≤ 0.5 exhibits a homogeneous NPs system, and PDI > 0.5 shows a heterogeneous NPs system. PDI > 0.5 is indicated an imbalance between the attraction forces and the repulsion forces, predicted as instability of NPs system. The stability prediction, efficiency, and safety of NPs depend on PDI because the size affects the target cells' uptake and the amount of NPs uptake. High-size distribution or heterogeneous NPs systems lead to efficiency decreasing and increased toxicity [51, 52]. CS-based NPs are the NPs system of CS and CS derivatives. It has synergies of advantages from the polymer and NPs systems for IN immunization [53]. Additionally, CS-based NPs are required nano-sized with positive surface charges for M cells uptake and mucoadhesion on the nasal mucosa. The positive surface charge is greater than 16 mV and PDI < 0.5 , which predicted the stability of CS-based NPs. However, the positive charge of CS-based NPs must be lower than 50 mV because the positive charge ≥ 50 induces hemolysis and thrombosis in *in vitro* studies and high compatibility with human monocytic cell lines [54].

Preparation methods for CS-based NPs are classified into two methods, physical and chemical. For the chemical method, organic solvents are used to prepare emulsions and then chemical crosslinking agents, such as glutaraldehyde and dehydroascorbyl palmitate, modify protein antigen for crosslinking resulting in antigen unstable. Additionally, organic solvent and crosslinking agents are cytotoxic agents and challenging to remove, possibly creating toxicity in formulations [55]. Spray drying and ionic crosslinking methods, physical methods, are interesting for NPs preparation without organic harm. Spray drying can be performed in large-scale products, but NPs product has various micron-sized, and high temperature in the preparation process may damage protein antigen. They are the major drawback of the spray drying method, which is challenging to optimize compared with the ionic gelation method as another physical method [55, 56].

The ionic gelation method is a successful preparation method for CS-based NPs delivery vaccine. NPs are prepared by a physical crosslink between the positive

charge of CS in an acidic solution and the negative charge of TPP, as shown in Figure 7. The gelation process spontaneously forms CS-based NPs under the addition of TPP with continuous mixing. The obtained CS-based NPs are collected via centrifugation or lyophilization before reconditioning in a suitable vehicle for administration. This process is blank or unloaded CS-based NPs preparation [57]. The antigen can be loaded into two methods: (1) incorporation method; protein antigen is premixed into CS or TPP solution before the gelation process and incorporated in the matrix structure of CS-based NPs. (2) incubation method; protein antigen is incubated with blank CS-based NPs in an acidic condition. The antigen is assorted on the surface of blank CS-based NPs due to physical absorption, an interaction between the carboxyl groups (COO^-) of antigen and amine groups (NH_3^+) of the CS backbone of CS-based NPs [56, 58]. Antigen-loaded CS-based NPs with incubation method exhibit strong immune responses when IN administration because antigen on the surface of CS-based NPs is easy for APCs detection and capture, then, presenting to other immune cells when compared with antigen loading with incorporation method [59, 60].

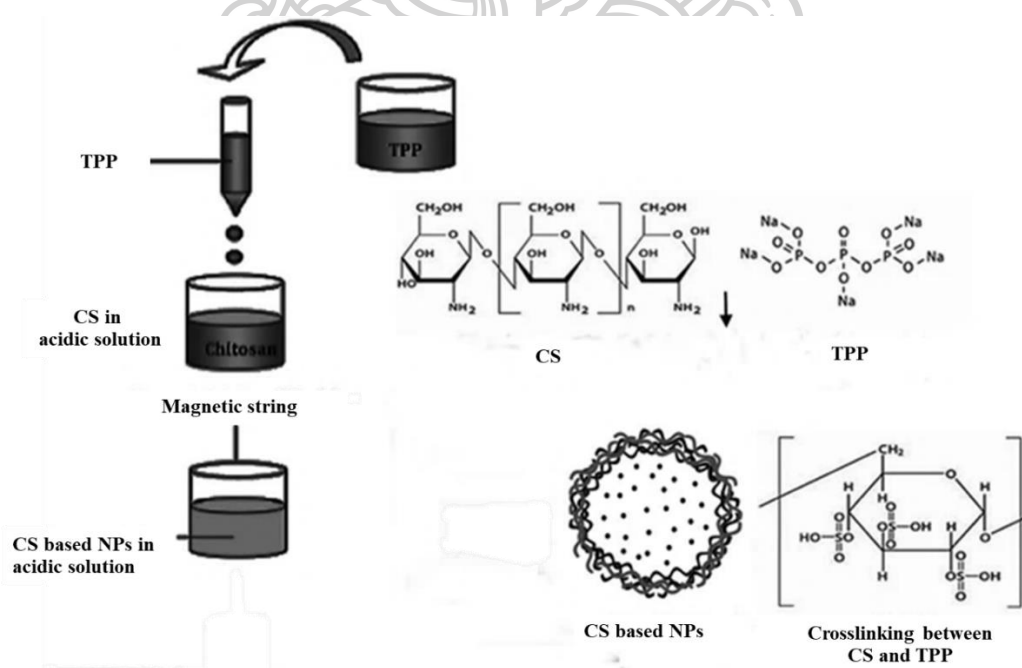


Figure 7 Structure of CS and TPP, and schematic illustration of CS-based NPs preparation procedure with ionotropic gelation method, modified from Caban et al., 2012 [57].

Several factors affect the incubation method's successful preparation of antigen-loaded CS-based NPs, such as size and surface positive charge of CS-based NPs depending on MWs and CS deacetylation, respectively. However, high MW with/without high CS concentration inhibited NPs formation because high viscosity destructs crosslinking between CS and TPP. Therefore, low MW CS with low CS concentration ($< 4 \text{ mg/mL}$ in 1 % v/v of acetic acid solution) is suggested for blank CS-based NPs preparation [58]. Additionally, the mass ratios of antigen: blank CS-based NPs and incubation time have the effect for LE and LC of antigen loading. Therefore, homogeneous NPs with positive charges with the highest LE and LC are required for IN formulation optimization [61].

2.2.2 CS-based MNs patch (CS-MNs patch) for ID immunization

2.2.2.1 Skin anatomy and immune response

Skin is the largest organ that covers and protects the body from the external environment, such as radiation, microorganisms, etc. It has various structures and functions to be divided into three layers: epidermis layer, dermis layer, and hypodermis layer or subcutaneous layer. The epidermis is the outermost layer. Keratinocytes and keratin production (hydrophilic compound) are major components of this layer. It is separated into five sublayers based on keratinization: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. Stratum basale is the deepest sublayer that proliferates keratinocytes for migrating toward the upper sublayer. The keratinocytes undergo programmed cell death, create corneocytes as keratin matrix, and release glycolipid into intracellular space that becomes bricks and mortar structure of stratum corneum [62]. Additionally, the five sublayers are classified based on the living cell into two groups (Figure 8); (1) non-viable epidermis (15-20 μm), a stratum corneum, and (2) viable epidermis (130-180 μm), the other sublayers containing APCs, especially, Langerhans cells [63]. The dermis layer (600 – 2,000 μm), the intermediate skin layer, contains several immune cells, such as dendritic cells, macrophages, mast cells, and T-lymphocytes. Moreover, the dermis has sensory nerves, blood vessels, and lymph vessels. The sensory nerve endings are intervened into the dermis layer from the hypodermis layer for

mechanical sensory detection (cold/warm, pain, and pressure forces, etc.). Hypodermis or subcutaneous layer is the layer under dermis layer. Adipocytes is mainly cells in this layer. It has energy storing function, control body temperature. Macrophages, APCs, and blood vessel are contained in subcutaneous but less dermis layer [62, 64].

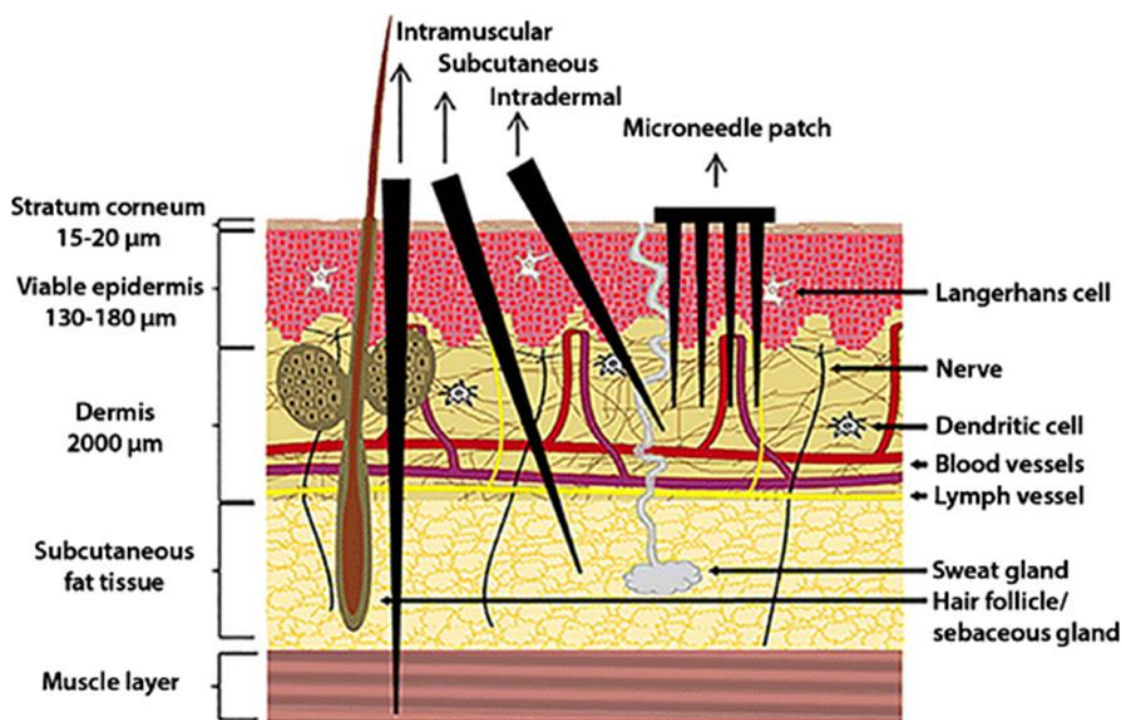


Figure 8 Skin structure and immunization with MNs, ID injection, and conventional injection (IM, SC) [52].

The principal function of the stratum corneum is the physical barrier, controlling water loss and preventing unwanted materials from entering the body. Only small molecules (< 500 Da) with log P about 1-3 are allowed permeation from the stratum corneum to the deeper sublayer. Vaccine antigen is a macromolecule restricted to pass through the stratum corneum, therefore, unable to stimulate the immune response cells located in the viable epidermis and deeper skin layers. Several physical transdermal drug delivery methods are developed, such as iontophoresis, electroporation, and sonophoresis. Unfortunately, they show no effectiveness with high cost when compared to conventional immunization methods (SC, IM) [65]. ID injection by hypodermic needles with the Mantoux technique into the viable-

epidermic and dermis layer elicited a stronger systemic immune response than conventional immunization (SC and IM) because the viable-epidermic and dermis layer has a higher population of APCs than subcutaneous and muscular layer as a delivery target of SC and IM (Figure 8). Moreover, APCs located in the viable epidermis and dermis layer can migrate via lymph vessels and present antigens to other lymph tissue/node sites to stimulate immune responses at the distal sites, especially the mucosal immune response. On the other hand, SC and IM immunization elicit a robust systemic immune response at a similar level compared with ID immunization but lacks mucosal immune responses because APCs from subcutaneous and muscular are restricted migration to mucosal tissues. These immune responses of ID immunization exhibit superior efficiency to conventional immunization. Practically, to stimulate immune responses at the same level, ID immunization can spare or reduce the amount of antigen vaccine by at least two times compared to conventional immunization [66, 67]. However, ID immunization faces complex technique limitations and requires trained healthcare personnel. Therefore, microneedles (MNs), micron-sized needles, were developed in 1971 to overcome the limitation. The morphology of MNs, similar to hypodermic needles but shorter in height, ranged between 25-2,000 μm for stratum corneum penetration and vaccine delivery into skin layers (Figure 9A and 9B). MNs are easy to apply and show the efficiency of vaccine delivery. It destroys the several limitations of conventional immunization as previously described; therefore, it gained interesting devices for vaccine delivery [68].

2.2.2.2 Classification of MNs

MNs are classified based on delivery strategy and fabrication into five types: solid, hollow, coated, dissolving, and hydrogel-forming MNs, as shown in Figure 9C.

Solid MNs is fabricated from non-degradable materials such as metal, silicon, and ceramics with strong mechanical strength property. It delivers vaccines with two-step processes called "poke and patch". In the first process, solid MNs are applied to the skin to create microholes before removing the patch. Then, the vaccine formulation is applied to the same area where solid MNs applied. The vaccine formulation diffuses into the skin via passive diffusion. Solid MNs have several

limitations, such as a lack of uniform delivery efficiency due to the application process and natural microhole healing and creating biohazardous sharps waste from applied solid MNs that requires particular disposal [65].

Hollow MNs are modified hypodermic needles by reducing the length and diameter size of needles to micron-sized. It can be a single MN array or multiple MN arrays on a patch. The MN is fabricated from non-degradable materials like solid MN but contains a hollow for vaccine solution injection via a hollow of MN array into the skin. This delivery strategy is called "poke and flow". Hollow MNs are an easy administration technique with accurate dose administration and are suitable for large dosing. Moreover, vaccine formulation does not require developing a commercial formulation. However, this MN type also creates biohazardous sharp waste [69].

Coated MNs, solid MNs coated with a mixture of vaccine solution and excipient/polymer and then dried. After administration, the coated solution is deposited in the skin layer. Then solid MNs are removed. This delivery strategy is called "coat and poke". The limitations of coated MN are the small loading capacity of the vaccine (less than 1 mg), the vaccine may be peel off during administration, creating biohazardous sharps waste, and coating formulation can decrease the sharpness of MN tip leading to a decrease in skin penetration [70].

In dissolving MNs, drugs/vaccines are mixed in dissolvable or degradable polymer then the mixture is used for MNs fabrication. When dissolving MNs is applied to the skin, the polymer dissolves/degrades in the skin, releases antigens from the polymer, and stimulates immune responses. This delivery strategy is called "poke and release". Dissolving MNs does not create biohazardous sharps waste, unlike previous MN types that can prevent contamination infection. Other advantages are a cost-effective fabrication process, high dose loading, and sustained delivery. The properties of the fabricated polymer are several factors that need to be concerned, such as dissolving/degrading rate, biocompatibility, and mechanical strength [71]. The widely-used polymers for fabricating dissolving MNs were reported, such as CS, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), sucrose, and hyaluronic acid. However, the mechanical strength and stability of dissolving MNs are factors that need to be considered before commercialization [69].

Hydrogel-forming MNs were first reported in 2012. They consist of swellable MNs obtained from crosslinked hydrogel polymer, and a vaccine reservoir may be incorporated in the swellable polymer or placed on the top of the MN patch. After administration, MNs patch rapidly uptakes the skin's interstitial fluid and forms a hydrogel. Then the vaccines are gradually released from swellable MNs into the skin. This MN type can load antigens in a higher amount than dissolving MNs, in a vaccine reservoir may be more stable than dissolving MNs. However, delivery efficiency (DE) may be reduced compared to the dissolving MNs, mechanical strength properties and complex fabrication method are factors concerned [72].

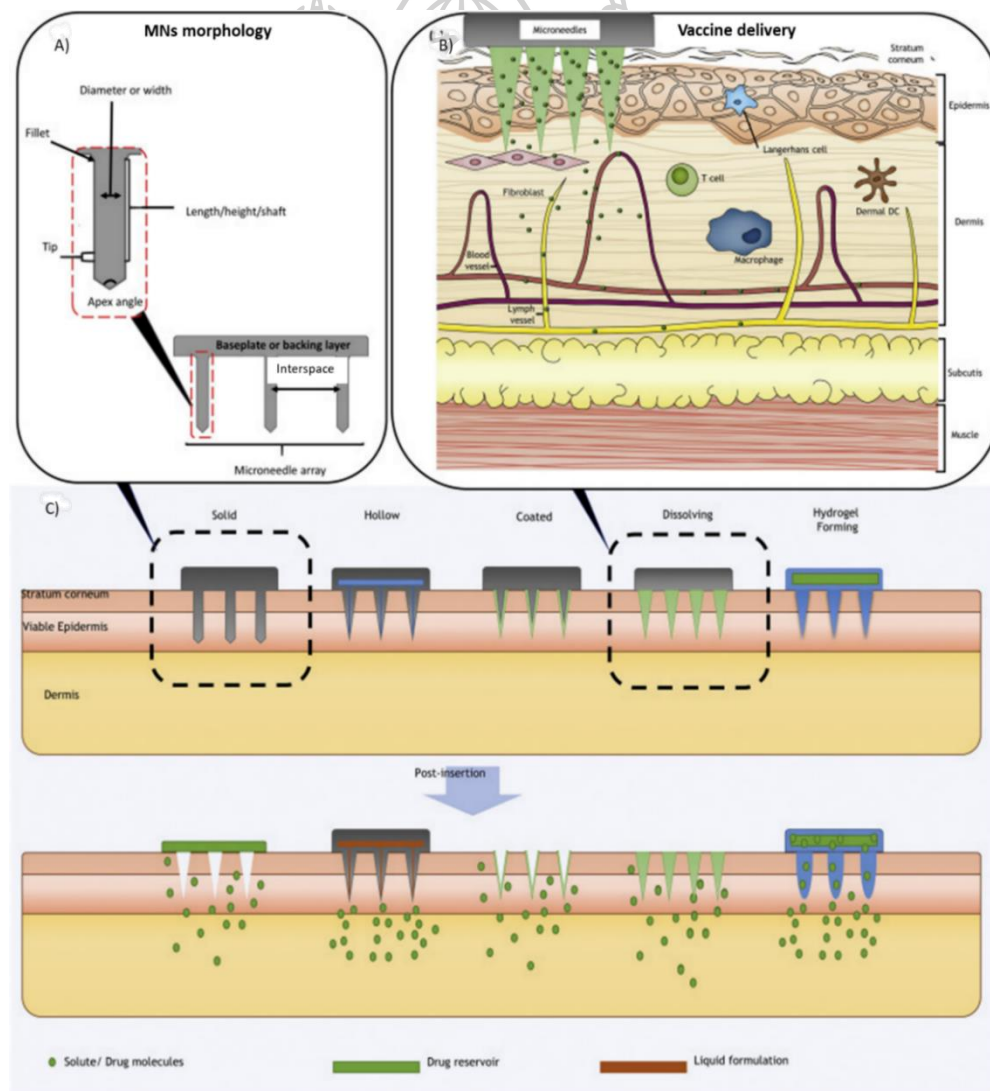


Figure 9 Schematic information of MNs: (A) MNs morphology, (B) vaccine delivery via MNs, and (C) delivery strategy of each MN type [65].

2.2.2.3 Fabrication methods of dissolving MNs

Based on several advantages of dissolving MNs for ID delivery systems as previously described. It is most interesting to develop as shown successful ID immunization in clinical studies [73-75]. Micromolding, droplet-born air drying, drawing lithography, and 3D printing were used for dissolving MNs fabrication, as shown in Figure 10. Micromolding (Figure 10A) is the most common fabrication method because it has many advantages, such as a convenient method, being available for several vaccines preparation, a variety of polymers selection, and offering cost-effectiveness. For the process, firstly, the mixture solution of drug/vaccine and polymers is poured onto a micromold. Then, the mixture is performed to remove the air bubble and fill into a micromold with centrifugation or vacuum pressure. After that, the solution in the mold is placed until completely dry. Finally, the dried mixture is gently peeled out from the micromold to obtain a dissolving MNs patch [76]. For droplet-born air drying (Figure 10B), the mixture solution of drug/vaccine and polymers is first dropped on the plate to obtain small droplets. Then it is shaped to the microneedle via air-warm blowing. The major drawback of this fabrication method is bioactivity lost via air-warm blowing and time consumption. However, it creates a minimal waste of drugs/vaccines and polymers [63]. Other fabrication methods are drawing lithography (Figure 10C) and 3D printing (Figure 10D). However, they are unsuitable for protein/vaccine MNs preparation because heat temperature during preparation may destroy protein/vaccine structure [76].

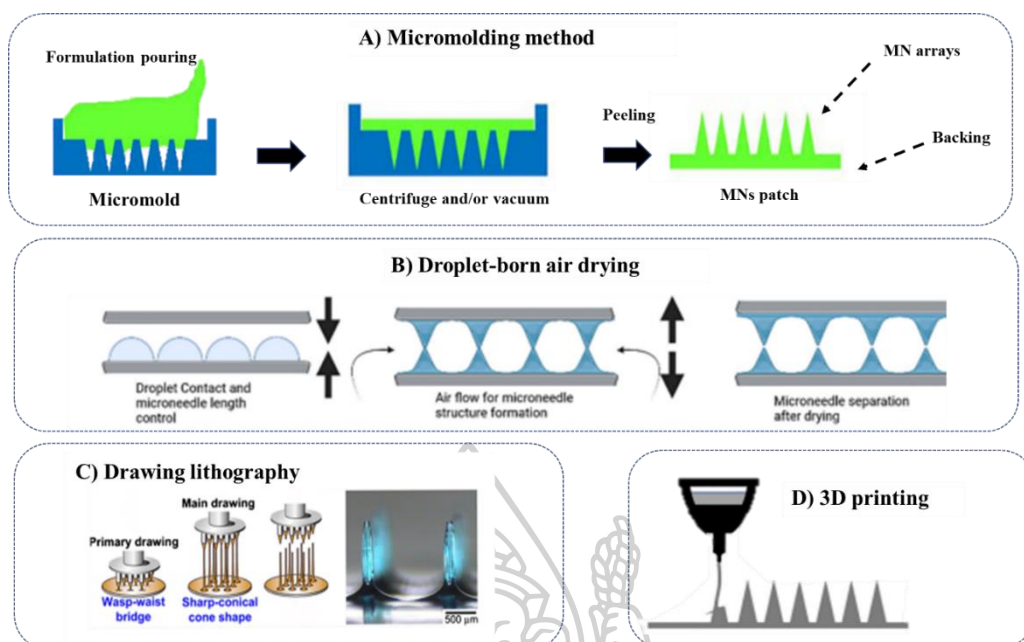


Figure 10 Methods of dissolving MNs fabrication [76, 77].

2.2.2.4 The fabricated polymer for dissolving MNs preparation

Both natural and synthetic polymers are widely used for MN fabrication due to their solubility, biodegradability, and biocompatibility for the skin and systemic. Natural polymers such as CS, trehalose, carboxymethylcellulose, and hyaluronic acid are commonly used for vaccine-loaded dissolving MNs because they are low-cost and non-toxic [77]. CS and trehalose exhibit stabilizer properties for vaccine antigens loading in MNs. Moreover, using CS as MNs patches shows the adjuvant property for vaccine delivery [78]. The major limitation of a natural polymer-forming dissolving MNs patch is poor mechanical strength or sharpness for skin insertion [79]. For synthetic polymers, such as poly(methyl vinyl ether-co-maleic anhydride) (GantrezTM, Ashland Corporation, UK), PVP and PVA are widely used for dissolving MNs patch preparation (Figure 11). The advantage of synthetic polymers-forming dissolving MNs compared to natural polymer-forming dissolving MNs is strongly resisted compression forces. PVA is an approved safe polymer for pharmaceutical excipients by the Food and Drug Administration of the United States of America. PVA MNs patch shows rapid and high water uptake for super-swelling lead to rapidly dissolve in a wide range of pH after administration for 20 min in *ex vivo* porcine skin and 15 min in monkeys [75, 80]. Therefore, PVA is the polymer that was used mostly, about

15.2% of total vaccine-loaded dissolving MNs formulations reported (PubMed, Embase, Web of Science and BioRxiv, 2021) [76]. Moreover, PVA is compatible with various natural polymers (such as CS and trehalose), enhances plasticity, and improves the mechanical strength of MNs patch [78, 81]. The mechanical strength, swelling properties, and dissolution rate of PVA MNs patch are depended on PVA molecular weight, the percentage of hydrolysis contained, and the ratio of PVA in the formulation [79, 82].

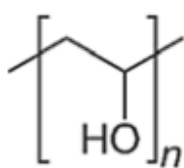


Figure 11 Structure of PVA [76].

2.2.2.5 Separating MNs patch

The conventional dissolving MNs patches are fabricated as the backing layer are the same and combined with MNs arrays, which is simple for fabrication. However, several publications found that conventional dissolving MNs need a long wearing time until the base of the patch completely dissolves for drugs/vaccine release, resulting in poor patient compliance and low efficacy [18, 83-85]. For example, a PVA MNs patch required a wearing time of 24 h for vaccination in mice [84], and CS MNs required four days of wearing time [18].

Separating-dissolving MNs patch or separating MNs patch is one type of developed dissolving MNs consisting of two parts separately, dissolving MN array and attachable backing layer (Figure 12). Drugs/vaccines are incorporated only in the dissolving MN array, not into the backing layer, which protects drugs/vaccines lost from the MN array to the outside, leading to accurate dosing without vaccine loss. After applying the MNs patch into the skin, the dissolving MN array absorbs the skin's interstitial fluid and gradually dissolves. The backing layer can detach from the arrays via various mechanisms, especially via different physical or polarity properties between the dissolving MN array and the backing layer. Then, the backing layer is

peeled off, leaving only the dissolving MN array inserted in the skin. This process can decrease the wearing time for MNs patch application and improve patient compliance [85]. An increase in the dissolving rate of the MNs array can reduce wearing time. Therefore, porous creation in a polymer matrix has been studied. The chemical substances that emit CO_2 in the polymer solution were used to induce porous in the polymer matrix after drying [13, 86]. NaHCO_3 is one of the common substances used in creating CO_2 . The number of porous produced in the polymer matrix depends on the concentration of NaHCO_3 added in the formulations. The high porous number increase surface area for water absorption resulting in increased swelling property and dissolving rate of MNs. NaHCO_3 mixing into CS matrix enhanced the dissolution property of CS in PBS pH 7.4 at 37°C , as a stimulated systemic interstitial fluid condition [17, 87]. Adding NaHCO_3 in the formulations is also a benefit in increasing the dissolving rate of the polymer matrix in separating MNs patch, reducing a wearing time [88]. However, porous containing in MNs polymer matrix decreases the mechanical strength property of the MNs array; therefore, the mechanical strength property and penetration ability need to be concerned, adjusting the suitable amount of the porous-creating substances [87].

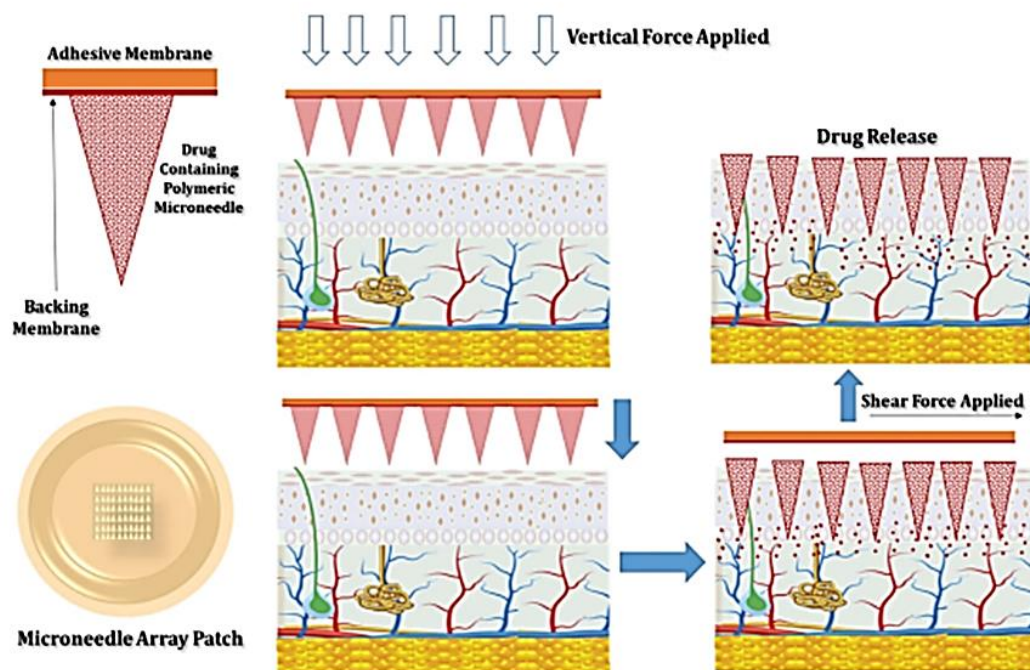


Figure 12 Structure and mechanisms of separating MNs patch [79].

2.2.2.6 Characterizations evaluation of dissolving MNs patch

Several publications demonstrated that not only vaccine contents and stability need to be evaluated, but characterizations and properties of dissolving MNs also affected vaccine delivery and immune stimulation. However, the standardized requirement of dissolving MNs patches is not specified, and no drug formulation monograph has been published in the pharmacopeias. Therefore, the reviews concluded the typical characterizations and properties of dissolving MNs patch reported in previously studies [14, 89]. We summarized the characterizations evaluation of dissolving MNs patch as follows;

2.2.2.6.1 Morphology

The morphology of MNs (Figure 9A), including shapes, height, base width, and the interspace between each MN array, are factors that affect skin insertion and vaccine delivery. For example, pyramidal and conical shapes showed suitable mechanical strength property against compression forces during MNs application into the skin. MN array should be sharp and strong enough to penetrate the skin easily. The height increase can increase vaccine loading but induce pain and extend skin-resealing duration after the application increasing the rate of skin infection. Minimal pain is generally obtained when MN height is less than 750 μm . The decrease in an interspace (base to base of the needles) as a densely MN array can create many microholes on the skin, enhancing delivery amount. However, the insertion force required increasing to achieve the skin insertion. Digital light microscopy and SEM are generally used to evaluate the MNs morphology. Digital light microscopy is a simple method typically used for pre-formulation screening. Then, SEM, a powerful magnification tool, is used to confirm the morphology and structure of a final formulation [89, 90].

2.2.2.6.2 Mechanical strength property

Mechanical strength properties must be analyzed to ensure they can resist desired compression forces. Generally, the MNs patches should be resisted the axial compression force at 0.089 N per MN array, as the maximum compression force of a human's thumb for applying MNs on the skin [91]. After the compression forces are applied, each compressed MNs is inspected for its morphology to assess any

deformation (bending or fracturing) and the reduction of needles height compared to morphology and height at the initial of the study. MN array with no fracture and slight height reduction, especially at 0.089 N/array indicating desirable mechanical strength property, shows the possibility to penetrate the skin. The mechanical strength property of MNs patch depends on the type of polymer used in MNs patch fabrication and the geometries of MNs [65]. For example, Aung *et al.* (2020) demonstrated that the cone-shaped dissolving MNs patches with $\sim 600 \mu\text{m}$ height were successfully inserted into porcine skin while they had 15-20% height reduction at 0.089 N/array without fracturing in mechanical strength evaluation [92].

2.2.2.6.3 Skin insertion property

The length of the MNs should be designed to penetrate the stratum commune and deliver vaccines into viable epidemic and dermis. The human skin is most suitable for evaluating transdermal delivery; however, its application has been limited by ethical approval and regulatory issues. Porcine and mice skin are alternatives as they have histologically (stratum corneum and viable epidemic) similar to human skin with dermis thickness of about 26 to 2,000 μm in porcine skin, 5 μm and 1,000 μm in mice skin, and 205 μm and 3,000 μm in human skin [64]. Therefore, porcine and mice skin are widely used as model skins for MNs penetration in *ex vivo* and *in vivo* studies. For skin insertion following the MN patch application is confirmed by colored dying, such as methylene blue, to stain the skin's surface and then imaging or measuring the color penetrated the skin. The microholes that appear will be compared to the total number of needles in the patch [63, 64]. However, in some cases, fluorescein was used to measure microholes number when the polymer interfered with the microhole detection [57].

2.2.2.6.3 Delivery efficiency and detaching time evaluation

For separated MNs patch, after MNs penetration into the skin, the MNs array absorbs skin's fluid, dissolves for drugs/vaccines release, and then detaches from the backing layer [15]. The percentages calculated from the amount of vaccine accumulated in the skin after the MNs patch application per the amount of vaccine loaded in separated MNs patch before application are shown "delivery efficiency, DE". However, measuring the amount of vaccines in the skin is challenging.

Therefore, the indirect method is an alternative. The total amount of vaccine in formulation before application minus the residue vaccine in formulation after an application was used to represent the amount of delivered vaccine in the skin [88].

The duration time of applying the MN patch has affected the DE. The duration time of applying that shows complete separation and exhibits the highest DE is called "detaching time". Clinically, a separated MNs patch is worn on the skin until complete separation. Therefore, if wearing time can be reduced, patient compliance will be increased [85, 88].

2.2.2.6.4 Safety evaluations

Not only immune stimulation efficiency is needed to evaluate, but safety is also required for adequate ID immunization with MNs patch. Skin resealing and AEs are major requirements. AEs of the skin are evaluated based on erythema and edema reactions after application. The level of AEs is indicated by the Draize method, as shown in Table 2 [90]. The Draize scores < 2 means mild, 2-5 means moderated, and >5 means severe in skin AEs [93]. The function of MNs is to penetrate the stratum corneum as a major skin barrier that may lead to a risk of skin infection and transepidermal water loss. The created microhole on the stratum corneum should quickly reseal to avoid toxic substances permeation, control skin infection, and prevent water loss from the skin. The skin resealing time after MNs patch removal depends on the polymer-forming MNs patch and geometries of MNs patch, such as length, width, shape, and number [90]. After MNs application, the lamellar body organelle of viable keratinocytes in the skin granulosum layer is stimulated and then secretes neutral lipids and antimicrobial peptides for skin resealing and infection protection [94]. Several previous studies demonstrated that skin resealing and recovery in skin AEs was completed recover within 24 to 48 h after MNs removal and did not show severe AEs or infection [95-97].

Table 2 Draize grading scores [93].

Description	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate-to-severe erythema	3
Severe erythema (beet redness) to slight eschar formations (Injuries in depth)	4
Edema	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edge of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

2.2.2.6.5 Stability

The stability of vaccine-loaded dissolving MNs kept in a suitable container at storage conditions of 4°C and accelerated condition storage of 25°C, or higher is evaluated in the sterile condition, following ICH and WHO guidelines [98, 99]. Moreover, the stability evaluation is divided into antigen stability and MNs stability. Antigen stability is determined by the vaccine content and immune stimulation efficacy of vaccine-loaded dissolving MNs [14]. Several methods, such as ELISA, plaque-forming assay, RT-qPCR, etc., are used to evaluate antigen stability. These methods are selected based on the types of vaccine antigens [78, 100]. In addition, for MNs stability, the physical morphology and mechanical strength properties are evaluated as previously described.

Chapter 3

MATERIALS AND METHODS

3.1 Materials

- 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDAC) (Sigma-Aldrich[®], St. Louis, MO, USA)
- 6-maleimidohexanoic acid (MHA) (Sigma-Aldrich[®], St. Louis, MO, USA)
- Ammonium-Chloride-Potassium (ACK) lysing buffer (GIBCO[™], New York, USA)
- Bovine serum albumin (BSA) (Sigma-Aldrich[®], St. Louis, MO, USA)
- Chitosan (CS, degree of deacetylation = 0.95, molecular weight = 11 KDa) (OliZac Technologies Co., Ltd., Pathumthani, Thailand)
- Dulbecco's modified eagle medium (DMEM) (GIBCO[™], Grand Island, New York, USA)
- Fetal bovine serum (FBS) (GIBCO[™], New York, USA)
- Fluorescein conjugated bovine serum albumin (FITC-BSA) (Sigma-Aldrich[®], St. Louis, MO, USA)
- Japanese encephalitis chimeric virus vaccine (JE-CV) (Imojev[®]) (GPO-MBP, Chachoengsao, Thailand)
- KAPA SYBR Universal One-Step qRT-PCR (Sigma-Aldrich[®], St. Louis, MO, USA)
- L-Cysteine hydrochloride monohydrate (Sigma-Aldrich[®], St. Louis, MO, USA)
- Mouse IFN- γ High Sensitivity ELISA kits (R&D Systems, Minnesota, USA)
- Mouse IL-4 High Sensitivity ELISA kits (R&D Systems, Minnesota, USA)
- N-hydroxysulfosuccinimide (NHS) (Sigma-Aldrich[®], St. Louis, MO, USA)
- Poly (vinyl alcohol) (PVA; MW = 85,000–124,000 Da) (Sigma-Aldrich[®] St. Louis, MO, USA)
- Recombinant E protein of Japanese encephalitis virus (MybioSource, California, USA).
- The goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (Invitrogen, New York, USA)

- The goat anti-mouse IgA horseradish peroxidase (HRP) conjugate (Invitrogen, New York, USA)
- TMB reagent, HRP substrate (Abcam[®], Cambridge, UK)
- Rhesus monkey kidney epithelial cells (LLC-MK2 cells) (American Type Culture Collection, ATCC, USA)
- Roswell Park Memorial Institute-1640 (RPMI-1640) medium (GIBCO[™], New York, USA)
- Sodium tripolyphosphate (TPP) (Sigma-Aldrich[®], St. Louis, MO, USA)
- Viral RNA extraction kit (Omega Bio-tek, Georgia, USA).
- All other chemicals were of molecular biology quality

3.2 Equipment

- Analytical balance (Sartorius CP224S; Scientific Promotion Co., Ltd., Bangkok, Thailand)
- Autoclave (Model:LS-2D; Scientific Promotion Co., Ltd, Thailand)
- Confocal laser scanning microscope (CLSM) (LSM900, Zewass, Germany).
- Cellulose membrane (CelluSep[®]; molecular weight cut-off 3500 Da)
- Centrifuge machine (5340R, Eppendorf, Germany)
- Centrifuge machine (PK 121R, ALC international, UK)
- Digital microscope (AM7915 series, Dino-Lite, Taiwan)
- Dino-capture 2.0 software (Dino-Lite, Hsinchu, Taiwan)
- Dynamic light scattering particle size analyzer (Zetasizer Nano ZS, Malvern Instrument, Worcestershire, UK).
- Fourier transform infrared spectroscopy (FTIR; Nicolet was5, Thermo electron Corporation, USA)
- Fluorescence stereomicroscope (Leica M60, Leica Microsystems, Germany)
- Franz diffusion cell; a volume of receiver 6 mL
- Freeze dryer (FreeZone^{2.5}, Labconco, USA)
- Incubator CO₂ chamber (Heraeus HERA Cell 240, Heraeus Holding GmbH., Germany)
- Laminar airflow (BIO-II-A, Telstar Life Science Solutions, Spain)
- LAS X software (3.6 version, Leica Microsystems, Germany)

- Leica CM1850 cryostat (Leica Biosystems, Germany).
- Nuclear magnetic resonance spectroscopy (NMR; AVANCE III HD, Bruker, USA)
- pH meter (Mettler Toledo, Switzerland)
- Polystyrene microplates, 96 wells (R&D Systems, Minnesota, USA)
- Scanning electron microscope (SEM; Mira TC, Czech Republic)
- Texture Analyzer TA-XT plus (Stable Micro System, Haslemere, UK)
- Universal microplate reader (Victor Nivo™, PerkinElmer Inc, USA)

3.3 Methods

3.3.1 Qualifications of live attenuated JE-CV in Vero cells

3.3.1.1 Monolayer cell culture

LLC-MK2 cells grown in six-well plates were cultured with completed DMEM supplemented with 10% FBS and penicillin/streptomycin in air containing 5% CO₂ at 37°C.

3.3.1.2 Plaque-forming assay

JE-CV (10^4 - $10^{5.8}$ PFU/0.5 mL/vial; Imojev®, GPO-MBP, Thailand) was weighted and evaluated the plaque content on LLC-MK2 cells monolayer with neutral red staining [98]. Briefly, JE-CV was serially diluted 10-fold with completed DMEM. One milliliter of each dilution was added into LLC-MK2 cells monolayer (confluency $\geq 90\%$) at 37°C, 5% CO₂ for 90 min. Then, each well was overlaid with 3% carboxymethyl cellulose with neutral red added. Plates were incubated at 37°C under the atmosphere containing 5% CO₂ for 5-7 days before plaque counting. Theoretically, in the neutral red assay, alive cells incorporate neutral red into their lysosomes, and the unstained plaques represent cell death (infected cells). Therefore, plaque formations (groups of dead cells on monolayer cells) appeared as clear circles against a pink background of live cells. The dilution of JE-CV that shows 60-40 PFU/well was measured and calculated accurately for vaccine content according to equation 1.

$$\text{Vaccine content (PFU/mL)} = P \times F \quad (1)$$

Where P is the appeared number of plaque-forming (PFU/mL), and F is the dilution factor.

3.3.2 Development of JE-CV-loaded CS-based NPs for intranasal immunization

3.3.2.1 Synthesis and characterization of CM

CM was synthesized by a chemical reaction between CS and MHA. The synthesized polymer was confirmed as previously described [9]. Briefly, 0.5 mg/mL of CS in 1%v/v HCl was reacted with an excess of MHA using NHS and EDAC as catalysts at room temperature for 18 h. The synthesized product was confirmed using NMR and FTIR after dialysis and lyophilization. The maleimide content on the CS backbone was determined by a modified Ellman's assay.

3.3.2.2 Preparation of FITC-BSA-loaded CS-NPs or CM-NPs

CS-NPs and CM-NPs were prepared by the ionic gelation method [59]. First, the polymers were separately dissolved in 1%v/v acetic acid to obtain 3 mg/mL of polymer solutions. Then, 1 mg/mL of TPP in deionized water was added into the polymer solutions with equal volume under gentle stirring at room temperature. After that, the NPs suspension was freeze-dried to obtain a dry powder. For loading, FITC-BSA as an antigen model was mixed with NPs at different mass ratios of 1:2 to 1:8 in simulated nasal fluid (SNF at pH 5.8 was composed of 8.77 mg/mL NaCl, 2.98 mg/mL KCl, and 0.59 mg/mL CaCl₂) [61, 99]. The mixtures were incubated at 4°C for 6 h and then collected for future studies.

3.3.2.3 Characterization of FITC-BSA-loaded CS-NPs or CM-NPs

3.3.2.3.1 Size and zeta potential measurements

The particle size and zeta potential of the NPs were investigated by photon correlation spectroscopy using Zetasizer Nano ZS. Each formulation was diluted with DI water to obtain 1 mL of the mixture. All measurements were carried out in triplicate under ambient conditions.

3.3.2.3.2 Loading efficiency and loading capacity

LE and LC were determined by an indirect method [61]. Briefly, 1.5 mL of FITC-BSA-loaded NPs were centrifuged at 14,000 rpm, 4°C for 30 min. The supernatant was then collected. The amount of free FITC-BSA (unloaded into NPs)

was determined using fluorescence spectrophotometry at $\lambda_{\text{excitation}} = 483 \text{ nm}$ and $\lambda_{\text{emission}} = 538 \text{ nm}$.

The percentage of FITC-BSA loading efficiency (%LE) and loading capacity (%LC) was calculated according to equations 2 and 3, respectively [58].

$$\% \text{ LE} = \left(\frac{A_{\text{total}} - A_{\text{free}}}{A_{\text{total}}} \right) \times 100 \quad (2)$$

Where A_{total} is the amount of the total FITC-BSA in the formulation (mg), and A_{free} is the amount of free FITC-BSA after loading (mg).

$$\% \text{ LC} = \left(\frac{A_{\text{total}} - A_{\text{free}}}{A_{\text{NPs}}} \right) \times 100 \quad (3)$$

Where A_{total} is the amount of the total FITC-BSA in the formulation (mg), A_{free} is the amount of free FITC-BSA after loading (mg), and A_{NPs} is the total weight of NPs (mg).

3.3.2.4 Preparation and characterization of JE-CV-loaded CS-NPs or CM-NPs

The FITC-BSA-loaded NPs with the highest %LE and %LC were selected to prepare JE-CV-loaded CM-NPs and CS-NPs. The particle size and zeta potential were characterized using Zetasizer Nano ZS. The amount of live attenuated JE-CV after loading into the CM-NPs and CS-NPs was evaluated using RT-qPCR [100]. Then, the %LE and %LC of each formulation were calculated as previously described in equations 1 and 2. The amount of FITC-BSA (mg) was changed to PFU, and in some cases, the unit of LC was explained as PFU/mg.

3.3.2.5 Mucoadhesive and uptake evaluation of FITC-BSA-loaded CS-NPs or CM-NPs

3.3.2.5.1 Preparation of *ex vivo* porcine nasal mucosa

Ex vivo porcine nasal mucosa was used for mucoadhesive and FITC-BSA uptake evaluation [101]. Briefly, the porcine nasal mucosa obtained from a local slaughterhouse was immediately removed, washed with cold PBS (pH 7.4), and refrigerated at 4°C until used (within 6 h).

3.3.2.5.2 Evaluation of mucoadhesive property

Mucoadhesive properties of FITC-BSA-loaded NPs on *ex vivo* porcine nasal mucosa were evaluated by a flow-through method using FITC-BSA as a model FITC-BSA [101, 102]. Briefly, 20 µl of FITC-BSA formulation was dropped on 1x1 cm² of porcine nasal mucosa attached to a plate and fixed at a 40° angle relative to the horizontal plane. SNF was then flushed over the nasal mucosa surface with a continuous rate of 5 mL/min to mimic the washing process of the nasal cavities [99]. At different time points and volumes of flushing, including 0 min (0 mL), 10 min (50 mL), 20 min (100 mL), 40 min (200 mL), 60 min (300 mL), and 80 min (400 mL), fluorescence images of the whole nasal mucosa were captured using a fluorescence stereomicroscope. LAS X software was used to measure FI, which linearly correlated with the amount of FITC-BSA remaining on the nasal mucosa [102].

The percentage of FITC-BSA remaining was calculated as follows:

$$\text{Remaining (\%)} = \frac{(FI_t - FI_{\text{blank}})}{(FI_0 - FI_{\text{blank}})} \times 100 \quad (4)$$

Where FI_{blank} is the FI of nasal mucosal before dropped FITC-BSA.

FI_0 is the FI of nasal mucosal after dropped FITC-BSA (at zero time point of washing).

FI_t is the FI of nasal mucosal after washing at different time points.

To compare the mucoadhesive property of FITC-BSA formulations, including FITC-BSA alone, FITC-BSA-loaded CS NPs, and FITC-BSA-loaded CM NPs, the Wash Out₅₀ (WO₅₀) quantitative method was used [103]. WO₅₀ is defined as the volume of SNF required to wash out 50% of the formula from the mucosa.

3.3.2.5.3 Evaluation of uptake property

Ex vivo FITC-BSA uptake was evaluated using Franz diffusion cells [104]. Briefly, *ex vivo* porcine nasal mucosa was prepared as described above and placed between the donor and the receptor compartments, and the cell was affixed using clamps. The receiver compartment was filled with degassed PBS and kept under gentle agitation at 50 rpm. FITC-BSA formulations, including FITC-BSA-loaded CS NPs, FITC-BSA-loaded CM NPs, and FITC-BSA alone, were separately added to the

donor compartment. After adding the FITC-BSA formulations for 15 min, the nasal mucosa was collected and then washed with SNF to remove excess formulation. The mucosa tissue was fixed with 10% formalin and embedded in a cryostat embedding medium. The fixed tissue was kept at a temperature lower than -30°C before performing microtomy using a cryostat (tissue thickness of $5\text{ }\mu\text{m}$). Tissue sections were mounted on the slides, and the amount of FITC-BSA uptake was evaluated using a CLSM.

3.3.3 Development of JE-CV-loaded separating CS-based MNs patch

3.3.3.1 Preparation of polymers blended solutions

A mixture of CS and PVA was prepared at the mass ratio of 1:4 and then mixed with NaHCO_3 to obtain the slurry of polymers solution at different mass ratios of 1:4:0, 1:4:0.25, and 1:4:0.5, respectively.

3.3.3.2 Fabrication of unloaded CS-based MNs patch (CS-MNs patch)

The fabrication process was carried out in the cleanroom, which maintained the temperature and percent relative humidity (%RH) at $5\pm 3^{\circ}\text{C}$ and $75\pm 2\%\text{RH}$, respectively. Each mass ratio of polymer blended solution (1 g) was weighed and cast onto a laser-engineered silicone micromold with a patch of 121 (11×11) cone-shaped needles (height $650\text{ }\mu\text{m}$, base width $300\text{ }\mu\text{m}$, and interspacing diameter $300\text{ }\mu\text{m}$). MNs micromolds were centrifuged at 4,000 rpm, 4°C , for 30 min to fill the polymer solution into the MN cavities. CS-MNs patches were then allowed to form gel overnight in a desiccator at 4°C . Afterward, the whole system was freeze-dried under 0.1 mBar at -50°C . Finally, the dried CS-MNs patch was gently peeled out of the mold and kept in a desiccator chamber at 4°C until use.

3.3.3.3 Fabrication of JE-CV-loaded CS-MNs patch

The fabrication processes were conducted the same as 3.3.3.2 (fabrication of unloaded CS-MNs patch); however, the processes were performed under biosafety cabinet class II to prevent the spreading of the viral vaccine into the environment and to keep the efficacy of the viral. The blended polymer solutions prepared were sterilized under UV light and cooled until they reached 4°C before mixing with JE-CV. JE-CV vaccine was mixed with blended polymer solutions (as previously described in 3.3.3.1) at various mass ratios, including 0:1, 1:3, 1:4, and 1:5, respectively. Finally, the dried JE-CV loaded CS-MNs patch was gently peeled out

from the mold and kept in a desiccator chamber at 4°C until use. In some formulations, FITC-BSA was used as an FITC-BSA model instead of JE-CV.

3.3.3.4 Fabrication of JE-CV-loaded separating CS-based MNs patch

JE-CV-loaded separating CS-based MNs patch (JE-CV-loaded sCS-MNs patch) were fabricated in the same process as JE-CV-loaded CS-MNs patch. Additionally, after allowing for the gel-forming of formulations in a desiccator at 4°C, residual gel on the micromold was removed using a spatula. The cellulose membrane as a backing layer was moistened with DI water and aligned on the surface of micromold. Afterward, the whole system was freeze-dried under 0.1 mBar at -50°C. Finally, the dried sCS-MNs patch was gently peeled out of the mold and kept in a desiccator chamber at 4°C until use. In some formulations, FITC-BSA was used as an FITC-BSA model instead of JE-CV.

3.3.3.5 Characterization of MNs patch

3.3.3.5.1 Morphology of MNs patch

The morphology of all MNs formulations was evaluated using a dino-lite digital microscope. Dimensions of needles (height, width, and interspace) were measured using Dino-capture software. Moreover, the scanning electron microscope (SEM) was used to confirm the structure of the needles.

3.3.3.5.2 Evaluation of mechanical strength of MNs patch

The compression mode of the texture analyser was used to evaluate the mechanical strength of MNs patch. The height of the needles was measured using a digital microscope before and after compression. Briefly, the MNs patch was carefully attached to the moveable probe using adhesive tape. The known axial compression forces of 3.3888 N to 13.31 N per 121 needles (0.028 N to 0.11N per needle) at a speed of 0.5 mm/s and the trigger force of 0.049 N are applied. The initial height of the needles (H_1) and changes in the height of the needles after compression (H_2) were evaluated using the digital microscope [95]. The mean percentages reduction height of needles was calculated as follows:

$$\text{Reduction height (\%)} = \frac{H_1 - H_2}{H_1} \times 100 \quad (5)$$

The percentage reduction height against applied forces was plotted to evaluate the mechanical strength.

3.3.3.5.3 *In vitro* skin insertion study

Neonatal porcine skin was used for insertion study as an alternative to human skin [95]. Briefly, excised whole neonatal porcine skin samples were obtained from stillborn piglets. The subcutaneous layer was carefully removed, and the skin was cleaned and then equilibrated with PBS (pH 7.4) for 1 h. MNs patches were separately inserted into the neonatal porcine skin using thumb pressure for 30 s. Following the removal of MNs patch from the skin, 1% methylene blue solution was stained on the application area for 10 min. Any excess methylene blue was removed using PBS. The microholes created by MNs were then captured and counted under a digital microscope. Finally, the penetration efficiency was calculated as follows:

$$\text{Penetration efficiency (\%)} = \frac{\text{Number of created microholes}}{\text{Number of needles}} \times 100 \quad (6)$$

3.3.3.5.4 Swelling and erosion determinations

Samples of each type of CS-MNs patch ($1 \times 1 \text{ cm}^2$) were weighed before immersion into a PBS buffer (pH 7.4) at 37°C under gentle agitation for 50 rpm. The samples were removed from the buffer at different intervals; 15 min, 30 min, 1, 2, 4, 8, 12, and 24 h. The absorbed excess moisture on the surface was removed using filter paper. The samples were weighted to determine the percentage swelling of CS-MNs patch, which was calculated as follows (7). Then, the samples were dried in the dedicator until saturated and weighted. The dried mass of each sample after immersion was calculated as the percentage of erosion [105] as shown in equation (8):

$$\text{Swelling (\%)} = ((W_t - W_i) / W_i) \times 100 \quad (7)$$

$$\text{Erosion (\%)} = \frac{(W_d - W_i)}{W_i} \times 100 \quad (8)$$

Where W_i is the initial dry mass of the CS-MNs patch.

W_t is the mass of the CS-MNs patch each time of immersion.

W_d is the dried mass of the CS-MNs patch after immersion.

3.3.3.5.5 Antigen content in separating CS-based MNs patch

The amount of FITC-BSA and JE-CV were evaluated from FITC-BSA-loaded MNs and JE-CV-loaded MNs. The sCS-MNs patch was dissolved for antigen content evaluation according to a previous study [106]. FITC-BSA-loaded sCS-MNs were dissolved in 10 mL of PBS maintained at 37°C and kept under gentle agitation at 50 rpm for 1 h. All samples were mashed through a 70 µm strainer and centrifuged at 14,000 rpm for 30 min at 4°C for supernatant collection. The concentration of dissolved FITC-BSA in PBS was measured using fluorescence spectrophotometry, and the amount of FITC-BSA in MNs patch was calculated. In the case of JE-CV-loaded sCS-MNs, the study was performed under sterile conditions. JE-CV-loaded sCS-MNs were dissolved in completed DMEM maintained at 4°C, then JE-CV was collected according to a previously described. JE-CV in each sCS-MNs patch was evaluated using RT-qPCR or plaque-forming assay as previously described. %LE and %LC of FITC-BSA or JE-CV in each formulation were then calculated.

3.3.4 *In vivo* study

For immunization study, C57BL/6 and ICR mice were used for *in vivo* studies. For C56BL/6, female-specific pathogen-free inbred mice aged 3-4 weeks were purchased from Nomura Siam International Co., Ltd., Thailand. On arrival, the mice are randomly divided into six groups, with six mice per group, as shown in Table 3. To adequate acclimation, mice were housed in a temperature-controlled room (24 ± 2°C) with 12/12 h of light/dark cycle and free access to food and water at least one week before the immunization study.

For the evaluation of MN patches study, female ICR inbred mice aged 3-4 weeks were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The mice were housed in the same conditions as C56BL/6 mice for *in vivo* insertion, skin reaction, and resealing studies.

All *in vivo* studies were approved by the animal studies ethics committee, faculty of pharmacy, Silpakon University (Project no. 004/2562).

Table 3 Immunization study (n=6).

Groups	Routes and formulations	PFU of JE-CV / mouse
1	IN, JE-CV solution	1×10^4
2	IN, JE-CV-loaded CS NPs	1×10^4
3	IN, JE-CV-loaded CM NPs	1×10^4
4	IN, Unloaded CS NPs (blank)	0
5	IN, Unloaded CM NPs (blank)	0
6	ID, JE-CV-loaded sCS-MNs patch	5×10^3
7	ID, Unloaded sCS-MNs patch (blank)	0
8	SC, JE-CV solution (half-dose)	1×10^4
9	SC, JE-CV solution (full-dose)	1×10^4
10	SC, 0.4%w/v NaCl (conventional vehicle)	0

3.3.4.1 *In vivo* insertion study of CS-MNs patch and sCS-MNs patch

The optimal formulation of CS-MNs patch and sCS-MNs patch were selected and then loaded with FITC-BSA for *in vivo* insertion study. Briefly, ICR mice were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal (IP) injection. Both FITC-BSA-loaded CS-MNs patch and FITC-BSA-loaded sCS-MNs patch, the formulations were applied to the hairless back of each mouse group by thumb pressing for 30 s. The adhesive film was then applied to prevent the mice from removing the sCS-MNs patch. Then, the formulations were worn on the skin at various time points (0.5, 1, 2, 4 and 8 h) after sCS-MNs patch application. At designated time points, the embedded MNs were removed, the microholes created on the skin were stained with methylene blue solution and then imaged. These microholes that appeared were calculated for *in vivo* penetration efficiency as previously described (equation 5). After applying on the mice's skin, the FITC-BSA-loaded CS-MNs patch was dissolved [95, 107] and the FITC-BSA-loaded sCS-MNs patch was separated from its backing [79]. The residue of both MN patches following the application was observed using a digital microscope. The time that needles of sMNs are completed separately from the backing layer is called "detaching time". The obtained detaching time was used for removing of MNs patch in the ID

immunization study. Moreover, after the administration, the residue MNs patch was evaluated FITC-BSA amount as a residual FITC-BSA by calculating the delivery efficiency of both the CS-MNs patch and sCS-MNs patch [88], as shown in equation 9.

$$\% \text{ Delivery efficiency} = [(A_{\text{total}} - A_{\text{residue}}) / A_{\text{total}}] \times 100 \quad (9)$$

Where A_{total} is the total amount of FITC-BSA in MNs patch before application, A_{residue} is the amount of FITC-BSA in MNs patch after application.

3.3.4.2 *In vivo* skin reaction and resealing

In this study, unloaded and JE-CV-loaded MNs were applied on each side of ICR mice with a similar procedure as *in vivo* insertion study [93, 95]. At detaching time points of 0, 1, 3, 6, 12, and 24 h, sCS-MNs patches were peeled. The administration sites after MNs patch application evaluated skin reaction and skin resealing. Briefly, the administration area was captured before and after to assess skin reaction using the Draize method based on erythema and edema [96, 97]. The application site was then stained with 1% methylene blue solution to observe microholes created. Finally, a digital microscope was used to capture and examine the skin recovery itself.

3.3.4.3 Immunization and sample collection

C56/BL6 mice were immunized with different formulations and administration routes, as shown in Table 3. The positive control group was C57/BL6 mice immunized with one dose of JE-CV (10^4 PFU) per mouse via SC. This immunization and sample collection schedule were performed according to a previous study [40], which demonstrated complete protection against JEV challenges after 28 days of SC immunization.

For IN immunization, mice were deprived of food for 2 h before anesthesia with IP injection of ketamine and xylazine combination. Briefly, mice were handled in a supine position. A micropipette was then inserted into the nostril to a depth of at least 0.2 cm to inject the vaccine formulation into the nasal cavity. The formulation (10 μ L/drop) was alternately administered through each side of the nostril. The new

drop was administered after a waiting time of 15 min following the previous administration. Mice were administered with JE-CV to obtain 10^4 PFU per mouse. For ID immunization, mice were anesthetized with a ketamine and xylazine combination prior to applying of sCS-MNs patch on mice.

Administration of JE-CV and 0.4% NaCl via SC were used as positive and negative control, respectively. After that, all mice were observed changing body weight every week and compared to negative control for safety evaluation.

On day 28, mice were anesthetized with ketamine/xylazine. The mice's saliva was stimulated with pilocarpine (0.5 mg/kg) by IP injection, and the saliva was collected within 15 min after the stimulating period. Blood was obtained by cardiac puncture. Then, mice were sacrificed by cervical dislocation to collect the spleen and nasal wash. For nasal wash collection, the nasal cavity was flushed with 200 μ L of 1% FBS in PBS (PBS-F), and the same PBS-F was reused 3 times flushing. Each mucosal sample (saliva and nasal wash) was separately mixed with a protease inhibitor at 1 mM phenylmethylsulphonyl fluoride and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and stored at -80°C until use for the IgA level determination [108]. Blood samples were clotted overnight at 4°C and then serum was separated from the blood with centrifugation at 10,000 rpm for 10 min at 4°C. The serum was collected and stored at -80°C until use for plaque reduction neutralization assay [38].

3.3.4.4 IgG antibody assay

The levels of specific anti-JEV IgG antibodies in serum were measured by enzyme-linked immunosorbent assay (ELISA) [38]. Briefly, recombinant E protein (5 μ g/mL) as a JE antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6) was added to polystyrene microplates (100 μ L/well). The plates were then placed at 4°C overnight. The wells were emptied and washed 3 times with 300 μ L PBS containing 0.05% Tween 20 (PBS-T). To block the non-specific protein-surface binding, the plate was filled with 300 μ L of 1% BSA in PBS (PBS-B) for 1 h at room temperature, followed by 3-times washing with PBS-T. Each mouse's serum samples were heat-inactivated (incubated at 56°C for 30 min) and initially 100-fold diluted. Next, one hundred microliters of 10-fold serially diluted serum were added to the wells and

incubated for 2 h at room temperature. Next, the wells were washed 3 times with PBS-T and incubated with 100 μ L of 1:5000 diluted solution of goat anti-mouse IgG horseradish peroxidase-conjugated antibody for 2 h at room temperature. After 3 washes, 100 μ L of TMB substrates were added to each well and incubated for 30 min at room temperature in a dark place. The reaction was stopped by adding 100 μ L of 4 N H₂SO₄, and the absorbance was then measured at a wavelength of 450 nm using a microplate reader. The end-point IgG titers were expressed as the highest dilution of the sample that had an optical density (OD) $\geq$ 0.1 relatives to the background's OD. The mice were considered seroconverted if the OD of initial serum dilution $\geq$ 2 times of pre-immune serum of each mouse [109]. The percentage of seroconversion was calculated according to the equation:

$$\text{Seroconversion (\%)} = N_s / N_t \times 100 \quad (10)$$

where N_s is the number of seroconverted mice
 N_t is the total number of mice.

3.3.4.5 Plaque reduction neutralization test

The level of PRNT in serum against the JEV Beijing P-3 strain, a wild-type virus, was measured as the PRNT₅₀ on a monolayer of Vero cells in 6-well plates. This method was performed at the center of vaccine development, Mahidol University [98]. Briefly, heat-inactivated sera are serially diluted 10-fold. Approximately 50 PFU of JEV was mixed with each serum dilution at 37°C for 60 min. Then, the mixtures were incubated in LLC-MK2 monolayer cells and incubated at 37°C for 90 min before covering with neutral red in 3.0% carboxymethyl cellulose as an overlay medium and incubation at 37°C for 5 days before plaque counting according to previously described in the plaque-forming assay. The highest dilution titer of serum reducing 50% of PFU, compared with the PFU number in a well without a serum sample, was the PRNT₅₀.

3.3.4.6 IgA antibody assay

The levels of specific anti-JEV IgA antibodies in oronasal fluids (saliva and nasal wash) are measured by ELISA [108]. These procedures were similar to the IgG assay. Except that, the goat anti-mouse IgG horseradish peroxidase-conjugated antibody (1:5000 dilution) was changed to goat anti-mouse IgA horseradish peroxidase-conjugated antibody (1:2000 dilution), and the serum (1:100 initial dilution) was changed to saliva (1:10 initial dilution) and nasal wash (1:2 initial dilution). Saliva and nasal wash were 2-fold separable diluted for IgA evaluation with ELISA.

3.3.4.7 Cytokine evaluation

Cytokine levels are produced by splenocytes of immunized mice [38]. Briefly, the harvested spleen was crushed through a 70 μm sterilized strainer for single-cell preparation. The red blood cells were lysed with ACK lysis buffer. The cell suspensions were centrifuged at 1,500 rpm for 10 min at 4°C, and the pellets (splenocytes) were washed with PBS 3 times before collecting. The splenocytes were re-suspended to obtain 5×10^6 cells/mL in RPMI medium, which was supplemented with 10% FBS and penicillin/streptomycin. Afterward, splenocytes are stimulated with JE antigen (1 $\mu\text{g/mL}$) and incubated in 24 well-plates at 37°C with 5% CO_2 for 3 days. Using cytokine ELISA kits, the media were collected and evaluated for IFN- γ and IL-4 cytokine levels.

3.3.5. Stability study

CM- or CS-based NPs and CS-based MNs are evaluated for stabilization following ICH guideline [110]. For NPs, CS and CM NPs powder were separately contained in a light-resistant container and were stored at $5^\circ\text{C} \pm 3^\circ\text{C}$ and $25^\circ\text{C} \pm 2^\circ\text{C}$ for 3 months. The characterization of NPs stability were evaluated depending on the periods of stability study (0, 1, 2, and 3 months).

For CS-based MNs, JE-CV-loaded separating MNs were contained in a light-resistant container with a sterile condition and were stored at $5^\circ\text{C} \pm 3^\circ\text{C}$ and $25^\circ\text{C} \pm 2^\circ\text{C}$ for 3 months. The morphology and mechanical strength were evaluated depending on the periods of stability study (0, 1, 2, and 3 months). After that, it was

dissolved in completed DMEM and evaluated live attenuated JE-CV as previously described in antigen containing section.

3.3.6. Statistical analysis

All experimental measurements were evaluated in triplicate ($n=3$). The results were expressed as mean $\pm$ standard deviation (SD). Statistical difference in the results was analyzed using one-way analysis of variance (ANOVA) following Tukey's post hoc. P-values of less than 0.05 (two-tailed) were considered to indicate the statistical significance.



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Qualifications of live attenuated JE-CV in Vero cells

4.2 Intranasal immunization of JE-CV-loaded CS-NPs and CM-NPs

4.2.1 Preparation and characterization of FITC-BSA-loaded CS-NPs and CM-NPs

4.2.2 *Ex vivo* mucoadhesive property and uptake study of FITC-BSA-loaded NPs

4.2.3 Preparation and characterization of JE-CV-loaded CS-NPs or CM-NPs

4.2.4 Intranasal immunization

4.2.5 Stability test

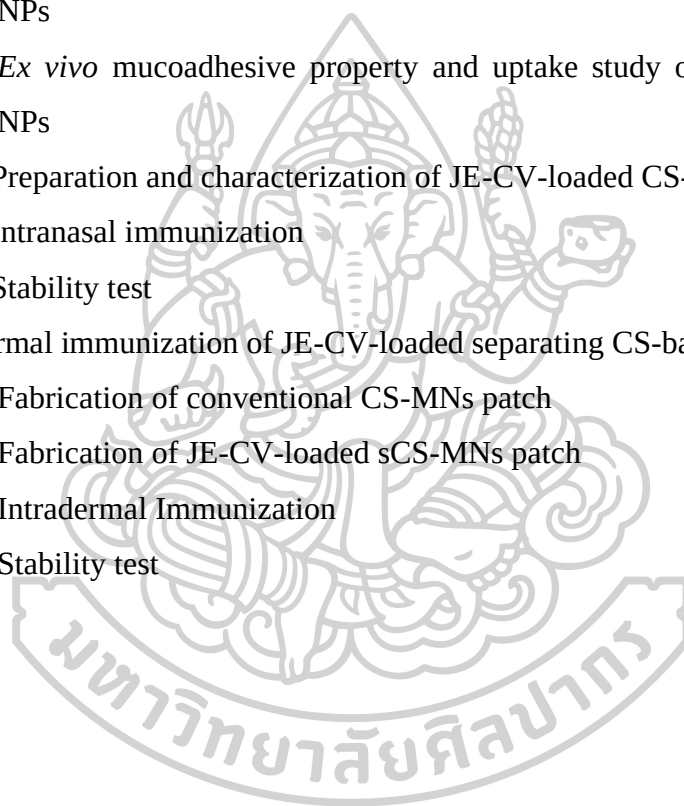
4.3 Intradermal immunization of JE-CV-loaded separating CS-based MNs patch

4.3.1 Fabrication of conventional CS-MNs patch

4.3.2 Fabrication of JE-CV-loaded sCS-MNs patch

4.3.3 Intradermal Immunization

4.3.4 Stability test



4.1 Qualifications of live attenuated JE-CV in Vero cells

The amount of live attenuated JE-CV was quantified in Vero cells as PFU. Generally, 0.5 mL of JE-CV (Imojev[®], GPO-MBP, Thailand) contains live attenuated JE-CV approximately 1×10^4 to $1 \times 10^{5.8}$ PFU/dose. Therefore, the accepted level of live attenuated JE-CV was not less than 10^4 PFU/dose [2]. The results show that JE-CV contained $51,000 \pm 9,000$ PFU/dose or approximately 5×10^4 PFU/dose that passed the criteria of JE-CV quantification. Therefore, JE-CV at the same manufacturing lot was kept at 4°C until use in all experiments.

4.3 Intranasal immunization with JE-CV-loaded CS-NPs and CM-NPs

4.2.1 Preparation and characterization of FITC-BSA-loaded CS-NPs and CM-NPs

CM, a CS derivative, was synthesized with chemical conjugation between the amino group (NH_3^+) of CS and the carboxylic group (COO^-) of maleimide. The successful synthesis of CM was confirmed by NMR and FTIR. The maleimide content on the CS backbone was quantified with a modified Ellman's assay that showed maleimide content as 463 ± 29.75 $\mu\text{mol/g}$ of the polymer. Compared with native CS, maleimide conjugated in the CS backbone resulted in higher molecular weight with a lower positive charge of synthesized CS [9].

FITC-BSA, a protein antigen model, was loaded into CS-NPs and CM-NPs preparations at different mass ratios of 1:2 to 1:8 by varying incubation times using a physical absorption technique. All formulations were characterized and measured loading efficiency and loading capacity. The characterization of all formulations is shown in Table 4. For CS- and CM-NPs, the sizes of unloaded NP were 193.1 ± 4.9 and 242.7 ± 4.0 nm, with positive surface charges of 26.3 ± 1.8 and 23.4 ± 0.7 mV, respectively. The sizes and zeta potential values of FITC-BSA-loaded CS-NPs were 352.6 ± 8.26 nm and 19.5 ± 0.9 mV and for FITC-BSA-loaded CM-NPs were 362.8 ± 7.1 nm and 18.4 ± 1.0 mV. According to the physical absorption method, NPs formed based on the interaction between amine groups (NH_3^+) as a positive charge of the CS backbone and the carboxyl groups (COO^-) as a negative charge of the protein antigen model, resulting in the size of nanoparticles increased with zeta potential decreased [61]. All NPs had a polydispersity index (PDI) of less than 0.5, indicating low varying

size distribution and without particle aggregation of all NPs formulations [58]. The morphology of NPs visualized with TEM showed that FITC-BSA-loaded CS-NPs and CM-NPs had a spherical shape, as shown in Figure 13.

Table 4 Characterizations of CS-NPs and CM-NPs unloaded and loaded with FITC-BSA.

Nanoparticles	Before loading			After loading FITC-BSA ¹		
	Size (nm)	PDI	Zeta potential (mV)	Size (nm)	PDI	Zeta potential (mV)
CS-NPs	193.1 ± 4.9	0.27 ± 0.02	27.3 ± 1.8	352.6 ± 8.3	0.32 ± 0.01	19.5 ± 0.9
CM-NPs	242.7 ± 4.0	0.32 ± 0.01	23.4 ± 0.7	362.8 ± 7.1	0.33 ± 0.03	18.4 ± 1.0

¹FITC-BSA-loaded NPs at 1:6 ratio for 1 h incubation

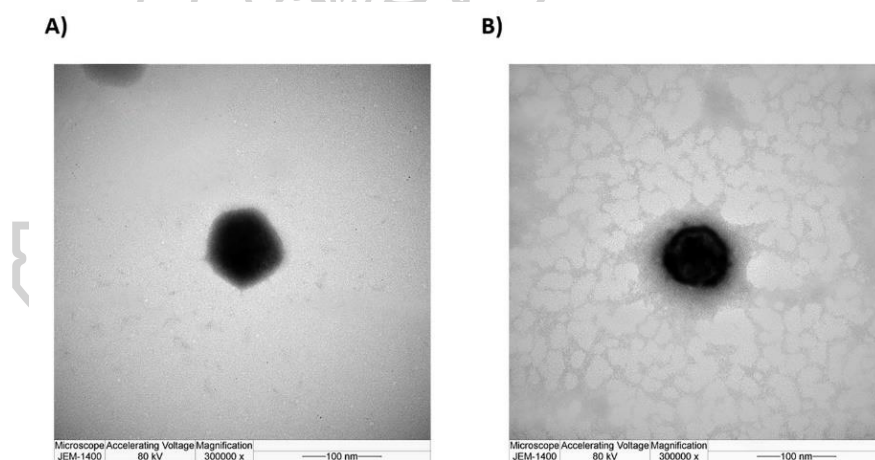
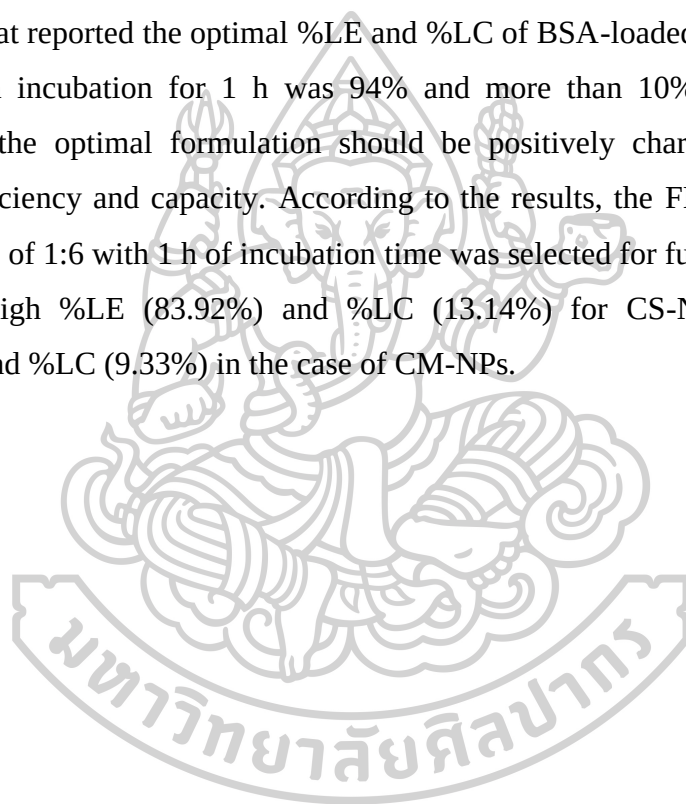


Figure 13 TEM images of (A) FITC-BSA-loaded CS-NPs and (B) FITC-BSA-loaded CM-NPs.

The model antigen loading into the NPs was performed using a physical adsorption technique by incubating NPs with FITC-BSA in SNF. The mass ratios of FITC-BSA:NPs and incubation time were examined to optimize the maximal loading. At 1 h after incubation, both CS-NPs and CM-NPs demonstrated saturated protein loading, as shown in Figures 14A and 14B, respectively. The result shows that %LE and %LC was affected by mass ratios of FITC-BSA:NPs. %LE of FITC-BSA-loaded

CS-NPs and FITC-BSA-loaded CM-NPs after 1 h incubation were in the range of 17.82% to 97.5% and 14.23% to 88.10%, respectively. The highest %LE of FITC-BSA-loaded CS- and CM-NPs was found at FITC-BSA:NPs at the ratio of 1:8. For loading capacity, %LC of FITC-BSA-loaded CS-NPs and FITC-BSA-loaded CM-NPs after 1 h incubation were in the range of 6.66% to 13.07% and 6.00% to 9.90%, respectively. The maximum %LC of FITC-BSA-loaded CS-NPs and CM-NPs were found at the mass ratios of FITC-BSA:NPs are 1:6 (13.14%) and 1:8 (9.33%), respectively, as shown in Figure 14C and 14D. These results were in line with the literature that reported the optimal %LE and %LC of BSA-loaded CS-NPs at the ratio of 1:7 with incubation for 1 h was 94% and more than 10%, respectively [61]. Therefore, the optimal formulation should be positively charged NPs with high loading efficiency and capacity. According to the results, the FITC-BSA:NPs at the weight ratio of 1:6 with 1 h of incubation time was selected for further study, which exhibited high %LE (83.92%) and %LC (13.14%) for CS-NPs and high %LE (61.76%) and %LC (9.33%) in the case of CM-NPs.



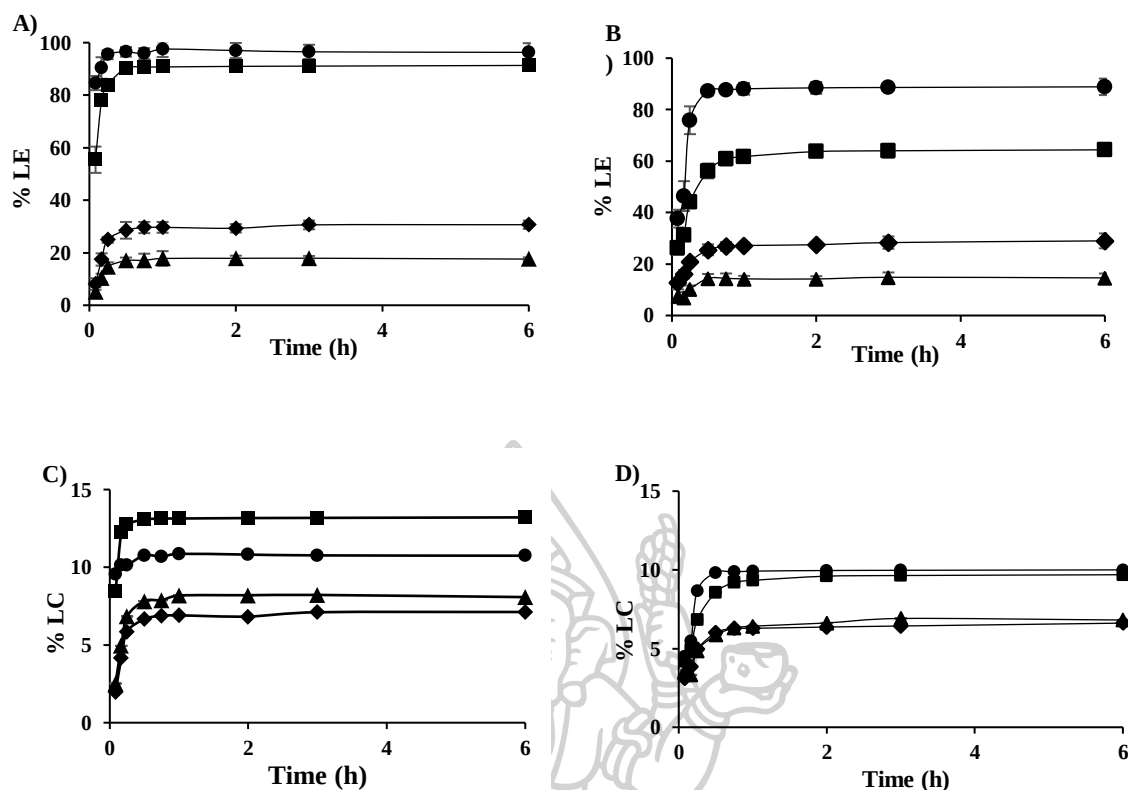


Figure 14 Percentage loading efficiency (%LE) of FITC-BSA-loaded (A) CS-NPs, (B) CM-NPs, and percentage loading capacity (%LC) of FITC-BSA-loaded (C) CS-NPs, (D) CM-NPs at various FITC-BSA:NPs ratios of 1:2 (▲), 1:4 (◆), 1:6 (■) and 1:8 (●).

4.2.2 *Ex vivo* mucoadhesive property and uptake study of FITC-BSA-loaded NPs

The flow-through method was used to evaluate the *ex vivo* mucoadhesive property of NPs under mimicking physiological conditions of the nasal cavity. The mucoadhesive evaluation was performed to determine the retention time and amount of different formulations retention on the mucosa after washing [101]. The fluorescence images of porcine nasal mucosa after washing with varying volumes of SNF are shown in Figure 15, which reveals that the FITC-BSA solution had lower fluorescence intensities (FI) than NPs.

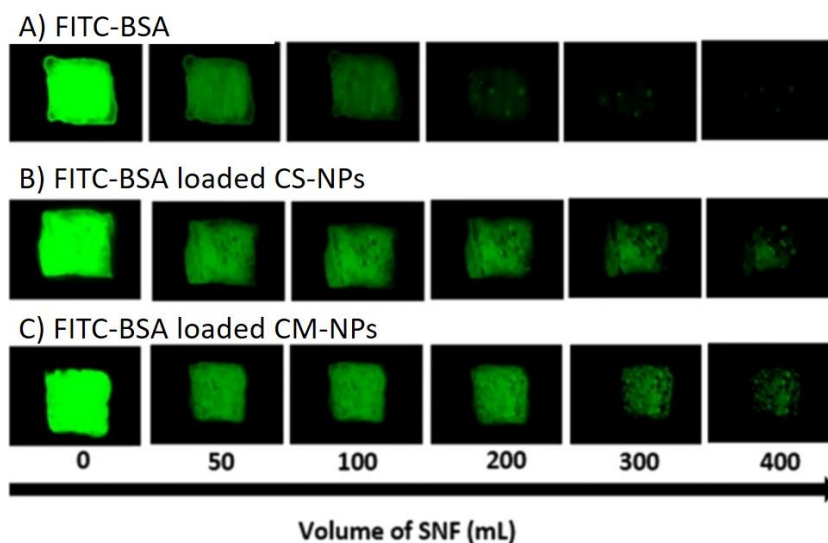


Figure 15 Fluorescence images of porcine nasal mucosa following application of different formulas, including A) FITC-BSA solution, B) FITC-BSA-loaded CS-NPs, and C) FITC-BSA-loaded CM-NPs and then washing with different volumes of SNF.

The FI of each formulation was used to calculate the percentage retention on the porcine nasal mucosa of both FITC-BSA-loaded NPs after being washed with SNF compared with the FITC-BSA solution. The results show that the FITC-BSA-loaded CS-NPs and CM-NPs retained on the nasal mucosa greater than the FITC-BSA solution ($p < 0.05$), as shown in Figure 16. After 100 mL of SNF washing at 20 min, the FITC-BSA solution was retained at approximately $42 \pm 6\%$, which is lower than that of FITC-BSA-loaded CS-NPs ($60 \pm 4\%$) and CM-NPs ($64 \pm 10\%$). However, no significant differences were found in the percentage retention of FITC-BSA-loaded CS-NPs and CM-NPs. The mucoadhesive properties of the formulations were assessed using the volume of SNF necessary to wash off 50% of the formulation from mucosal surfaces, defined as WO_{50} [102]. Figure 16 illustrated that WO_{50} of FITC-BSA solution, FITC-BSA-loaded CS-NPs, and FITC-BSA-loaded CM-NPs were 102 ± 8 mL, 154 ± 24 mL, and 208 ± 28 mL, respectively. They demonstrated that the FITC-BSA-loaded CS-NPs and CM-NPs had good mucoadhesive properties with high FITC-BSA retention on the porcine nasal mucosa, in contrast with the FITC-BSA solution. The interaction between the formulation and mucin dictated the mucoadhesive properties of liquid formulations. The greater the contact, the longer

the formulation remains on the mucosa. The addition of maleimide groups to the NPs increased mucoadhesion relative to non-functionalized NPs via various interaction mechanisms: (1) Interaction between hydrogen atoms. The amine (NH_2) group of CS (pKa 6.3) is protonated (NH_3^+) in the nasal cavity, which includes 3% mucin with a pH of 5.5-6.5, resulting in hydrogen bonding between itself and hydroxyl groups (OH^-) [101, 111]; (2) Electrostatic interaction, the positive charge of CS interacts with the negative charge of sialic acid residues in mucin. Both CS-NPs and CM-NPs demonstrate interaction processes. These interactions are pH-dependent and consist of non-specific mucosal binding [9, 111]; (3) Michael-type addition reaction, a pH-independent reaction of CM with mucin thiols. Strong covalent connections are formed between maleimide groups in CM-NPs and cysteine in the mucous membrane as a result of this reaction [9].

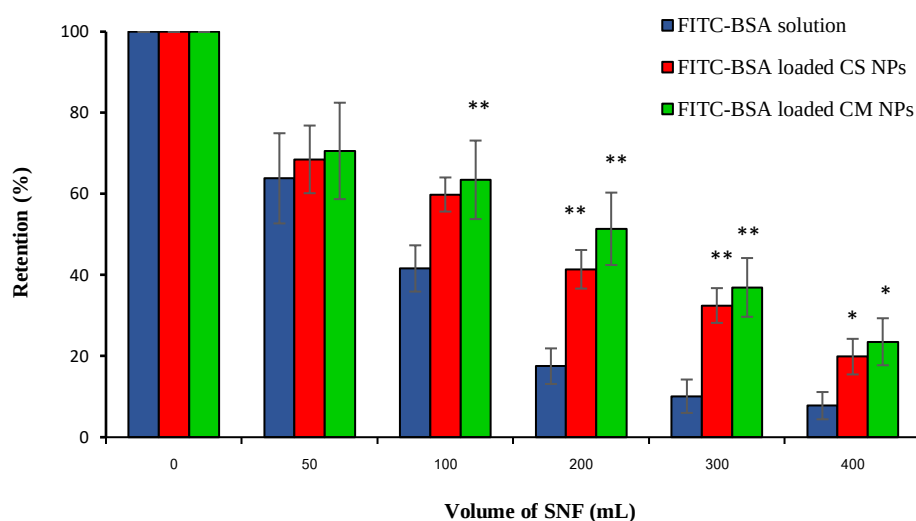


Figure 16 Percentages of FITC-BSA retention on porcine nasal mucosa after washing with various volumes of SNF: FITC-BSA solutions (■), FITC-BSA-loaded CS-NPs (■), and FITC-BSA-loaded CM-NPs (■). * $p < 0.05$, ** $p < 0.01$ compared with FITC-BSA solution.

According to *ex vivo* uptake evaluation, as shown in Figure 17, the FITC-BSA formulations were taken into the porcine nasal mucosa within 15 min. CLSM micrographs of the apical side showed that FITC-BSA-loaded CS-NPs and FITC-BSA-loaded CM-NPs deposited on the nasal mucosa surface (X and Y sections) and

uptake deep in the nasal mucosa (Z-section, depth of uptake) more than that of FITC-BSA solution, which is in accordance with a previous study [3]. The drug delivery via transnasal mucosa pathways is influenced by the physicochemical characteristics of the drug compounds. Transnasal mucosa routes have both intracellular and extracellular pathways. Small molecules (~ 1 kDa) can diffuse freely across extracellular pathways. In contrast, macromolecules, such as BSA (MW 66 kDa) and E protein of JEV (MW 54 kDa), are transported mainly by M-cell absorption, an intracellular pathway requiring dipole-dipole interactions. According to negative charges of protein antigens, therefore, positively charged NPs deliver protein antigens via M-cell uptake more effectively than the protein-antigen solution [38, 112].

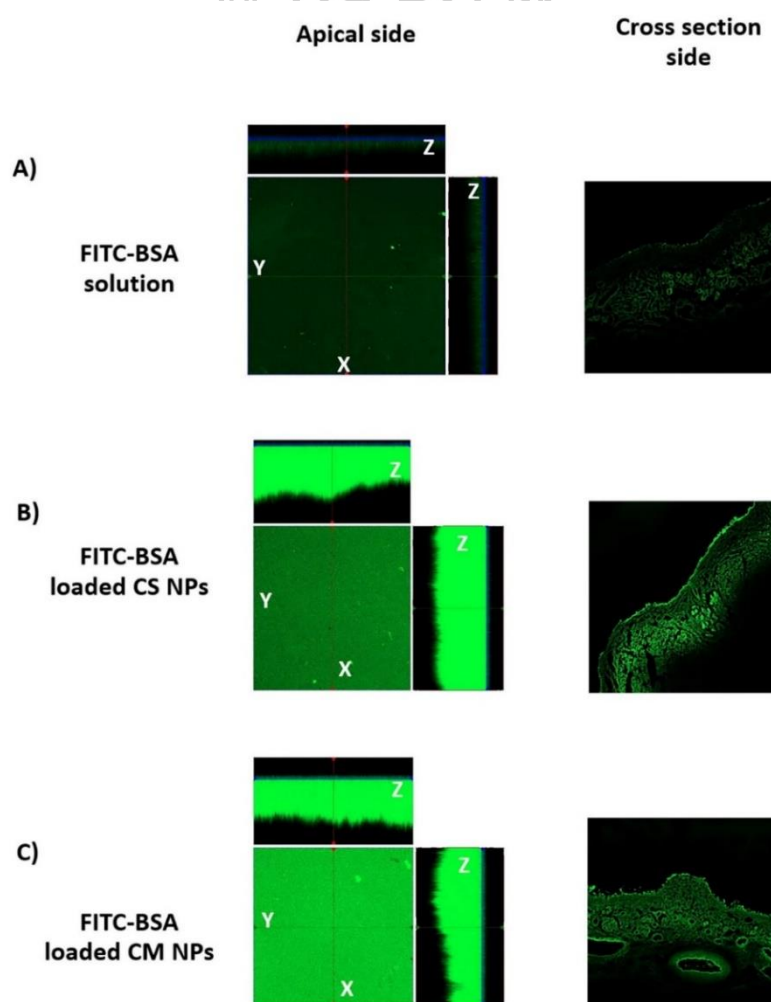


Figure 17 CLSM images (apical (X, Y, and Z sections) and cross-sections) of FITC-BSA formulations, including A) FITC-BSA solutions, B) FITC-BSA-loaded CS-NPs,

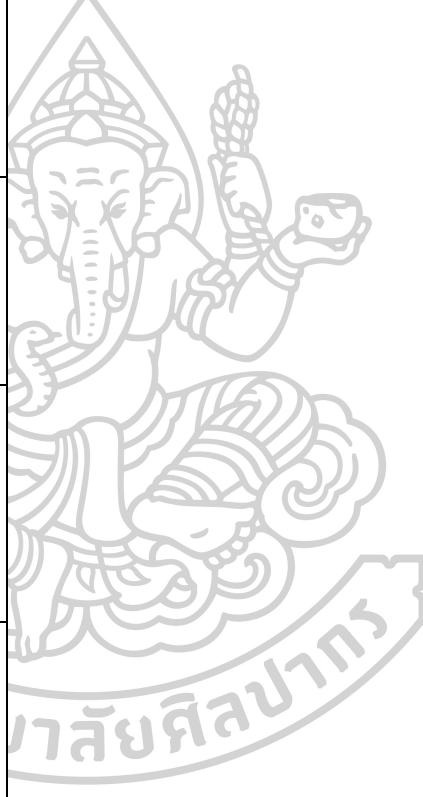
and C) FITC-BSA-loaded CM-NPs uptake into porcine nasal mucosa at 15 min of *ex vivo* study.

4.2.3 Preparation and characterization of JE-CV-loaded CS-NPs or CM-NPs

CS- and CM-NPs were prepared by the ionic gelation method and then loaded JE-CV using a physical absorption technique with optimal conditions as described. All formulations were successfully prepared and characterized, as shown in Table 5. The mean particle sizes of blank CS-NPs and CM-NPs (without JE-CV) were 183.4 ± 7.6 nm and 247.4 ± 8.1 nm, respectively, with positive charges of about 26.6 ± 1.9 mV and 23.5 ± 0.9 mV. When the vaccine was incorporated, the mean particle sizes of JE-CV-loaded NPs increased to 218.7 ± 22.8 nm and 333.0 ± 44.1 nm for CS-NPs and CM-NPs, respectively. Furthermore, the surface charges of JE-CV-loaded NPs were decreased to 22.94 ± 1.99 mV and 19.67 ± 1.17 mV, respectively. The absolute values of zeta potential ≥ 16 mV were utilized to indicate the dispersion stability of the NPs system. The size distribution indicated their monodisperse nature in the absence of particle aggregation ($PDI < 0.5$) [56]. The %LE of JE-CV loaded into CS-NPs and CM-NPs were 85.3% and 86%, and the LC of JE-CV-loaded CS-NPs and CM-NPs were 1.20×10^4 PFU/mg and 1.23×10^4 PFU/mg, respectively. According to the results, their particle sizes were less than 1 μ m with positive charges, which is in accordance with the expected characteristic of the CS-based NPs to have a mucoadhesive property and antigen absorption through M-cells in the nasal cavity.

Table 5 Characterization of JE-CV-loaded CS- or CM-NPs.

Nanoparticles	Before loading			After loading			%LE	LC (10 ⁴ PFU/mg)
	Size (nm)	PDI	Zeta potential (mV)	Size (nm)	PDI	Zeta potential (mV)		
CS-NPs	183.4 ± 7.6	0.20 ± 0.02	26.6 ± 1.8	218.7 ± 22.8	0.29 ± 0.04	22.9 ± 2.0	85.3 ± 3.9	12.1±0.6
CM-NPs	247.4 ± 8.1	0.26 ± 0.04	23.5 ± 0.9	333.0 ± 24.1	0.35 ± 0.07	19.7 ± 1.2	86.0 ± 8.0	12.3±1.1



4.2.4 Intranasal immunization

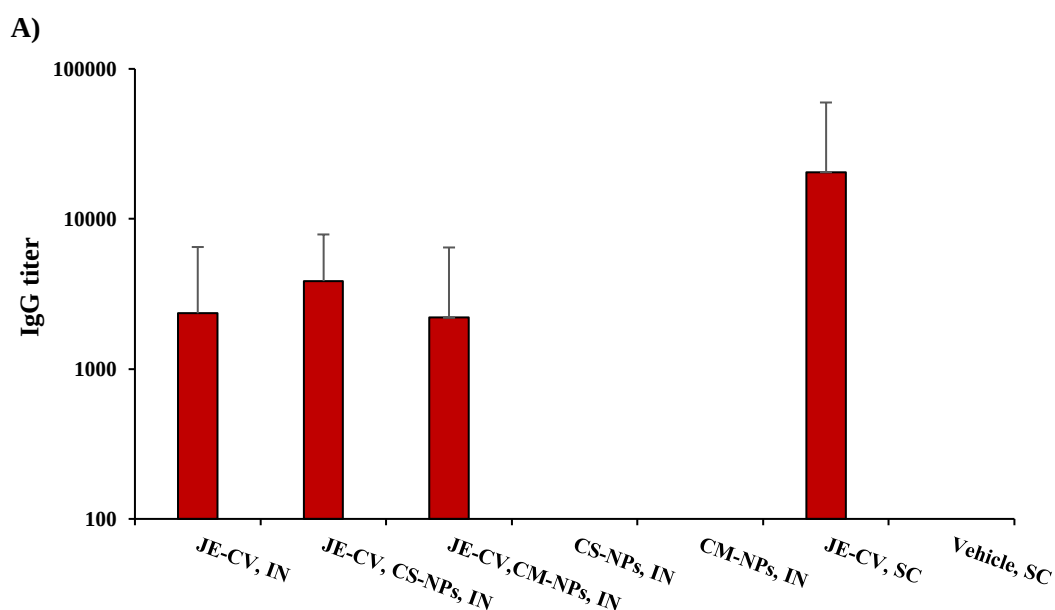
C57/BL6 mice (4-5 weeks old) were immunized by different formulations and routes to achieve 1×10^4 PFU of JE-CV per animal, except for negative control, as shown in Table 6. The immunization regimen was performed following a previous study, which found that 28 days of SC immunization provided complete protection against JEV challenges [40]. The weight of mice in all experimental groups was 18-22 g/mouse. The body weight of mice did not significantly change during the study when compared with the negative control group. Therefore, that indicated no AEs occurred in all immunization groups.

Table 6 Intranasal immunization study for mice in each group (n = 6).

Groups	Routes and formulations
1	IN immunization of JE-CV solution (JE-CV, IN)
2	IN immunization of JE-CV-loaded CS NPs (JE-CV, CS-NPs, IN)
3	IN immunization of JE-CV-loaded CM NPs (JE-CV, CM-NPs, IN)
4	IN immunization of CS NPs (CS-NPs, IN)
5	IN immunization of CM NPs (CM-NPs, IN)
6	SC immunization of JE-CV solution (JE-CV, SC; positive control)
7	SC immunization of 0.04% NaCl (Vehicle, SC; negative control)

After immunization for 28 days, HIR in serum (IgG) as a systemic immune response and secretory samples (saliva and nasal wash) as a mucosal immune response were evaluated. In addition, spleens were harvested to investigate CMIR from splenocytes. All groups of mice immunized with JE-CV elicited IgG that exhibited 100% seroconversion (6/6 mice). Both NPs without JE-CV and the negative control group were not seroconverted (0/6 mice). Major immunological defense against JEV infection is provided by neutralizing antibodies as a function of serum IgG, and PRNT₅₀ is used to define the neutralizing titer (NT) as an activity of neutralizing antibodies against JEV [113]. Moreover, the PRNT₅₀ at seroprotection (PRNT₅₀ titers ≥ 10) correlated with protection against JE in humans accepted by the WHO [2]. The results showed that mice immunized with JE-CV formulations via SC and IN exhibited seroprotection with PRNT₅₀ titers ≥ 10 . Compared with IN

immunization, mice immunized by SC significantly improved PRNT₅₀ more than 45 times ($p < 0.05$). However, there were no significant differences in PRNT₅₀ levels between the groups of IN immunization of JE-CV, and no mice in both NPs formulations without JE-CV and the negative control group elicited PRNT₅₀ and IgG, as shown in Figure 18. Obviously, SC immunization produced a higher serum IgG level and effective NT (as the systemic immune response) than IN immunization as a mucosal administration [114]. However, immunization by SC injection has several limitations, such as it causes pain resulting in poor patient compliance, contamination, and infection. Therefore, immunization via IN route can overcome these limitations of SC immunization [3]. To achieve the desired efficacy of this platform, IN immunization requires an adjuvant or delivery platform to enhance the systemic immune response against mosquito-borne flavivirus [109]. Several studies investigated the efficiency of mucoadhesive NPs as vaccine delivery systems for IN immunization. The studies demonstrated that mucoadhesive NPs enhanced antigen uptake and HIR for mosquito-borne flavivirus [100, 115, 116]. Our results exhibited that using IN immunization effectively induced both seroconversion (100%) and seroprotection (PRNT₅₀ titers ≥ 10). Hence, IN immunization may be an alternative approach with a painless way for JE-CV immunization.



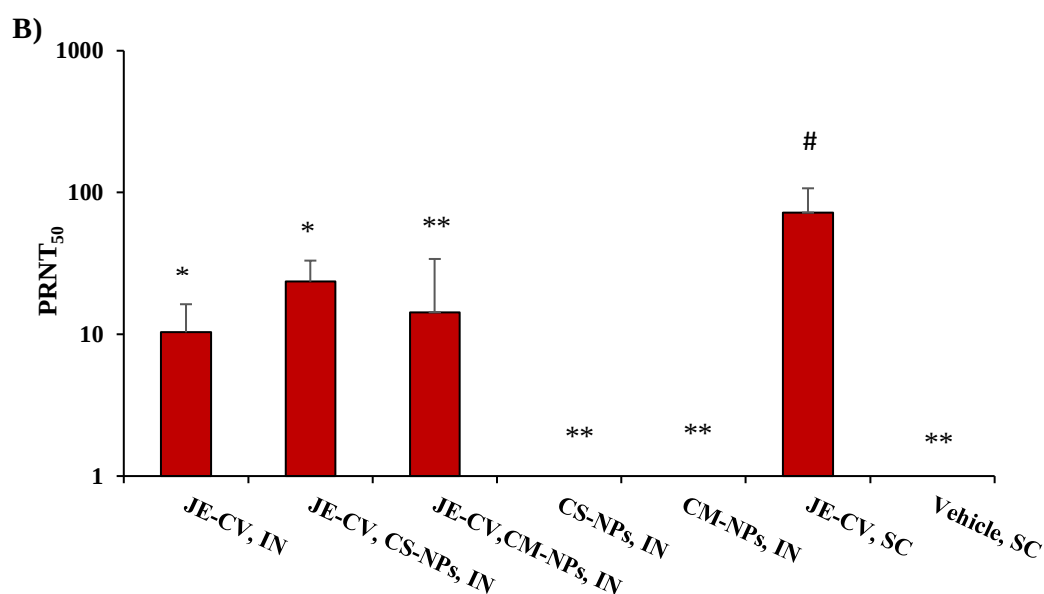


Figure 18 Humoral immune responses (HIR) in mice on day 28 after JE-CV immunization with different formulations and routes. A) Specific anti-JEV IgG antibody titer and B) 50% plaque reduction neutralization titer (PRNT₅₀). Results are expressed as mean $\pm$ SD ($n = 6$), * $p < 0.05$, ** $p < 0.01$ compared with JE-CV solution immunized via SC, and # $p < 0.05$ compared with JE-CV solution immunized via IN.

As shown in Figure 19, mice immunized with the different formulations of the JE-CV vaccines via IN stimulated specific anti-JEV IgA antibody titers in saliva and nasal wash higher than in SC immunization. For IN immunization groups, JE-CV-loaded NPs can stimulate more IgA than that JE-CV solution ($p < 0.05$). For IgA in the saliva, JE-CV-loaded CS-NPs and CM-NPs can trigger 2.35-fold and 2.72-fold more significantly than the JE-CV solution. In the case of IgA in the nasal wash, JE-CV-loaded CS-NPs and CM-NPs evoked 2.11-fold and 2.34-fold greater levels than the JE-CV solution. Moreover, blank CS-NPs and CM-NPs did not elicit mucosal immune responses, which is in line with a negative control group.

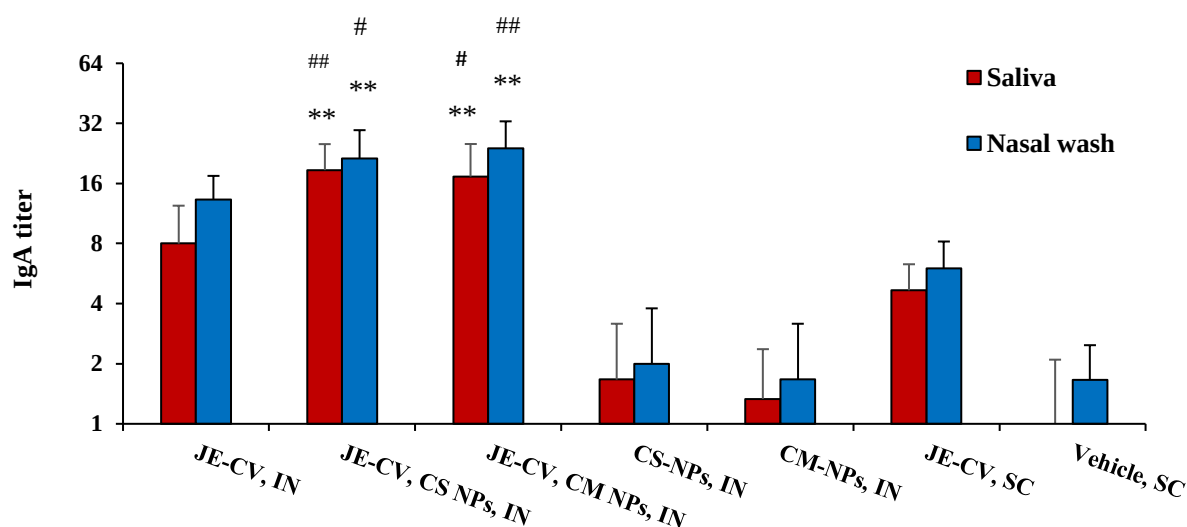


Figure 19 Specific anti-JEV IgA antibody titer in saliva (■) and nasal wash (■) on day 28 after JE-CV immunization in mice with different formulations and routes. Results are expressed as mean $\pm$ SD ($n = 6$), * $p < 0.05$; ** $p < 0.01$ compared with JE-CV solution immunized via SC and # $p < 0.05$; ## $p < 0.01$ compared with JE-CV solution immunized via IN.

CS-based NPs were studied for mucoadhesive properties as IN vaccine delivery adjuvants. Several studies reported that CS-based NPs are feasible for inactivated and subunit immunizations because they can overcome the limitations of IN immunization by improving low immunogenicity and limited nasal cavity absorption. Our findings are consistent with these studies; using positively charged NPs as a vaccine delivery system for IN immunization demonstrated a better adjuvant property for both systemic and mucosal immune responses than mice immunized with antigen alone (without NPs). The mucoadhesive and antigen absorption abilities could be related to the enhanced adjuvant property of positively charged NPs. APCs capture and deliver antigens from the nasal cavity to other mucosal locations, such as saliva and nasal mucus in the oronasal cavity, via the common lymphatic pathway, causing mucosal immune reactions. The limitation of IN for APCs trafficking to the spleen showed weak systemic immune responses with strong mucosal immunological responses [3, 117]. JE-CV-loaded CS- and CM-NPs showed more potent mucoadhesive properties, although their NT was not significantly different compared

to IN immunization with JE-CV solution. Our findings are in accordance with a study by Wang et al. (2016) that found calcium phosphate NPs-loaded with a live attenuated chimeric dengue virus immunization did not significantly enhance NT compared to the vaccine solution [100]. According to our study, high levels of IgA in saliva and nasal secretions indicate an effective stimulation of mucosal immune responses.

CMIR stimulation was confirmed by determining cytokine levels of IL-4 and IFN - γ from splenocytes of mice immunized. IL-4 is a major Th₂ cytokine that stimulates HIR against exogenous pathogens in the systemic circulation via IgG. Figure 20 shows that all JE-CV immunization groups stimulated IL-4 levels of approximately 40-70 pg/mL, with no significant differences between the SC and IN immunization groups. This study demonstrated that there was no change in IgG levels in all groups of JE-CV inoculated mice.

IFN- γ is a major Th₁ cytokine produced by T lymphocytes and NK cells in response to viral or vaccination stimulation. It has the ability to prevent flavivirus replication and amplification. As a result, it is considered that the persistence of IFN- γ levels following JE vaccination is required for protection against JEV infection [31, 118]. Figure 20 illustrates that mice immunized with JE-CV solution via SC produced IFN- γ (543.4 ± 54.4 pg/mL) approximately 1.9-fold higher than the IN immunization with JE-CV solution (285.4 ± 46.2 pg/mL). IFN- γ levels after IN immunization with JE-CV-loaded CS-NPs (424.2 ± 61.7 pg/mL) and JE-CV-loaded CM-NPs (407.4 ± 64.5 pg/mL) were 1.5- and 1.4-fold higher than those obtained with JE-CV solution (285.4 ± 46.0) ($p < 0.05$). Moreover, in mice immunized with blank CS-NPs and blank CM-NPs, the negative group were not elicited CMIR. Practically, vaccination via SC as a parenteral injection will attract systemic immune cells and activates cytotoxic T lymphocytes, resulting in high IFN- γ production and apoptotic induction of infected cells (programmed cell death). Several investigations on IN immunization found that positive charge NPs as mucoadhesive NPs enhanced M cell and APCs uptake efficiency via non-specific absorption and increased cytotoxic T lymphocyte activity, resulting in improved IFN- levels. Examples are the JE strain SA-14-14-2 vaccine and the chimeric West Nile virus vaccine. The viral vaccines have negative charges on the protein structure, which results in poor absorption compared to vaccine-loaded NPs, which generally have higher positive charges. Our results

demonstrated that IN immunization of JE-CV in mice with CS-NPs and CM-NPs produced more IFN- γ than that of the JE-CV solution. Theoretically, antigen uptake depends on the interaction of carrier compounds with specific cells. In general, positively charged NPs are more rapidly taken up into cells with stronger interactions than negatively charged NPs due to electrical interactions between positively charged NPs and the negative charge of cell membranes. The results exhibited the CMIR efficiency of JE-CV-loaded NPs, which was related to the efficiency of JEV protection.

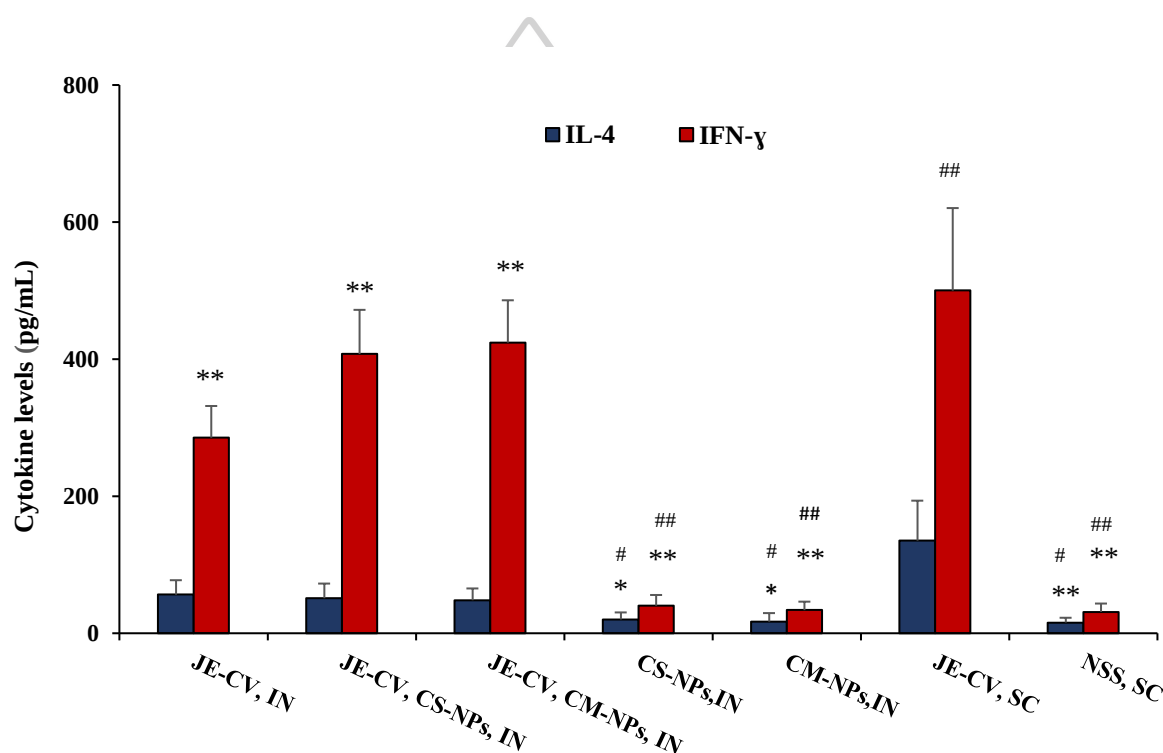


Figure 20 Cytokine levels of IL-4 and IFN- γ from splenocytes of immunized mice with different JE-CV formulations. Results are expressed as mean $\pm$ SD ($n = 6$), * $p < 0.05$; ** $p < 0.01$ compared with JE-CV solution of mice immunized via SC and # $p < 0.05$; ## $p < 0.01$ compared with JE-CV solution of mice immunized via IN.

4.2.5 Stability test

The physical stabilities of CS-NPs and CM-NPs after storage at 4°C and 25°C for 3 months were evaluated by observing the changes in characteristics, including size, PDI, and zeta potential. As shown in Table 7, the size and PDI of CS-NPs after being kept at 4°C for 3 months slightly increased from 183.4 ± 7.6 nm to 189.7 ± 7.6

nm and 0.20 ± 0.02 to 0.23 ± 0.07 , in contrast, the zeta potential was increased from 26.6 ± 1.8 mV to 24.9 ± 1.4 mV compared with initial CS-NPs. The tendency characteristics of CS-NPs after being kept at 25°C for 3 months were similar compared with initial CS-NPs (size ranged between 185.1 ± 4.6 and 190.1 ± 12.5 , PDI ranged between 0.20 ± 0.02 and 0.25 ± 0.06 , and zeta potential ranged between 24.9 ± 1.4 to 26.4 ± 1.5 mV). The results indicated that the physical stabilities of CS-NPs lyophilized powder at 4°C and room temperature were consistent with the previous studies [119, 120]. In the case of CM-NPs, after keeping the particles at 4°C for 3 months, the size tended to slightly increase from 247.4 ± 8.1 nm to 282.6 ± 8.7 nm, PDI increased from 0.26 ± 0.04 to 0.31 ± 0.04 , and charges were decreased from 23.5 ± 0.9 mV to 20.8 ± 2.3 mV compared with initial CM-NPs ($p > 0.05$). On the other hand, after keeping the CM-NPs at 25°C for 3 months, all characteristics were significantly changed when compared with initial CM-NPs (0 month) ($p < 0.01$). The CM-NPs particle sizes and PDI have increased from 247.4 ± 8.1 nm to 423.8 ± 17.6 nm and 0.26 ± 0.04 to 0.46 ± 0.08 , while the zeta-potentials decreased from 23.5 ± 0.9 mV to 13.2 ± 1.3 mV for 3-month storage. The instability might depend on the CM being conjugated with residue excess of the maleimide group from the conjugation process. Leading to a decrease in the positive charge of NPs, the zeta potential of the formulations reduced to 13 mV close to zero (about ± 10 mV) means the aggregation of NPs may occur. In accordance with the zeta potential, less in charges after the storage at 25°C, the NPs tend to agglomerate. Moreover, there is a higher PDI value as the size distribution increases due to their aggregates in the formulation. Therefore, to avoid the instability of both CS-based NPs, they were recommended to be stored at 4°C.

Table 7 Effects of storage conditions on the characteristics of CS-NPs and CM-NPs.

Formulations	Storage conditions	Characterizations		
		Size (nm)	PDI	Zeta potential (mV)
CS-NPs	Initial time (0 month) at 4°C	183.4 ± 7.6	0.20 ± 0.02	26.6 ± 1.8
	1 month at 4°C	185.0 ± 5.4	0.21 ± 0.03	26.4 ± 1.2
	2 months at 4°C	187.1 ± 3.8	0.23 ± 0.02	24.6 ± 1.3
	3 months at 4°C	189.7 ± 7.6	0.23 ± 0.07	23.8 ± 1.7
	1 month at 25°C	185.1 ± 4.6	0.20 ± 0.02	26.4 ± 1.5
	2 months at 25°C	190.1 ± 12.5	0.22 ± 0.04	25.7 ± 1.6
	3 months at 25°C	187.0 ± 10.1	0.25 ± 0.06	24.9 ± 1.4
CM-NPs	Initial time (0 month) at 4°C	247.4 ± 8.1	0.26 ± 0.04	23.5 ± 0.9
	1 month at 4°C	246.3 ± 7.2	0.28 ± 0.02	23.5 ± 1.7
	2 months at 4°C	263.8 ± 8.1	0.27 ± 0.03	21.6 ± 1.3
	3 months at 4°C	282.6 ± 8.7	0.31 ± 0.04	20.8 ± 2.3
	1 month at 25°C	305.4 ± 10.7 [#]	0.34 ± 0.05 [#]	19.3 ± 1.8 [#]
	2 months at 25°C	383.3 ± 14.8 [#]	0.39 ± 0.06 [#]	17.3 ± 1.2 [#]
	3 months at 25°C	423.8 ± 17.6 [#]	0.46 ± 0.08 [#]	13.2 ± 1.3 [#]

Results are expressed as mean ± SD (n = 3). [#] $p < 0.01$ when compared with the same formulation at the initial time point.

4.3. Intradermal immunization with JE-CV-loaded separating CS-based MNs patch

4.3.1 Fabrication of conventional CS-based MNs patch (CS-MNs patch)

4.3.1.1 Morphology of conventional CS-based MNs patch (CS-MNs patch)

The CS-based MNs patch (CS-MNs patch) fabricated from CS and PVA at a 1:4 mass ratio was successfully prepared with good mechanical properties [82]. To modify the characteristic of MNs patch, in this study, we added NaHCO₃ to improve a swelling property and decrease the dissolution time of the patch. The morphology of CS-MNs is summarized in Table 8. The CS-MNs patch showed fully-formed MN

arrays (121 needles/patch) with sharp needles. The dimensions of MNs patch were $629.0 \pm 3.7 \mu\text{m}$ in needle height, $297.5 \pm 1.5 \mu\text{m}$ in needle base width, and $290.3 \pm 8.3 \mu\text{m}$ in the interspace (base to base) on a patch area of approximately 0.5 cm^2 . To determine the maximum amount of NaHCO_3 added in the CS-MNs patch formulation, various mass ratios of NaHCO_3 :CS:PVA, including 0:1:4, 0.25:1:4, and 0.5:1:4, were evaluated. The results showed that NaHCO_3 :CS:PVA at the mass ratio of 0.25:1:4 was a maximum ratio that can prepare fully-formed MN arrays with a height of $624.7 \pm 6.9 \mu\text{m}$ and a base width of $304.7 \pm 6.6 \mu\text{m}$, and was interspaced by $285.0 \pm 2.0 \mu\text{m}$ interspace. However, the morphology of the CS-MNs patch with NaHCO_3 (0.25:1:4 mass ratio) and without NaHCO_3 (0:1:4 mass ratio) was not different. NaHCO_3 produced CO_2 in the matrix polymer, creating porous in the structure. The porous size and number in matrix stature depended on the amount of NaHCO_3 . In the case NaHCO_3 was added too much, CO_2 was over-produced and high porosity porous, causing the MNs structure to be fragile [17, 121].

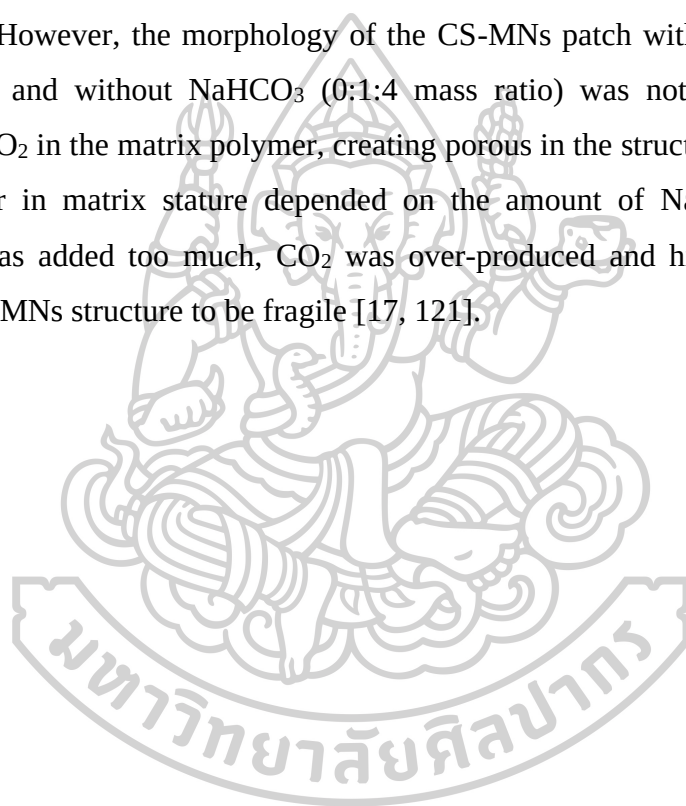
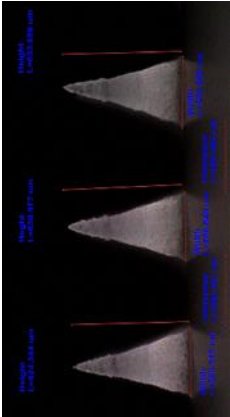
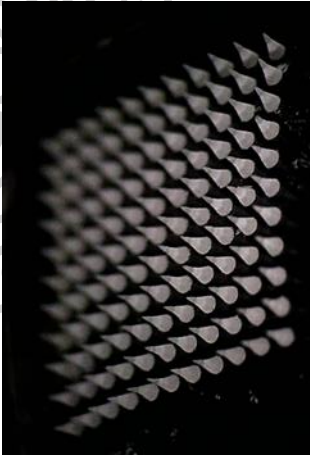
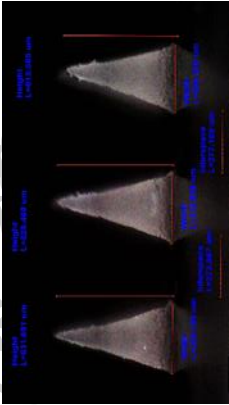
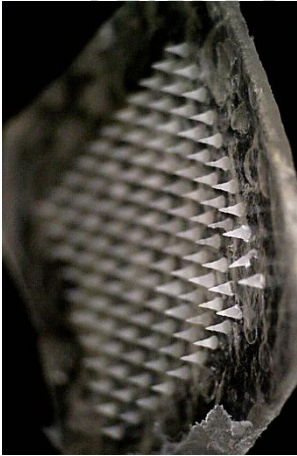


Table 8 The morphology of CS-MNs patch at various mass ratios of NaHCO₃:CS:PVA.

Formulations (NaHCO ₃ :CS:PVA)	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
0:1:4	 MNs patch was completely formed with a total of 121 needles.	 Height: 629.0 ± 3.7 µm Base width: 297.5 ± 1.5 µm Interspace: 290.3±8.3 µm
0.25:1:4	 MNs patch was completely formed with a total of 121 needles.	 Height: 624.7 ± 6.9 µm Base width: 304.7 ± 6.6 µm Interspace: 285.0 ± 2.0 µm

Formulations (NaHCO ₃ :CS:PVA)	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
0.50:1:4	 MNs patch was incompletely formed.	 MN arrays were not fully-formed and fragile.

4.3.1.2 Mechanical strength and skin insertion of conventional CS-based MNs patch (CS-MNs patch)

For mechanical strength, the CS-MNs patches were applied with different compression forces of 3.3888 N to 13.31 N per patch (0.028 N to 0.11 N per needle) using a texture analyzer. The percentage needles reduction height of the CS-MNs patch is shown in Figure 21. In all compression forces studied, the reduction height of needles in the formulation without NaHCO_3 ranged between 2% to 7% and 3% to 10% in CS-MNs patch containing NaHCO_3 (0.25 mass ratio). Moreover, all formulations maintained their shape without cracking as they became compressed instead. Therefore, these CS-MNs patches exhibited desirable mechanical strength to resist the application forces [95]. The results noted that at 10.77 N/patch (0.089 N/needle), a maximum compression force of the human using thumb pressure for applying the MNs [91], the needle reduction height of CS-MNs patch with NaHCO_3 was significantly increased compared to MNs patch without NaHCO_3 ($p < 0.05$). It is because the porous in the matrix polymer causes a decrease in the mechanical strength of the MNs patches [86]. For the insertion study, after the MNs patches were applied to neonatal porcine skin, both formulations were successfully inserted into the skin with 100% penetration (Figure 22). The results indicated an insertion ability of CS-MNs patch (with NaHCO_3 0.25 mass ratio) or without NaHCO_3 .

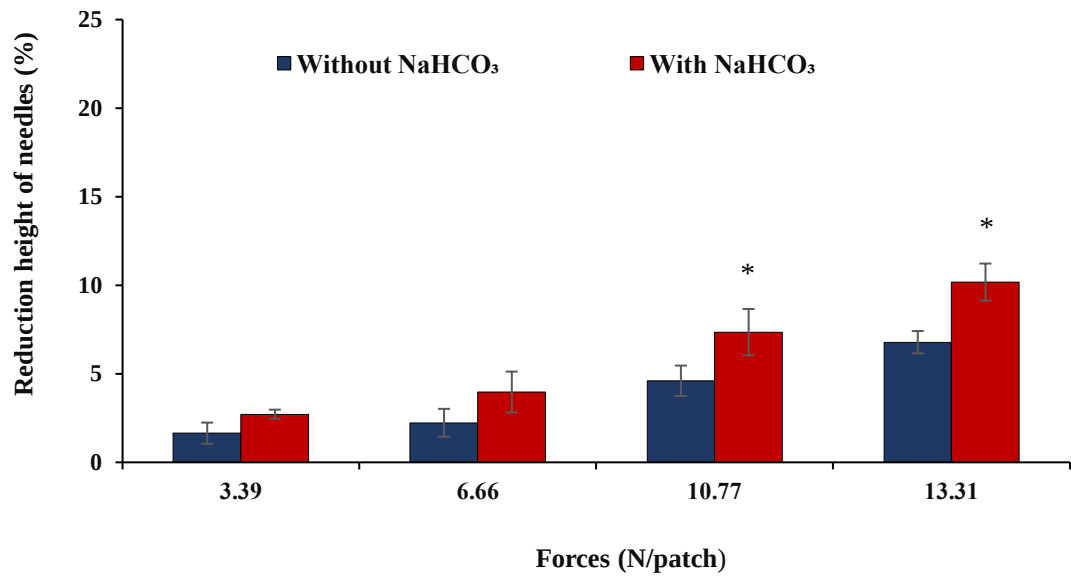


Figure 21 The percentage needles reduction height of CS-MNs patch without NaHCO₃ (■) and CS-MNs patch with NaHCO₃ (■). All data are presented as mean $\pm$ SD (n = 3). * $p < 0.05$ compared with CS-MNs patch without NaHCO₃ at the same force.

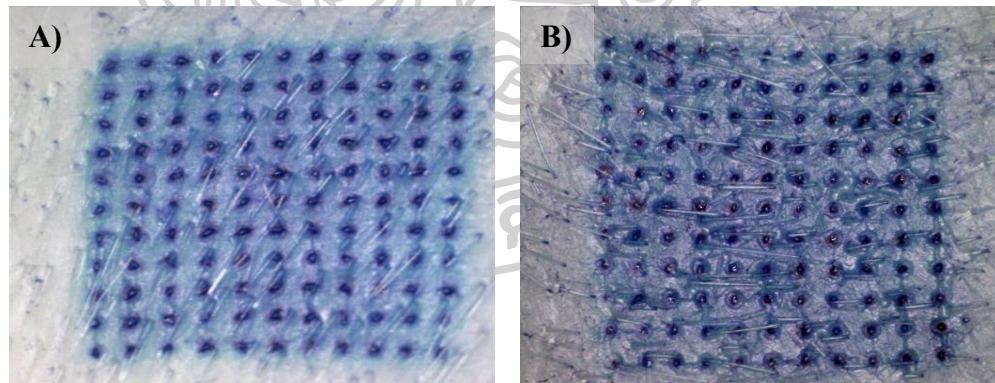


Figure 22 Images of neonatal porcine skin stained with methylene blue following the application of A) CS-MNs patch without NaHCO₃ and B) CS-MNs patch with NaHCO₃.

4.3.1.3 Dissolution and swelling of conventional CS-based MNs patch (CS-MNs patch)

Dissolution property is an important factor for dissolving MNs, dependent on the water solubility or biodegradable property of fabricated matrix polymer [122]. The fast dissolution of needles in the skin is an ideal property that decreases wearing time and enhances patient compliance [123, 124]. CS and PVA, biodegradable polymers, were used to fabricate CS-MNs patches in this study due to swelling and dissolving properties when contacted with the body's interstitial fluid, then release the encapsulated compound [122]. Both CS-MNs patch with or without NaHCO_3 exhibited similar swelling and dissolution kinetics (Figure 23 and Figure 24). The swelling from both formulations indicated an initial rapid swelling from 0.25 h to 0.5 h (15-30 min) after the patches were immersed (Figure 23). At 15 min, the swelling of the CS-MNs patch without NaHCO_3 was 950% and the CS-MNs patch with NaHCO_3 was 1,050%. After 30 min, the swelling properties were then significantly decreased over time (1 to 24 h) compared to the swelling at the initial period of 15 min (0.25 h) ($p < 0.05$). The percentage erosions of the CS-MNs patch without and with NaHCO_3 were 30% to 76% and 38% to 83%, respectively (Figure 24). The percentage erosion of the CS-MNs patch without and with NaHCO_3 significantly increased over a period of 0.25 to 2 h (about 1.1- for the patch without NaHCO_3 and 1.2-fold for the patch with NaHCO_3) and then the erosion seems to be stable in the range of 88% and 92% over 4 to 24 h. Several studies reported that rapid water uptake leads to the fast erosion of matrix polymers [107, 125]. It might be because the porous formed by NaHCO_3 increased the surface area of the polymer matrix in contact with the water resulting in high swelling and erosion rate of CS-MNs patch with NaHCO_3 [126, 127]. Therefore, the mixture of NaHCO_3 :CS:PVA, at the mass ratio of 0.25:1:4, was selected for further studies.

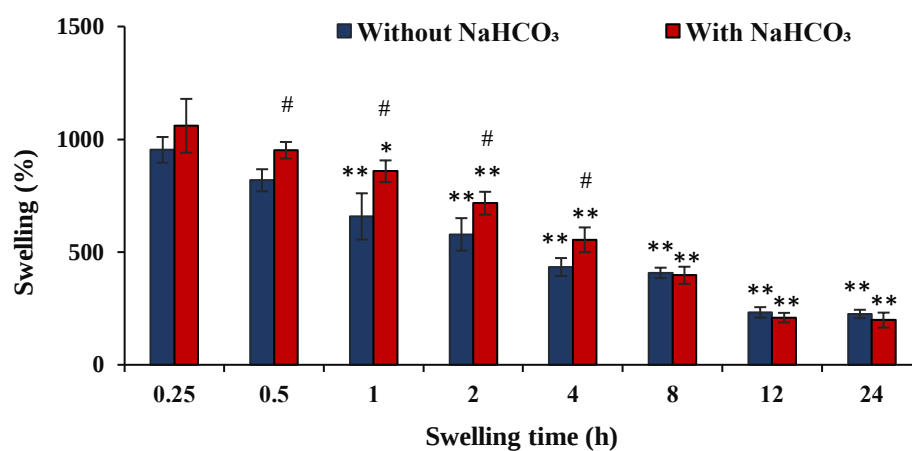


Figure 23 The percentage swelling of CS-MNs patch without NaHCO₃ (■), and CS-MNs patch with NaHCO₃ (■). All data are presented as mean ± SD (n = 3). * $p < 0.05$, ** $p < 0.01$ compared with the swelling at 0.25 h in the same formulation. # $p < 0.05$ compared with the swelling between each formulation at the same time.

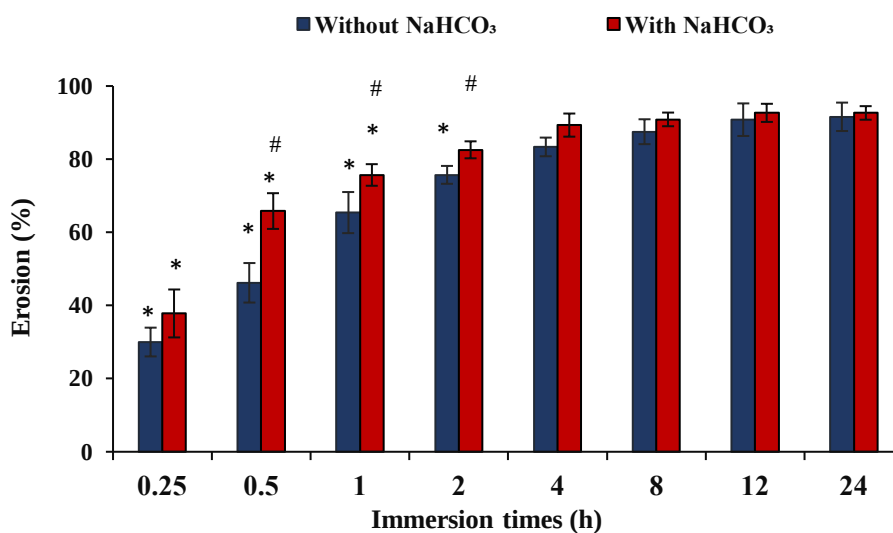


Figure 24 The percentage erosion of conventional CS-based MNs patch without NaHCO₃ (■) and CS-based MNs patch with NaHCO₃ (■). All data are presented as mean ± SD (n = 3). * $p < 0.01$ compared to the degradation at 0.25 h in the same formulation. # $p < 0.05$ compared the swelling of each formulation at the same time.

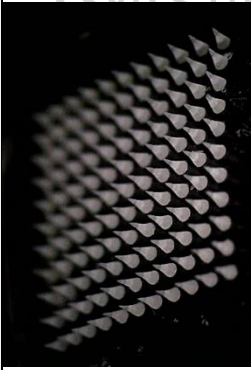
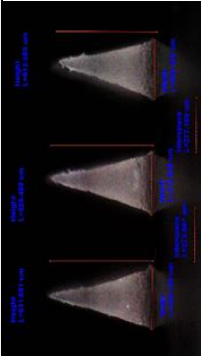
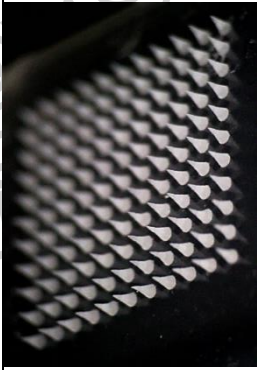
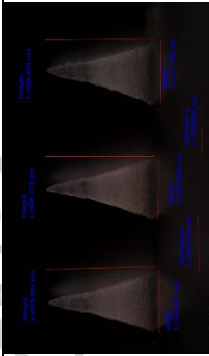
4.3.2 Fabrication of JE-CV-loaded conventional CS-MNs patch

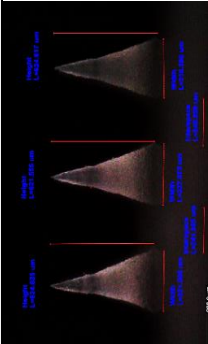
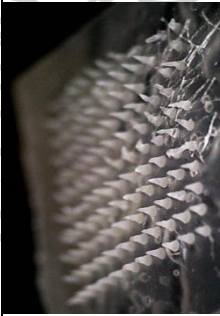
4.3.2.1 Morphology of JE-CV-loaded conventional CS-MNs patch

Maximum loading of JE-CV into the CS-MNs patch was studied at various mass ratios of JE-CV: blended solutions (1:3, 1:4, and 1:5). The morphologies of JE-CV-loaded CS-MNs patches were imaged by Dino-Lite microscope, as shown in Table 9. It was found that the maximum loading of JE-CV in the CS-MNs patch was observed at 1:4 of mass ratio. As a result, the JE-CV-loaded CS-MNs patch was successfully fabricated comprising 121 (11×11) cone-shaped arrays with 623.0 ± 1.4 μm in height, 321.0 ± 4.9 μm in base width, and 275.0 ± 2.0 μm in interspacing.



Table 9 Morphology of JE-CV-loaded CS-MNs patch at various mass ratios of JE-CV:blended polymers.

Formulations (JE-CV:polymers)	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
Unloaded Vaccine	 MNs patch was completely formed with a total of 121 needles.	 Height: $624.7 \pm 6.9 \mu\text{m}$ Base width: $304.7 \pm 6.6 \mu\text{m}$ Interspace: $275.0 \pm 2.0 \mu\text{m}$
1:5	 MNs patch was completely formed with a total of 121 needles.	 Height: $626.7 \pm 1.9 \mu\text{m}$ Base width: $327.0 \pm 2.8 \mu\text{m}$ Interspace: $269.0 \pm 6.0 \mu\text{m}$

Formulations (JE-CV:polymers)	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
1:4	 MNs patch was completely formed with a total of 121 needles.	 Height: $623.0 \pm 1.4 \mu\text{m}$ Base width: $321.0 \pm 4.9 \mu\text{m}$ Interspace: $275.0 \pm 2.0 \mu\text{m}$
1:3	 MNs patch was incompletely formed.	 MN arrays were not fully-formed and fragile.

4.3.2.2 Mechanical strength and skin insertion of JE-CV-loaded conventional CS-MNs patch

The mechanical strength property of CS-MNs patches is shown in Figure 25. It was found that the unloaded vaccine CS-MNs patch and JE-CV-loaded CS-MNs patch exhibited a similar resistant property to compression force. Moreover, Figure 26 demonstrated a complete penetration (100%) of the JE-CV-loaded CS-MNs patch into neonatal porcine skin, shown by stained microholes as blue dots. All characteristics evaluation of JE-CV-loaded CS-MNs patch at 1:4 mass ratio revealed the optimized properties to use this formulation to develop a separating CS-based MNs patch (sCS-MNs patch) in the further study.

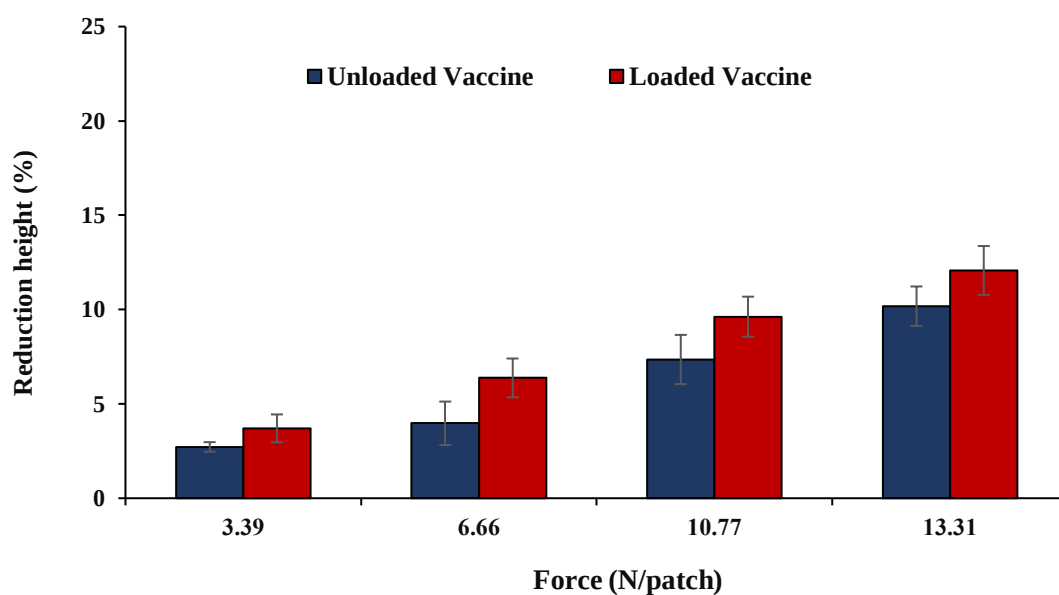


Figure 25 The percentage of needles reduction height of unloaded JE-CV in the CS-MNs patch (■) and JE-CV-loaded CS-MNs patch (■). All data are presented as mean $\pm$ SD (n = 3).

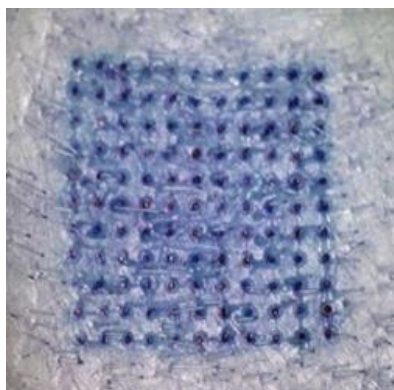


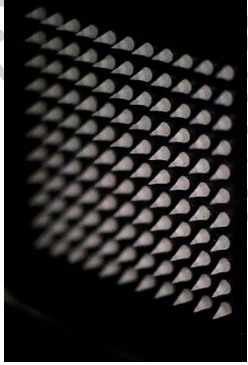
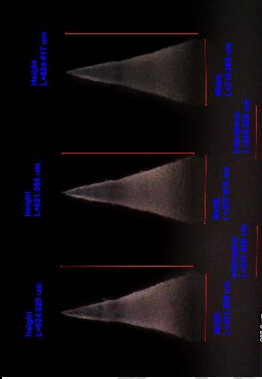
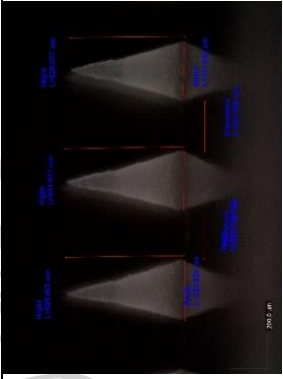
Figure 26 Image of neonatal porcine skin stained with methylene blue after applying JE-CV-loaded CS-MNs patch.

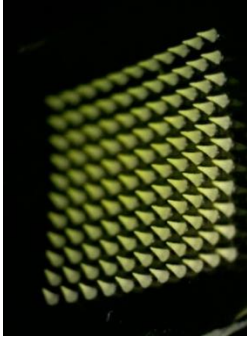
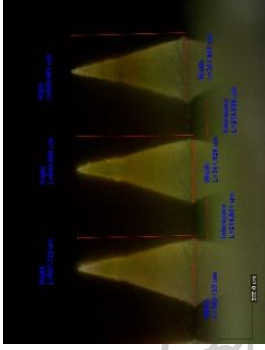
4.3.2 Fabrication of JE-CV-loaded separating CS-based MNs patch (JE-CV-loaded sCS-MNs patch)

4.3.2.1 Morphology of JE-CV-loaded sCS-MNs patch

A Blank sCS-MNs patch was successfully prepared by attaching the blend polymer solution on a nitrocellulose membrane as a base of MNs patch. FITC-BSA and JE-CV were loaded into the sCS-MNs patch for the FITC-BSA-loaded sCS-MNs patch and JE-CV-loaded sCS-MNs patch, respectively. Both formulations were successfully prepared and presented a similar morphology with the blank sCS-MNs patch, as shown in Table 10. Furthermore, the morphology of the JE-CV-loaded sCS-MNs patch was confirmed with SEM that exhibited successfully preparing (Table 11). Moreover, the fluorescent image of the FITC-BSA-loaded sCS-MNs patch (Figure 27) exhibited that FITC-BSA was deposited only in needles but not in a nitrocellulose base. Therefore, it was indicated the successful preparation of the sCS-MNs patch.

Table 10 Morphology of sCS-MNs patch.

Formulations	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
Blank sCS-MNs patch	 MNs patch was completely formed with a total of 121 needles.	 Height: $626.7 \pm 1.9 \mu\text{m}$ Base width: $327.0 \pm 2.8 \mu\text{m}$ Interspace (base to base): $269.0 \pm 6.0 \mu\text{m}$
JE-CV-loaded sCS-MNs patch	 MNs patch was completely formed with a total of 121 needles.	 Height: $630.5 \pm 11.0 \mu\text{m}$ Base width: $315.82 \pm 4.7 \mu\text{m}$ Interspace (base to base): $272.6 \pm 6.6 \mu\text{m}$

Formulations	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
FITC-BSA-loaded sCS-MNs patch	 MNs patch was completely formed with a total of 121 needles.	 Height: $639.6 \pm 11.5 \mu\text{m}$ Base width: $352.0 \pm 9.3 \mu\text{m}$ Interspace (base to base): $213.4 \pm 2.1 \mu\text{m}$

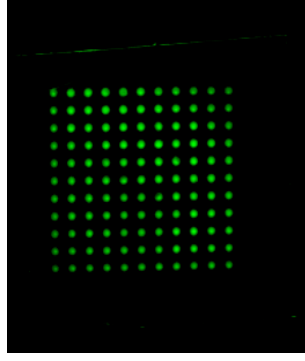


Figure 27 Fluorescent image of FITC-BSA-loaded sCS-MNs patch (top view).



4.3.2.2 Mechanical strength and skin insertion of JE-CV-loaded sCS-MNs patch

All sCS-MNs formulations show good mechanical properties without needle fracture (Figure 28). The height reduction of needles increased when the compression forces increased. Among all formulations, the percentage of needles height reduction of the JE-CV-loaded sCS-MNs patch was slightly higher than the other two formulations; however, there is no significant difference ($p>0.05$). Figure 29 showed that all formulations could penetrate the ICR mice's skin (~90%), which confirmed the ability of the MNs for skin insertion.

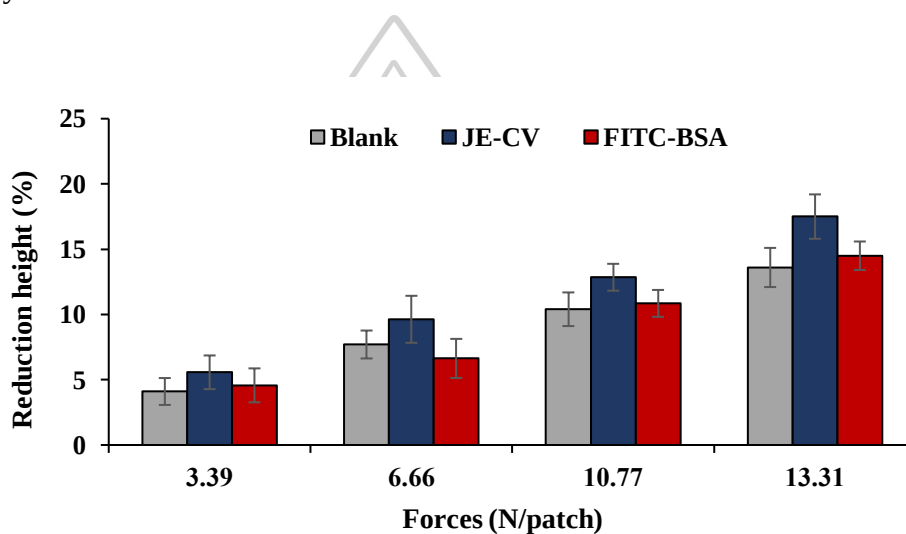


Figure 28 The percentage of needle reduction height of the blank sCS-MNs patch (□), JE-CV-loaded sCS-MNs patch (■), and FITC-BSA-loaded sCS-MNs patch (■). All data are presented as the mean $\pm$ SD ($n = 3$).

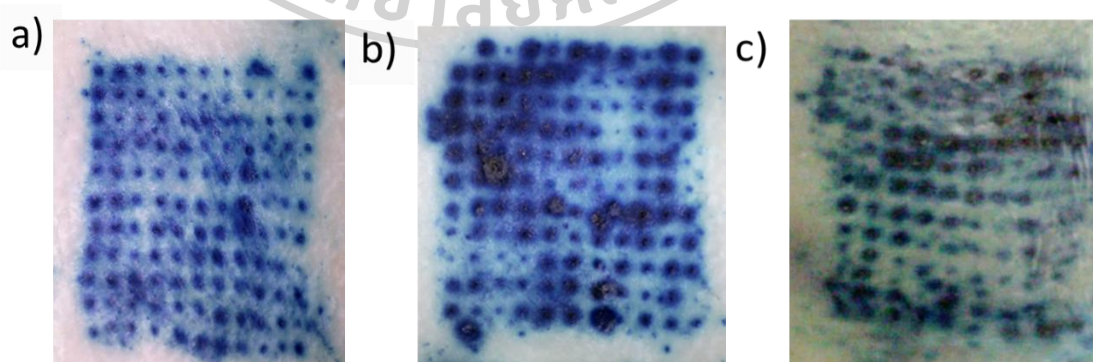


Figure 29 Images of ICR mice skin stained with methylene blue following the application of the different formulations, including a) blank sCS-MNs patch, b) JE-CV-loaded sCS-MNs patch, and c) FITC-BSA-loaded sCS-MNs patch.

4.3.2.3 Loading of FITC-BSA and JE-CV into sCS-MNs patch

FITC-BSA-loaded sCS-MNs patch had %LE of $80.4 \pm 4.5 \%$ and LC of 130 ± 40 mg/g. In the case of the JE-CV-loaded sCS-MNs patch, %LE and LC were $68.0 \pm 10.4 \%$ and $1.0 \pm 0.3 \times 10^4$ PFU/g using the plaque assay method, and $79.0 \pm 3.6 \%$ and $1.2 \pm 0.6 \times 10^4$ PFU/g when using RT-qPCR assay (Table 12). JE-CV quantified with both methods showed no significant differences in the level of JE-CV antigen. The result indicated that the vaccine preparation process did not inactivate the live-attenuated JE-CV. Moreover, the variation of the mean in plaque assay, a new qualification method for the live attenuated vaccine, was in the acceptable range compared with RT-qPCR, confirming the sensitivity and reproducibility of this analysis [128].

Table 12 Loading efficiency (LE) and loading capacity (LC) of FITC-BSA and JE-CV in sCS-MNs patch.

Formulations	Loading efficiency (%LE)	Loading capacity (LC)
FITC-BSA-loaded sCS-MNs patch	$80.4 \pm 4.5 \%$	130 ± 40 mg/g
JE-CV-loaded sCS-MNs patch	$68.0 \pm 10.4 \%$ (Plaque assay)	$1.0 \pm 0.3 \times 10^4$ PFU/g
	$79.0 \pm 3.6 \%$ (RT-qPCR assay)	$1.2 \pm 0.6 \times 10^4$ PFU/g

4.3.2.4 *In vivo* insertion study of CS-MNs patch and sCS-MNs patch

The formulations of both FITC-BSA-loaded CS-MNs patch and -loaded sCS-MNs patch were prepared with the same mixture of FITC-BSA and fabricated polymers, but differed kinds of dissolving MNs patch, including a classical dissolving MNs patch and separating-dissolving MN patch, respectively. Both FITC-BSA-loaded CS MNs patch and -loaded sCS-MNs patch exhibited similar properties of morphology, mechanical strength, and *in vivo* insertion that showed the penetration into ICR mice's skin of more than 90 percent, as shown in the Appendix.

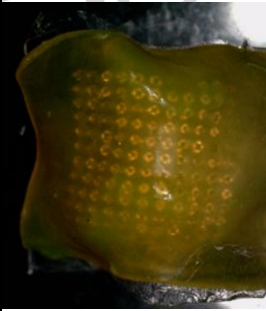
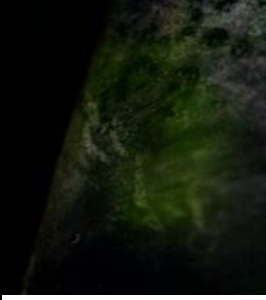
Following the application of both FITC-BSA-loaded CS MNs patch and -loaded sCS-MNs patch into the IRC mice were used to investigate the delivery efficiency of the model antigen of the sCS-MNs patch when compared with FITC-BSA-loaded CS MNs patch. The delivery efficiency at various administration periods

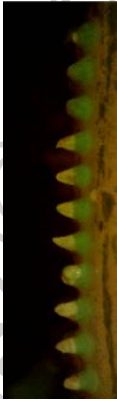
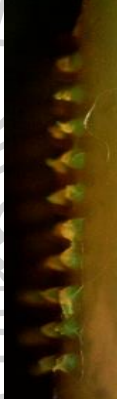
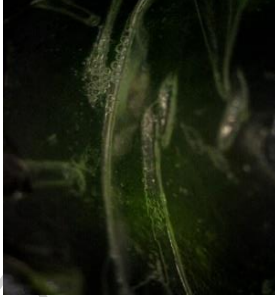
is shown in Table 13. The results found that after wearing the FITC-BSA-loaded sCS-MNs patch for 1 h, the percentage delivery efficiency of FITC-BSA was significantly improved from ~39% (0.5 h of wearing) to ~91% (1 h of wearing) ($p < 0.05$), which is incline towards the saturation. Because the MN array of the sCS-MNs patch could penetrate and separate completely at 1 h of wearing. Therefore, one hour of wearing time was selected for JE-CV delivery using the sCS-MNs patch.

For the FITC-BSA-loaded CS-MNs patch, the delivery efficiencies were ~11% (0.5 h of wearing) to ~94% (8 h of wearing, the limited time of study) ($p < 0.05$). At 1 h of wearing, the FITC-BSA-loaded CS-MNs patch did not dissolve completely in mice's skin with a delivery efficiency of 28% (3-fold lower than the FITC-BSA-loaded CS MNs patch at the same time); instead, it dissolves in mice's skin with a delivery efficiency of 61% at 2 h. Therefore, wearing time should be more than 1 h after MNs patch application.

Our results confirmed the separating property of the sCS-MNs patch in the case that whether the needles can separate from the backing membrane led to its detachable property. Although the dissolving time of the needles of the FITC-BSA-loaded CS MNs patch was more than 1 h, the complete separation of the needles from the FITC-BSA-loaded sCS-MNs patch was 1 h. Those results indicated that the needles of the FITC-BSA-loaded sCS-MNs patch separated and embedded in the skin after peeling at 1 h of wearing time. Then embedded needles were dissolved in the skin to release FITC-BSA. This property could decrease the wearing time of MNs patches applied on the skin, which is in line with several studies of separating MNs patches [129, 130].

Table 13 Images of FITC-BSA residual and percentage delivery efficiency of FITC-BSA from both CS-MNs patch and sCS-MNs patch delivered into ICR mice.

Wearing times (h)	FITC-BSA-loaded CS-MNs patch		FITC-BSA-loaded sCS-MNs patch	
	Images of FITC-BSA residual	Delivery Efficiency (%)	Images of FITC-BSA residual	Delivery Efficiency (%)
0.5		 10.8 ± 2.4		38.7 ± 12.1
1		 28.2 ± 5.9		91.4 ± 7.5

Wearing times (h)	FITC-BSA-loaded CS-MNs patch		FITC-BSA-loaded sCS-MNs patch	
	Images of FITC-BSA residual	Delivery Efficiency (%)	Images of FITC-BSA residual	Delivery Efficiency (%)
2		 60.6 ± 8.5		92.7 ± 3.4
4		 77.5 ± 10.1		94.7 ± 2.3
8		 93.8 ± 6.7		96.0 ± 2.0

4.3.2.5 Skin resealing and irritation of JE-CV-loaded sCS-MNs patch

In this study, the skin's ability to reseal after an application of MNs patch was investigated to confirm its safety. The holes on the skin created by the MNs should be closed as fast as possible after removing the patch to avoid infection by pathogenic microorganisms and increase the healing rate [131]. The resealing is a natural skin-repairing mechanism to recover stratum corneum related to lipids synthesis in the lamellar body section. [131, 132]. JE-CV-loaded sCS-MNs patch created ~90% of micropores on the skin surface at the initial of the study (0 h). The skin resealing after detaching the JE-CV-loaded sCS-MNs patch at 0, 1, 6, 12, 24, and 48 h was evaluated, as shown in Table 14. The results revealed that the skin resealing ability depended on the recovery times, which is the skin resealed over time. The percentage of skin resealing was ~ 15% at 1 h of recovery time point and increased to ~ 40% (6 h), ~ 65% (12 h), ~ 75% (24 h), and ~ 100% (48 h) as a complete resealing. According to the previous study, the microhole that appeared on the mice's skin from the dissolving MNs patch required at least 24 h of recovery time for completion of skin resealing [92, 95].

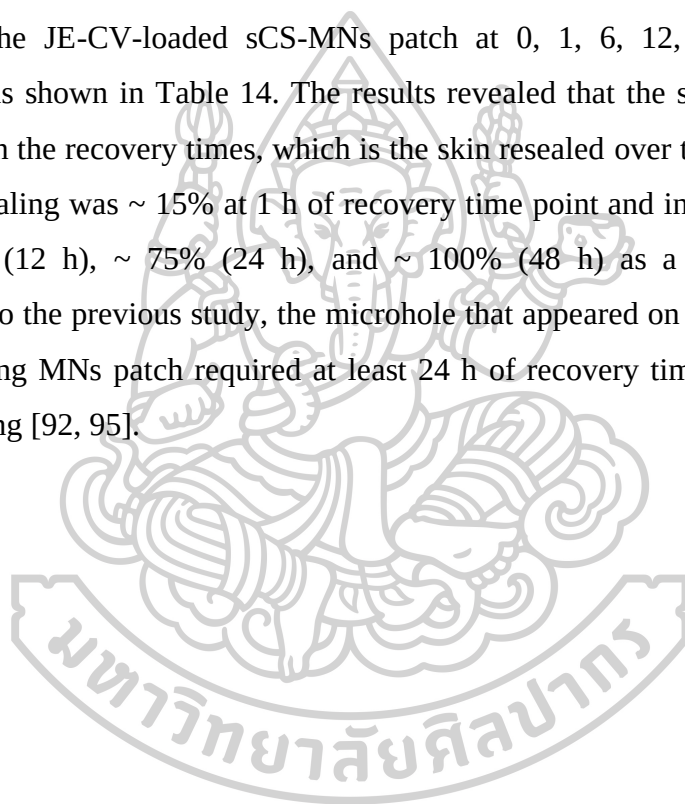
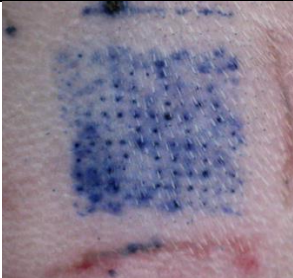
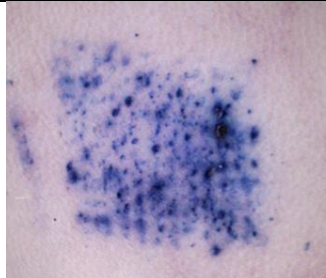
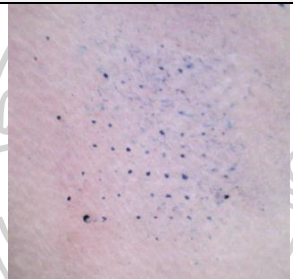
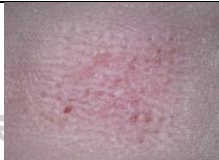


Table 14 Skin resealing at various time points after detaching the JE-CV-loaded sCS-MNs patch from the ICR mice skin.

0 h	1 h	6 h
		
12 h	24 h	48 h
		

Skin irritation was also evaluated after detaching the blank sCS-MNs patch and JE-CV-loaded sCS-MNs patch from the skin (Table 15). The results found that both formulations induced erythema at the site of administration when evaluated with the Draize scale ($p > 0.05$), indicating a moderate skin reaction without systemic reaction. The results implied that the JE-CV did not induce skin irritation but was induced by as sCS-based MNs patch. However, the skin irritation disappeared after removing the MNs patch for 48 h, similar to the previous studies [96, 97]. As a result, using a JE-CV-loaded sCS-MNs patch would be safe for ID administration due to the natural skin resealing of the body.

Table 15 Skin irritation at various time points after detaching the JE-CV-loaded sCS-MNs patch from the ICR mice skin.

Time (h)	Blank sCS-MNs	JE-CV-loaded sCS-MNs
0		
1		
6		
12		
24		
48		

4.3.3 Intradermal Immunization

ID immunization studies were determined in C57/BL6 mice (4-5 weeks old, n=6 per group). In this study, the mice were divided into 5 groups, as shown in Table 15.

Table 16 Intradermal immunization study for mice in each group (n = 6).

Groups	Routes and formulations
1	SC immunization of JE-CV solution 1×10^4 PFU/mouse (Full-dose JE-CV, SC)
2	SC immunization of JE-CV solution 5×10^3 PFU/mouse (Half-dose JE-CV, SC)
3	ID immunization of JE-CV-loaded sCS-MNs patch, 5×10^3 PFU/mouse (Half-dose JE-CV-loaded sCS-MNs patch, ID)
4	ID immunization of sCS-MNs patch (Blank sCS-MNs patch, ID)
5	SC immunization of 0.04% NaCl (Vehicle, SC)

All immunized mice with JE-CV formulations elicited IgG after immunization for 28 days, as shown in Figure 30A. The mice immunized with the full-dose of JE-CV via SC injection stimulated PRNT₅₀ with approximately 46.2 ± 28.6 titers which were significantly higher than the half-dose of JE-CV-loaded sCS-MNs via ID immunization (20.4 ± 6.0 titers, $p < 0.01$) and via SC injection (9.5 ± 2.7 titers, $p < 0.01$), respectively. The full-dose SC immunization and half-dose ID immunization with sCS-MNs patch stimulated PRNT₅₀ ≥ 10 titer as a protective level. It was demonstrated that using a half-dose of JE-CV via ID immunization using sCS-MNs patch successfully induced humoral immune responses.

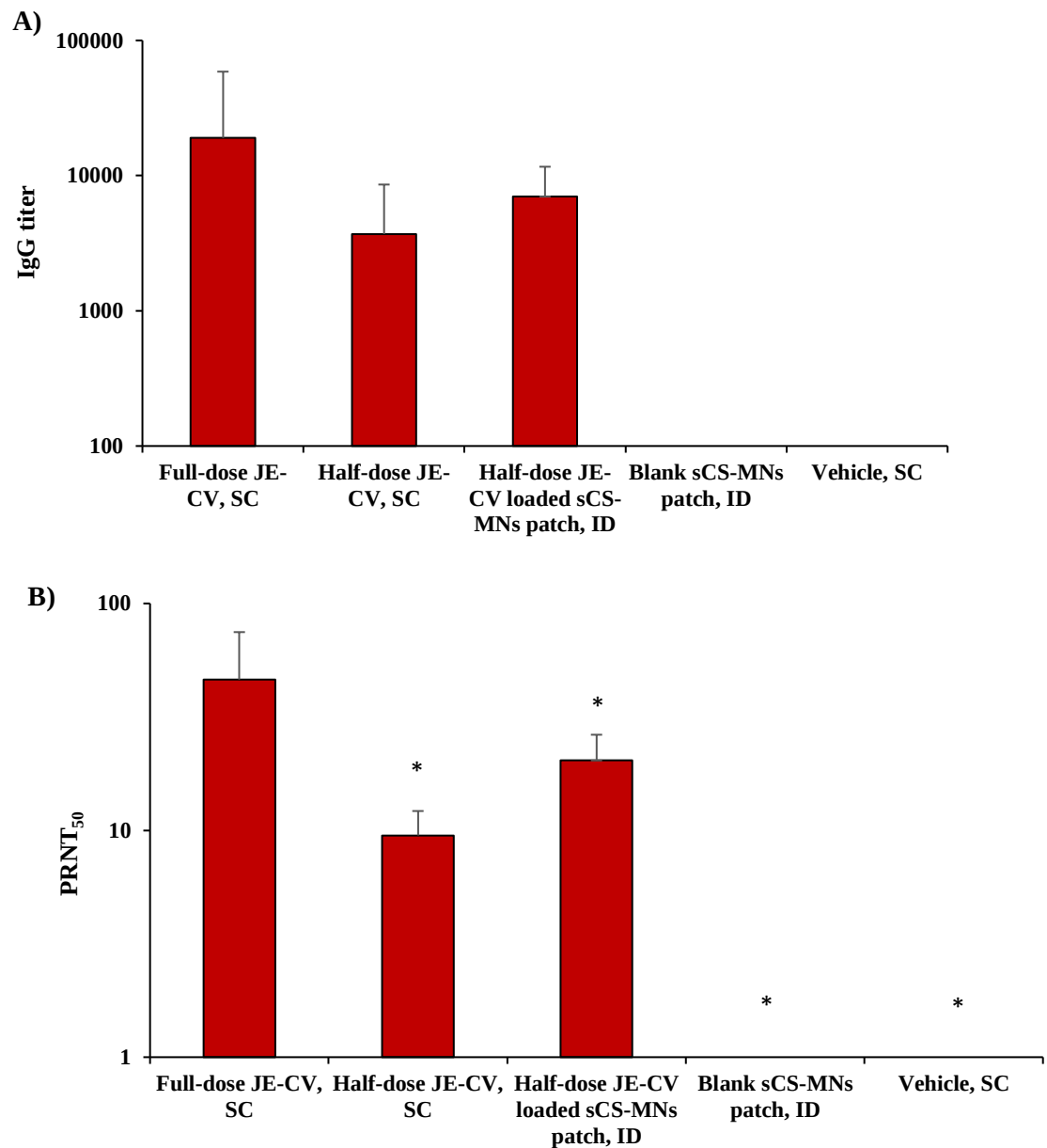


Figure 30 HIR at day 28 after JE-CV immunization in mice. A) Specific anti-JEV IgG antibody titer and B) 50% PRNT₅₀. All data are expressed as mean $\pm$ SD ($n = 6$), * $p < 0.01$ compared with full-dose of JE-CV solution (1×10^4 PFU/mouse) immunized via SC injection, and # $p < 0.05$, ## $p < 0.01$ compared with a half-dose JE-CV (5×10^3 PFU/mouse) solution immunized via SC injection.

Specific IgA, an antibody that plays a role in the immune function of the mucosal immune responses, was evaluated in saliva and nasal wash. The results showed that mice immunized with half-dose of JE-CV-loaded sCS-MNs via ID

stimulated IgA levels (saliva 6.7 ± 2.1 titers and nasal wash 8.4 ± 4.1 titers), which was higher than immunization with full-dose (saliva 4.7 ± 1.6 titers and nasal wash 6.0 ± 2.2 titers) and half-dose (saliva 2.4 ± 0.8 titers and nasal wash 3.4 ± 1.0 titers) of JE-CV via SC injection (Figure 31). In the case of half-dose immunization, ID immunization with JE-CV-loaded sCS-MNs patch obtained IgA levels in saliva and nasal wash that were 2.8-fold and 2.5-fold significantly higher than those of SC injection. The antigen vaccine is captured by APCs at the inductive site and then migrates to mucosal lymph nodes and systemic lymph organs for immune stimulation. The skin contains a number of APCs, dendritic cells and Langerhans cells, higher than the subcutaneous layer or muscle, resulting in strong immune responses in both mucosal and systemic immune responses after immunization via ID compared with SC or IM [92, 133].

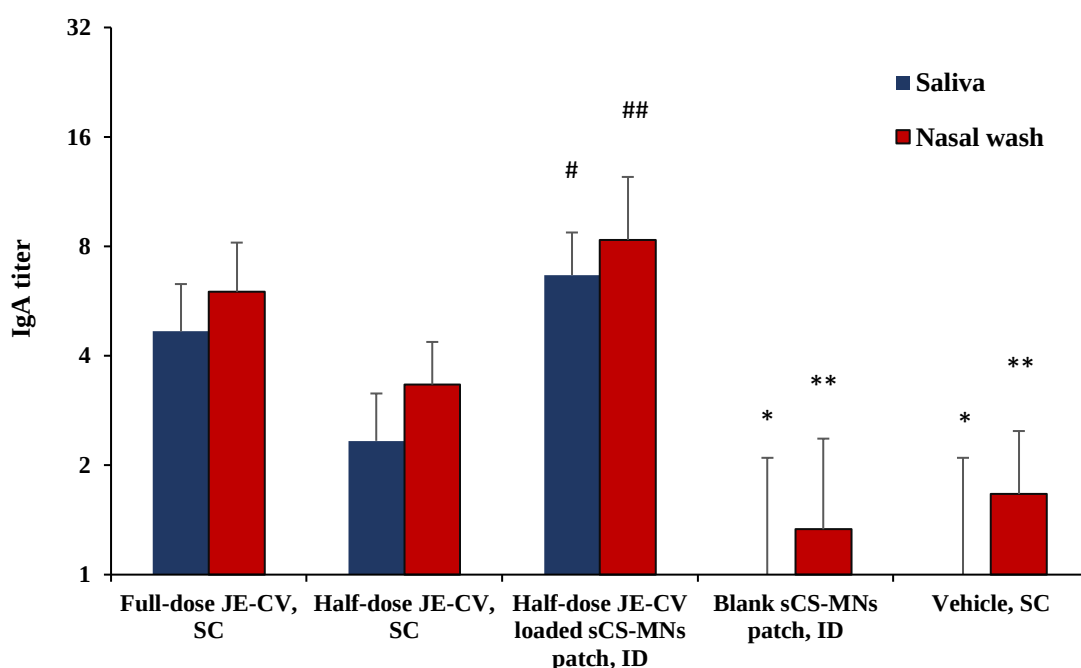


Figure 31 Specific anti-JEV IgA antibody titer in saliva (■) and nasal wash (■) after immunized mice with JE-CV formulations for 28 days. Results are expressed as mean $\pm$ SD ($n = 6$), * $p < 0.05$, ** $p < 0.01$ compared with full-dose of JE-CV solution (1×10^4 PFU/mouse) immunized via SC, and # $p < 0.05$, ## $p < 0.01$ compared with a half-dose of JE-CV (5×10^3 PFU/mouse) solution immunized via SC.

CMIR stimulation was confirmed by determining cytokine levels, including IL-4 and IFN- γ . The levels of IL-4 in JE-CV immunization groups ranged from 50 to 70 pg/mL; the highest IL-4 was found in the group of full-dose JE-CV SC immunization (70 pg/mL), as shown in Figure 32. Compared with full-dose JE-CV SC immunization, mice immunized with half-dose JE-CV ID immunization showed slightly decreased IL-4 levels with no significant differences. The results demonstrate the potential of the ID administration with the MNs patch for enhancing the immune responses, even if immunized with only a half-dose of the vaccine. For IFN- γ cytokine, full-dose JE-CV solution immunized by SC injection induced about 543.40 ± 54.39 pg/mL, while using half-dose JE-CV-loaded sCS-MNs patch via ID stimulated approximately 511.40 ± 62.19 pg/mL ($p > 0.05$), which is revealed the potential of the ID immunization with MNs patch. Comparing a half-dose of JE-CV, ID immunization elicited IFN- γ 1.4-fold greater than SC immunization ($p < 0.05$). The results were consistent with the previous studies, which compared ID immunization with conventional immunization by SC [134, 135].

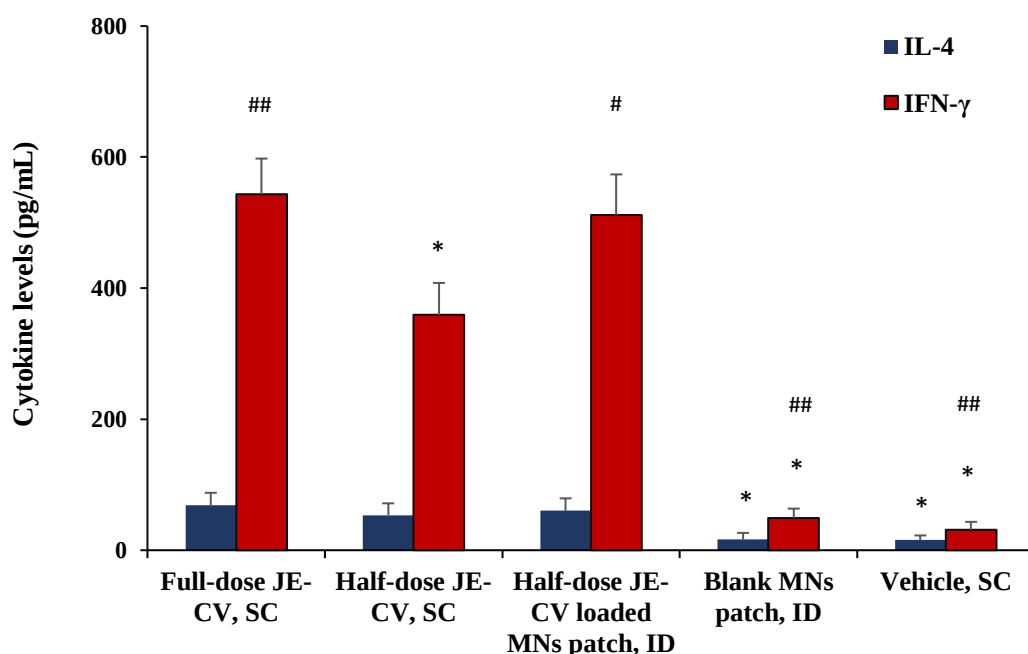


Figure 32 Cytokine levels of IL-4 (■) and IFN- γ (■) from splenocytes of mice immunized with various formulations. Results are expressed as mean $\pm$ SD ($n = 6$), * $p < 0.01$ compared with full-dose JE-CV solution (1×10^4 PFU/mouse) SC

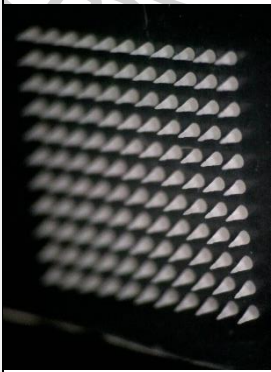
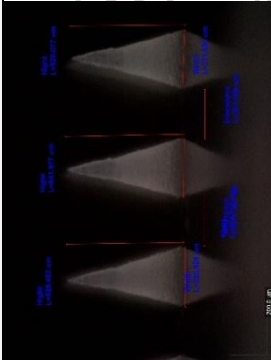
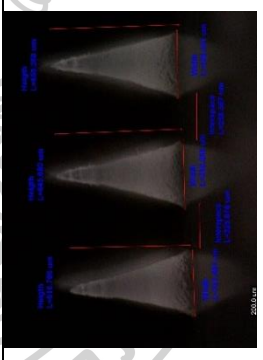
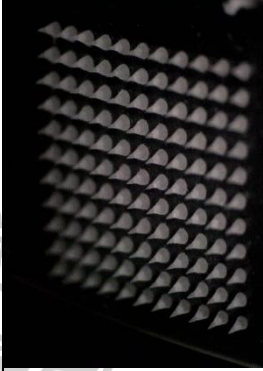
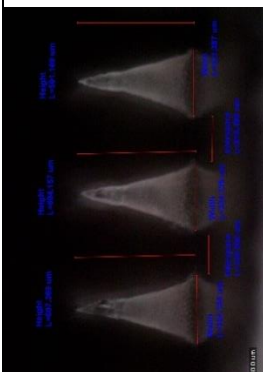
immunization, and [#] $p < 0.05$, ^{##} $p < 0.01$ compared with a half-dose JE-CV (5×10^3 PFU/mouse) solution SC immunization.

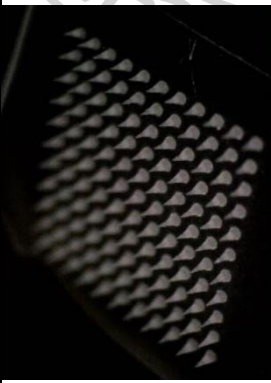
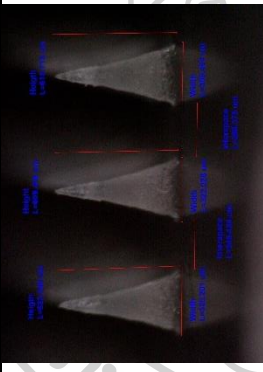
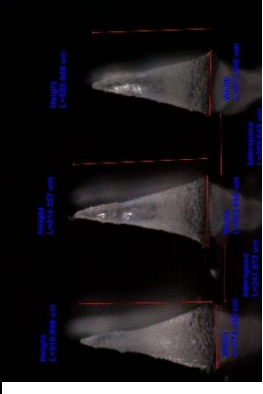
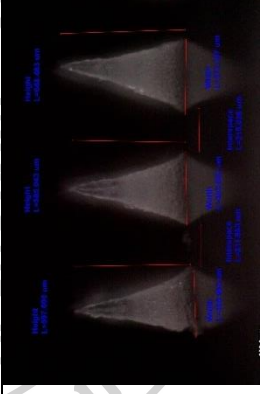
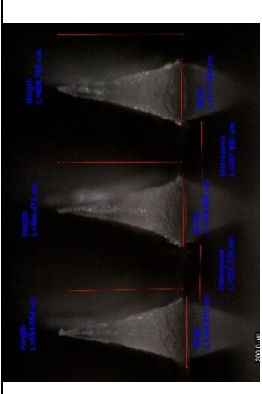
4.3.4 Stability test

The stability of the JE-CV-loaded sCS-MNs patch was studied by evaluating the physical properties, including morphology and mechanical strength, and the amount of vaccine remaining in the patch after being stored at 4°C and 25°C over 3 months. The morphology of the JE-CV-loaded sCS-MNs patches after storing at 4°C and 25°C for 3 months were not changed (Table 17).



Table 17 Morphology of JE-CV-loaded separating CS-MNs patch after storing at 4°C and 25°C for 3 months.

Storage time (Month)	Morphology after storing at 4°C		Morphology after storing at 25°C	
	Tilt-view	Dimensions of the Array (215 X)	Tilt-view	Dimensions of the Array (215 X)
0		 Height: 630.5 ± 11.0 µm Base width: 315.82 ± 4.7 µm Interspace: 272.6 ± 6.6 µm		 Height: 630.5 ± 11.0 µm Base width: 315.82 ± 4.7 µm Interspace: 272.6 ± 6.6 µm
1		 Height: 640.2 ± 21.3 µm Base width: 348.7 ± 6.6 µm Interspace: 232.0 ± 9.0 µm		 Height: 600.9±8.6 µm Base width: 329.9±7.4 µm Interspace: 204.0 ± 20.5 µm

Storage time (Month)	Morphology after storing at 4°C		Morphology after storing at 25°C	
	Tilt-view	Dimensions of the Array (215 X)	Tilt-view	Dimensions of the Array (215 X)
2		 Height: $615.9 \pm 6.45 \mu\text{m}$ Base width: $318.9 \pm 8.3 \mu\text{m}$ Interspace: $258.9 \pm 13.4 \mu\text{m}$		 Height: $593.4 \pm 33.3 \mu\text{m}$ Base width: $285.3 \pm 16.2 \mu\text{m}$ Interspace: $252.3 \pm 14.6 \mu\text{m}$
3		 Height: $611.2 \pm 32.7 \mu\text{m}$ Base width: $355.3 \pm 23.3 \mu\text{m}$ Interspace: $213.5 \pm 2.2 \mu\text{m}$		 Height: $598.6 \pm 7.1 \mu\text{m}$ Base width: $336.8 \pm 16.9 \mu\text{m}$ Interspace: $261.9 \pm 8.7 \mu\text{m}$

The percentage reduction height of needles from JE-CV-loaded sCS-MNs patch after being stored at 4°C and 25°C over 3 months were evaluated at the compression force of 10.77 N/patch, which is a maximum compression force that humans can achieve by thumb pressure in order to apply MNs into the skin [91]. The storage time over 3 months at both temperatures did not affect the reduction in needle height of the JE-CV-loaded sCS-MNs patch (Figure 33), ranging from 11% to 14%.

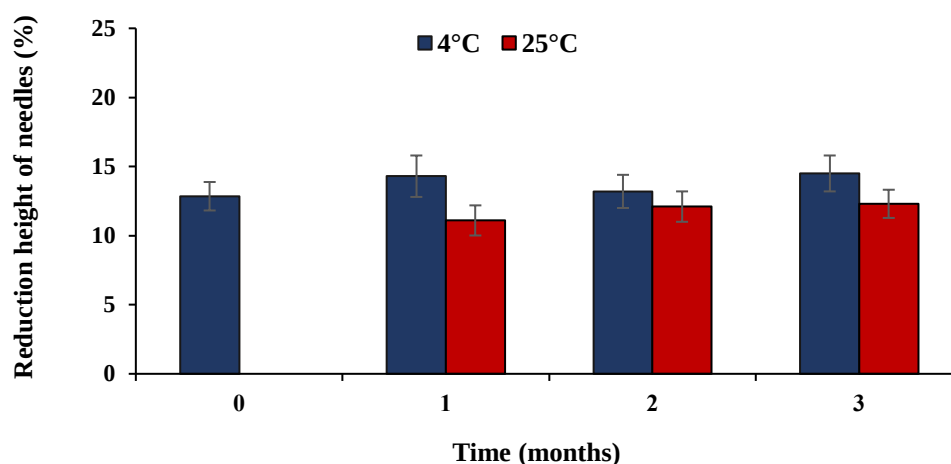


Figure 33 The percentage of reduction height of needles from JE-CV-loaded sCS-MNs patch after storing for 3 months at 4° and 25 °C. The compression force was used at 10.77 N/patch. All data are presented as the mean $\pm$ SD (n = 3).

For vaccine stability evaluation, the amounts of JE-CV in sCS-MNs patches after being stored at 4°C and 25°C for 3 months were evaluated using RT-qPCR. The percentage of JE-CV remaining was calculated by comparing the amount of JE-CV in sCS-MNs patch at different storage time points with the initial of the study (JE-CV=100%). The results revealed that the amount of JE-CV-loaded sCS-MN patch after storing at 4 °C for 1 month was $87.0 \pm 6.1\%$, and the amount of JE-CV decreased to $75.4 \pm 6.6\%$ and $56.0 \pm 7.6\%$ after storing for 2 and 3 months, respectively (Figure 34). It can be implied that a JE-CV-loaded sCS-MN patch may stabilize antigens and protect against a vaccine loss at short-term protection; however, it might be insufficient for long-term protection following ICH Q5C guideline. The percentages of JE-CV that remained in the patch after being stored at 25°C

(accelerated condition) were significantly reduced when compared to 4°C ($p < 0.001$). Besides the acceptable properties of sCS-MNs patch for JE-CV immunization, stability is also a factor that needs to concern. As reviewed, JE-CV-loaded sCS-MNs patch may improve the stability by adding other stabilizers such as disaccharides stabilizers. Saengkrit et al. (2018) demonstrated that trehalose enhanced stabilizing property of CS to prevent live attenuated influenza vaccine loss for vaccine-incorporated CS dried powder under refrigeration conditions for 12 months [136]. Theoretically, the structure of disaccharides such as trehalose and sucrose had a high ability to accommodate the irregular surface structure of the vaccine resulting in structure protection. [78, 106]. Therefore, improving the stability of JE-CV-loaded sCS-MNs patch by using a disaccharide stabilizer and then optimizing for the amount needed in the formulations were recommended for further study.

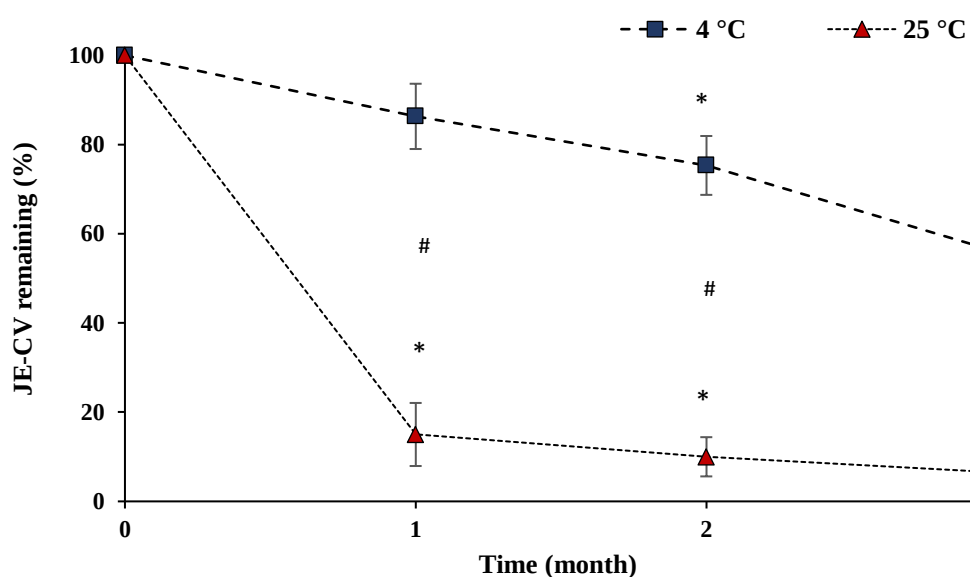


Figure 34 The percentage of JE-CV remaining in the sCS-MNs patch after storage at 4°C (■) and 25°C (▲) at 0, 1, 2, and 3 months. The data are expressed as mean ± SD ($n = 3$), * $p < 0.01$ compared to the percentage of JE-CV remaining in the sCS-MNs patch at the beginning of the study (0 month) at 4°C, and # $p < 0.01$ compared to the percentage of JE-CV remaining in the sCS-MNs patch after storage at 25°C at the same time of the study.

Chapter 5

Conclusion

In this study, CS-based NPs for IN immunization and CS-based MNs patches for ID immunization, a non-invasive alternative approach for flavivirus vaccination, were developed to improve the immune responses of the JE-CV compared with traditional SC immunization. This work, therefore, demonstrates the formulations, characteristics, and efficacies of using JE-CV-loaded CS-based NPs for IN immunization and JE-CV-loaded sCS-MNs patches for ID immunization. The results of this study could be concluded as follow:

5.1 Development of JE-CV loaded CS-based NPs for IN immunization

In this study, CS-NPs-loaded JE-CV and CM-NPs-loaded JE-CV were successfully developed. The optimal loading condition was found from the mass ratio of FITC-BSA: NPs at 1:6, incubation time 1 h at 4°C. This condition produced positive charge NPs, which was necessary for mucoadhesive and uptake properties of vaccine-loaded CS-based NPs, with high LE (>85%) and LC ($>1.2 \times 10^4$ PFU/mg) in all formulations. FITC-BSA-loaded NPs exhibited superior mucoadhesive and uptake properties on *ex vivo* nasal swine mucosa, compared to FITC-BSA alone without NPs. Mice immunized with JE-CV at 1×10^4 PFU (full dose of vaccine) via IN stimulated systemic and mucosal immune responses in mice, while SC immunization only stimulated both systemic immune responses but lack of mucosal immune responses. Moreover, immunized mice with JE-CV loaded CS- and CM-NPs via IN immunization elicited PRNT₅₀ at seroprotection level (PRNT₅₀ titers ≥ 10 , as recommended by WHO), similar to SC immunization with JE-CV solution. In the case of IFN- γ , a major CMIR for preventing flavivirus replication and amplification, the results revealed that IN immunization of JE-CV in mice with CS-NPs and CM-NPs produced more IFN- γ than that of the JE-CV solution; however, there are lower than those obtained from SC immunization with JE-CV solution. Regarding safety, all immunized mice were not found to have adverse drug reactions. Therefore, this study exhibited the potential of IN immunization with JE-CV-loaded CS-based NPs (CS-

NPs and CM-NPs) in stimulating the immune responses, which were in accordance with JE vaccine requirements.

5.2 Development of JE-CV-loaded sCS-MNs patch for ID immunization

The blended polymer of NaHCO_3 :CS:PVA (0.25:1:4) was fabricated for the CS-based MNs patch that showed an elegant appearance, able to resist sufficient compression forces with good ability for skin insertion. In addition, JE-CV was loaded into a CS-MN patch to obtain the optimal mass ratio of 1:4. The JE-CV-loaded CS-MNs patch was then successfully prepared using a cellulose membrane as an MN base to achieve a separating MNs patch. The morphology, mechanical strength, and penetration properties of the JE-CV loaded sCS-MNs patch were evaluated and showed acceptable results similar to JE-CV loaded CS-MNs patch. The percentages LE and LC of JE-CV loaded sCS-MNs patch were approximately 79% and $1.2 \pm 0.6 \times 10^4$ PFU/g, respectively. For safety, after applying MNs, the skin appeared the microholes and was naturally resealed without skin infection after peeling and completely recovered at 48 h. ID immunization in mice with the JE-CV-loaded sCS-MNs patch (5×10^3 PFU/mouse; a half dose of vaccine) effectively elicited HIR (serum and secretions), CMIIR, and PRNT₅₀, similar to SC immunization at the full dose of vaccine. However, JE-CV loaded sCS-MNs patch was stable at 4 °C only for a month; therefore, to expand for practical use, the stability enhancement was recommended for future study.

Based on safety and immune stimulation efficiency results, immunization with the CS-based NPs (CS-NPs and CM-NPs) via IN immunization and JE-CV-loaded sCS-MNs patch via ID immunization could be a promising approach for effective JE-CV immunization.

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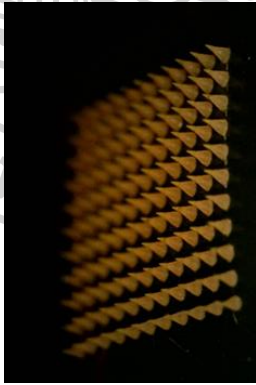
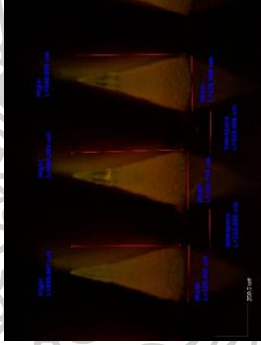
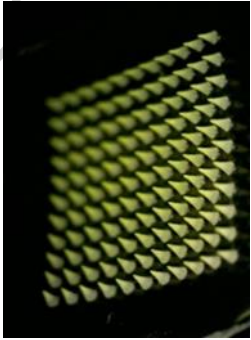
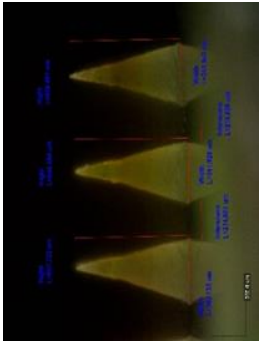
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APPENDIX

Table 18 Morphology of FITC-BSA-loaded CS-MNs patch and -loaded sCS-MNs patch

Formulations	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
FITC-BSA-loaded CS-MNs	 MNs patch was completely formed with a total of 121 needles.	 Height: $658.6 \pm 8.2 \mu\text{m}$ Base width: $336.3 \pm 8.5 \mu\text{m}$ Interspace: $213.4 \pm 2.1 \mu\text{m}$
FITC-BSA-loaded sCS-MNs		

Formulations	Morphology	
	Tilt view of MNs patch	Dimensions of the Array (215X)
	MNs patch was completely formed with a total of 121 needles.	Height: $639.6 \pm 11.5 \mu\text{m}$ Base width: $352.0 \pm 9.3 \mu\text{m}$ Interspace: $213.4 \pm 2.1 \mu\text{m}$

Table 19 Loading efficiency (LE) and loading capacity (LC) of FITC-BSA-loaded CS-MNs patch and JE-CV-loaded sCS-MNs patch.

Formulations	Loading efficiency (%LE)	Loading capacity (LC)
FITC-BSA-loaded CS-MNs patch	$83.6 \pm 2.3 \%$	$149 \pm 51 \text{ mg/g}$
FITC-BSA-loaded sCS-MNs patch	$80.4 \pm 4.5 \%$	$130 \pm 40 \text{ mg/g}$

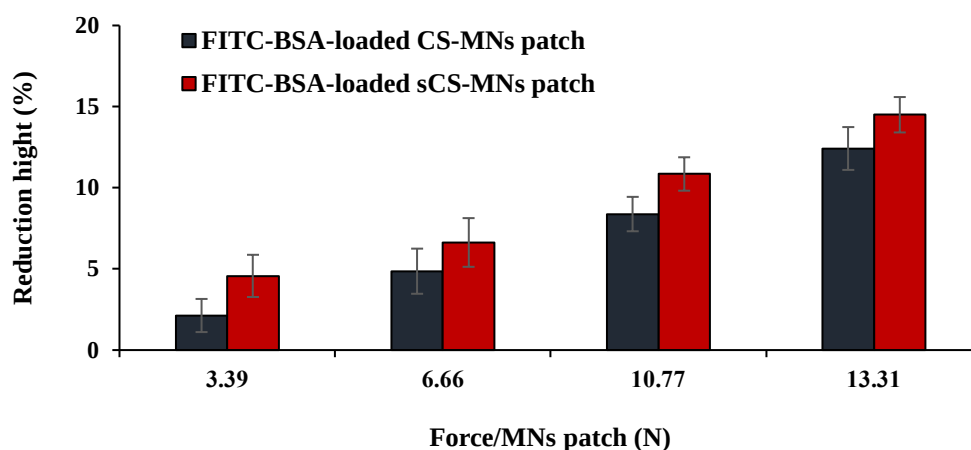


Figure 35 The percentage of needle reduction height of the FITC-BSA-loaded CS-MNs patch (■), and FITC-BSA-loaded sCS-MNs patch (■). All data are presented as the mean $\pm$ SD ($n = 3$).

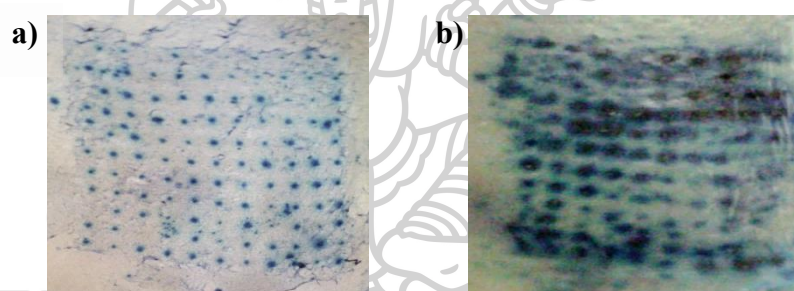


Figure 36 Images of ICR mice skin stained with methylene blue following the application of the different formulations, including a) FITC-BSA-loaded CS-MNs patch, and b) JE-CV-loaded sCS-MNs patch.

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