DEVELOPMENT OF NANOPARTICLE-IN-MICROSPHERE(NIMOS) SYSTEM FOR ORAL DELIVERY OF VACCINE AND ANTIBODY AGAINST PED VIRUS FOR SWINE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biomedicinal Chemistry
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การพัฒนาระบบนาโนพาร์ทิเคิลในไมโครสเฟียร์(นิมอส)สำหรับนำส่งวัคซีนและแอนติบอดีต่อไวรัสพีอีดีทางปากสำหรับสุกร

น.ส.รชตพรรณ จันทร์ฉาย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	DEVELOPMENT OF NANOPARTICLE-IN-MICROSPHERE(NIMOS) SYSTEM FOR ORAL DELIVERY OF VACCINE AND ANTIBODY AGAINST PED VIRUS FOR SWINE
By	Miss Rachatapan Junchay
Field of Study	Biomedicinal Chemistry
Thesis Advisor	Associate Professor WARANYOO PHOOLCHAROEN, Ph.D.
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รชตพรรณ จันทร์ฉาย : การพัฒนาระบบนาโนพาร์ทิเคิลในไมโครสเฟียร์(นิมอส)สำหรับนำส่งวัคซีนและแอนติบอดีต่อไวรัสพีอีดีทางปากสำหรับสุกร . (DEVELOPMENT OF NANOPARTICLE-IN-MICROSPHERE(NIMOS) SYSTEM FOR ORAL DELIVERY OF VACCINE AND ANTIBODY AGAINST PED VIRUS FOR SWINE) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ, อ.ที่ปรึกษาร่วม : อ. ดร.ธีรพงศ์ ยะทา

เชื้อไวรัสพีอีดีเป็นอุปสรรคสำคัญในทำอุตสาหกรรมการเลี้ยงสัตว์และเป็นสาเหตุหลักที่ทำให้เกิดอัตราการตายลูกสุกรแรกเกิดสูงถึง 95% จากการติดเชื้อในลำไส้ ทูซีเท็นโมโนโคลนอลแอนติบอดี (2C10 mAb) เป็นชีววัตถุที่ได้จากการผลิตแบบชั่วคราวของต้นใบยาสูบ (*Nicotiana benthamiana*) ซึ่งถูกระบุว่าเป็นหนึ่งในชีววัตถุที่มีศักยภาพเป็นแอนติบอดีต้านเชื้อไวรัสพีอีดีได้โดยการนำส่งทางปาก ชีววัตถุเป็นเวชภัณฑ์คลื่นลูกใหม่และได้รับการพัฒนาอย่างต่อเนื่องซึ่งมีประโยชน์หลากหลายประการในด้านความเป็นพิษต่ำและมีความจำเพาะสูง อย่างไรก็ตามชีววัตถุไม่เหมาะสำหรับใช้ร่วมกับการให้ทางปากเนื่องจากข้อจำกัดของความเป็นกรดสูงและน้ำย่อยต่างๆของทางเดินอาหาร ดังนั้นเพื่อเอาชนะข้อจำกัดเหล่านี้การพัฒนาการให้ชีววัตถุทางปากจึงมีความจำเป็นอย่างยิ่งเพิ่มความเสถียรให้กับชีววัตถุ ไมโครบีดที่ตอบสนองต่อค่าพีเอช ถูกใช้เป็นพาหนะสำหรับการนำส่งทางปากและอัลจิเนต - ไคโตซานเป็นวัสดุที่รู้จักกันดีคุณสมบัติช่วยป้องกันชีววัตถุจากสภาพแวดล้อมที่ไม่เหมาะสมของทางเดินอาหาร ความเข้มข้นของอัลจิเนต - ไคโตซานได้รับการปรับให้เหมาะสมที่สุดสำหรับการสร้างไมโครบีดที่ตอบสนองต่อค่าพีเอช ซึ่งใช้อัลจิเนตที่มีความเข้มข้น 2% และไคโตซานที่มีความเข้มข้น 1% ซึ่งเป็นคุณสมบัติที่เหมาะสมที่สุด จากนั้นการวิเคราะห์ลักษณะทางกายภาพของไมโครบีดที่ตอบสนองต่อค่าพีเอช รวมถึงการวัดขนาดเส้นผ่านศูนย์กลางด้วยเวอร์เนียดิจิทัลพบว่าการกระจายของขนาดเส้นผ่านศูนย์กลางอยู่ระหว่าง 1.5-1.6 มม. ซึ่งมีมากถึง70% กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดแสดงให้เห็นว่าภาพพื้นผิวของไมโครบีดที่ตอบสนองต่อค่าพีเอช มีลักษณะเป็นโครงสร้างตาข่ายที่มีความพรุนมากมาย ในขณะเดียวกัน กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านแสดงให้เห็นถึงอนุภาคนาโนที่ถูกเก็บไว้ในไมโครบีดนอกจากนี้พฤติกรรมการปลดปล่อยของไมโครบีดที่ตอบสนองต่อค่าพีเอช ในสารละลายจำลองทางสรีรวิทยา (SPF) ได้รับการตรวจสอบภายใต้กล้องจุลทรรศน์เรืองแสงพบว่าไมโครบีดที่ตอบสนองต่อค่าพีเอช สามารถปกป้องและรักษาอนุภาคนาโนจากสารละลายจำลองทางสรีรวิทยา (SPF) ได้ ในขั้นตอนสุดท้ายชีววัตถุที่ห่อหุ้มด้วยไมโครบีดได้รับการทดสอบด้วยสารละลายจำลองทางสรีรวิทยา (SPF) หลังจากนั้นจะทำการประเมินประสิทธิภาพของชีววัตถุด้วยเทคนิคเรียลไทม์พีซีอาร์ (Real-Time PCR) จากผลลัพธ์ทั้งอนุภาคคล้ายไวรัสและ ทูซีเท็นโมโนโคลนอลแอนติบอดี (2C10 mAb) ยังคงมีความเสถียรแม้ว่าจะผ่านการทดสอบด้วยสารละลายจำลองทางสรีรวิทยา (SPF) โดยสรุปแล้วอัลจิเนต - ไคโตซานมีประสิทธิภาพเพียงพอที่จะกักเก็บอนุภาคนาโนไว้และไมโครบีดที่ตอบสนองต่อค่าพีเอช สามารถใช้เป็นพาหนะในการส่งยาทางปากได้เนื่องจากสามารถป้องกันชีววัตถุจากสภาพแวดล้อมที่มีความเป็นกรดสูงและปล่อยอนุภาคนาโนไปยังพื้นที่เป้าหมายได้

สาขาวิชา ชีวเวชเคมี ลายมือชื่อนิสิต .....

ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6076112933 : MAJOR BIOMEDICINAL CHEMISTRY

KEYWORD: chitosan-alginate, Porcine epidemic diarrhea virus, 2C10 monoclonal antibody, microencapsulation

Rachatapan Junchay : DEVELOPMENT OF NANOPARTICLE-IN-MICROSPHERE(NIMOS) SYSTEM FOR ORAL DELIVERY OF VACCINE AND ANTIBODY AGAINST PED VIRUS FOR SWINE. Advisor: Assoc. Prof. WARANYOO PHOOLCHAROEN, Ph.D. Co-advisor: TEERAPONG YATA, Ph.D.

PED virus is a major barrier to the animal farming industry, and it is the main cause of the mortality of suckling piglets up to 95% by enteric infection. The 2C10 monoclonal antibody (mAb) obtained from *Nicotiana benthamiana* produced by transient expression was indicated as one of the potential candidates is an antibody against PEDV by oral delivery. The biologics are a new wave of pharmaceuticals and have been continuously developing, which has various benefits of low toxicity and high specificity. However, it unsuitable for use with oral drug delivery owing to limitations of the strong acidity and protease of the GI tract. Therefore, to overcome these limitations, the development of oral drug delivery is essential for biologics to enhance stability. The pH-responsive microbeads were used as a carrier for oral delivery and the alginate-chitosan is a well-known material, some properties protect biologics from an inappropriate environment of the GI tract. The concentration of alginate-chitosan was optimized for pH-responsive beads construction, which the 2% alginate and 1% chitosan is a suitable qualification. Then the physical characterization of pH-responsive microbeads includes the diameter size measurement by digital vernier, found that the distribution of diameter size was between 1.5-1.6 mm which was more than 70%. The SEM showed that the surface of pH-responsive microbeads was a network structure with varying porosity, meanwhile, the TEM demonstrates to the nanoparticles kept in microbeads. Further, the release behaviour for pH-responsive microbeads in simulated physiological fluids (SPF) was investigated under a fluorescent microscope, it was found that the pH-responsive microbeads could protect and retain the nanoparticles from SPF. In the last step, the biologics encapsulated with microbeads were tested with SPF, after that, it evaluated the efficacy by Real-Time PCR. From the result, both the viral-like particles and the 2C10 mAb still stable, even though, was tested with SPF. In summary, the alginate-chitosan is efficient enough to retain the nanoparticles, and the pH-responsive microbeads can use as a carrier for oral drug delivery owing to could be protected from an acidic environment and released the nanoparticles to the target area.

Field of Study:	Biomedicinal Chemistry	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

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Rachatapan Junchay

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
%	=	percentage
Ba^{2+}	=	Barium ions
Ca^{2+}	=	Calcium ions
$CaCl_2$	=	Calcium chloride
cells/well	=	cells per well
cDNA	=	complementary DNA
Cq	=	quantification cycle
DNA	=	Deoxyribonucleic Acid
DI water	=	distilled water
kV	=	kiloVolt
min, mins	=	minute, minutes
µL	=	microlitre
mg/mL	=	milligram per milliliter
mL	=	milliliter
mm	=	Millimeter
M	=	molar
mAb	=	monoclonal antibody
mM	=	millimolar
MBS	=	Microbead Broken Solution

µm	=	micrometer
µg	=	microgram
NiMOS	=	Nanoparticles-in-microsphere
NaCl	=	Sodium Chloride
nm	=	nanometer
PEDV	=	Porcine Epidemic Diarrhea Virus
pH	=	Potential of Hydrogen ion
rpm	=	revolutions per minute
Real-Time PCR	=	Real-Time Polymerase Chain Reaction
S.D.	=	standard deviation
SEM	=	Scanning Electron Microscope
Sec	=	*seconds*
SGF	=	Simulated Gastric Fluid
SIF	=	Simulated Intestinal Fluid
siRNA	=	interfering Ribonucleic Acid
SPF	=	Simulated Physiological Fluids
TBP	=	TATA binding protein
TEM	=	Transmission Electron Microscope
TGE	=	Transmissible Gastro Enteritis
v/v	=	volume by volume
w/v	=	weight by volume

CHAPTER I
INTRODUCTION

Swine is one of the most important livestock species produced in animal farming. Inevitably, several infectious diseases can cause major financial damage to the producers of farmed pigs. Among several viruses, Porcine Epidemic Diarrhea has been now identified as one of the most serious infectious diseases. Porcine Epidemic Diarrhea Virus (PEDV), the causative agent of porcine epidemic diarrhea, was firstly discovered in Belgium in the early 1970s (Pensaert and De Bouck 1978). The symptoms of infected animals include intense diarrhea, vomiting, weight loss, and dehydration (Chasey and Cartwright 1978). PEDV infection also affects the growth rate of swine and newborn piglets (Pensaert and De Bouck 1978) leading to severe economic losses.

Among the effective prevention and control strategies, vaccination is one of the most effective approaches. Vaccines against infectious pathogens for the swine industry can be broadly categorized as traditional and modern vaccines. The traditional vaccine includes inactivated or killed vaccines and attenuated vaccines. The modern vaccine includes recombinant technology vaccines and synthetic peptide vaccines as well as DNA vaccines, which are in progress intensively (Correia, Bates et al. 2014). Generally, all of these vaccines have shown both advantages and disadvantages. Although there are several efforts trying to develop effective vaccines against various infectious diseases using recombinant vaccines and recombinant DNA technology, the inherent limitation of these modern antigens is their low immunogenicity in comparison to the traditional vaccines. The poor immunogenicity frequently observed in recombinant antigens is associated with a lack of immune activating components (Jorge and Dellagostin 2017). Most licensed vaccines in the swine industry are in the form of live attenuated or inactivated microorganisms (McVey and Shi 2010), (Unnikrishnan, Rappuoli et al. 2012). Traditional vaccines are generally more effective than modern vaccines because mostly the protection is mediated by the combination of multiple antigens composed of lipopolysaccharides,

lipoproteins, complex polysaccharides as well as proteins (LaFrentz, LaPatra et al. 2008), (Shoemaker, Klesius et al. 2009), (Shoemaker, Klesius et al. 2011).

Another approach to prevent or inhibit viral infections is the use of neutralizing antibodies to inhibit direct viral cell-to-cell spread of PED viruses. A previous study developed a plant-produced monoclonal antibody (mAb) 2C10 as a prophylactic candidate to prevent the PEDV infection (Rattanapisit, Srijangwad et al. 2017). The 2C10 mAb was transiently expressed in tobacco and lettuce using a geminiviral vector. Interestingly, the plant-produced 2C10 mAb can efficiently bind to the virus and inhibit PEDV infection *in vitro*, suggesting excellent potential for a plant-expressed 2C10 as PEDV prophylaxis for PEDV infection.

Orally administration of biopharmaceutical agents is more advantageous over other routes of administration due to its easy handling and low cost of production (Ferraro, Morrow et al. 2011). However, oral delivery of biopharmaceutical agents, such as vaccines and monoclonal antibodies, still has some limitations. The harsh conditions in the gastrointestinal tract (e.g., to the low pH and enzymes in the stomach) have the potential to damage biopharmaceutical agents (Silin, Lyubomska et al. 2007). Additionally, inefficient delivery to the active site causes the administration of large doses of biopharmaceutical agents. Therefore, the development of safe and cheap carriers capable of efficient and selective delivering biopharmaceutical agents to the intestine is important.

Recently, we have successfully developed the formulation of pH-responsive microspheres as a potential delivery of biopharmaceutical agents (Ma, Pacan et al. 2008). As shown in Figure 1, our preliminary study suggested that the pH-responsive microparticles maintain their structure in an acidic environment and stimulated the release of encapsulated cargos in alkaline conditions, indicating that this microparticle system has potential to use for oral delivery of biopharmaceutical agents to the intestine.

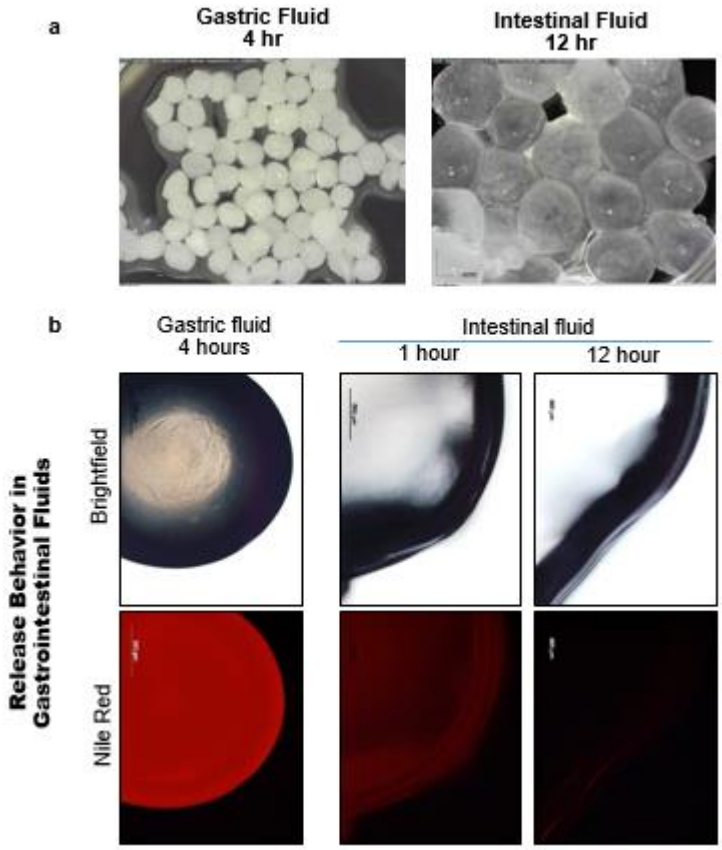


Figure 1; pH responsive behavior of alginate microspheres

a) Size changes of spherical microbeads in intestinal and gastric fluids. Representative stereo microscope images of microbeads after treatments. The samples of microbeads were collected at a given interval. **b)** Release behavior of alginate microspheres in simulated physiological fluids. In our preliminary study, Nile Red, a fluorescent lipophilic dye was used as a model drug to evaluate the drug release behaviors of the pH-responsive alginate microparticles, and subsequently a comparison on the release behaviors is made between the gastric and intestinal fluid. The samples of microbeads were collected at a given interval. The remaining Nile Red-labelled nanoparticles entrapped in alginate beads as a function of time was determined using a fluorescent microscope.

In this study, we proposed to use a combination of pH sensitive polymers and a timed-release approach to optimize the formulation of alginate microbeads for the release of active ingredients at specific areas in the intestine. This could be

achieved by varying polymer types (alginate and chitosan) and concentrations of polymer based on transit time through the small intestine as shown in Figure 2.

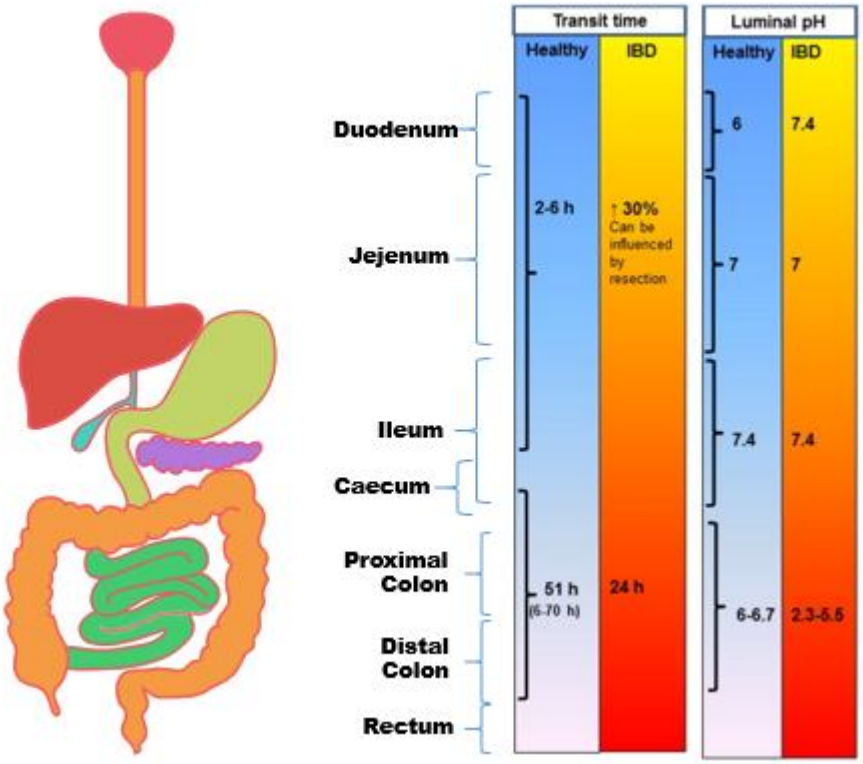


Figure 2; Transit time and luminal pH changes to the gastrointestinal tract. (Modified from (Hua, Marks et al. 2015). Reference: (Hua, Marks et al. 2015).

Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue (Hua, Marks et al. 2015).

Next, the encapsulation of biopharmaceutical agents in pH-responsive microspheres will be exploited to improve the stability and efficacy of two different biopharmaceutical agents. These include i) lived attenuated PEDV as a potent vaccine for the PED prevention and ii) 2C10 monoclonal antibody as PEDV prophylaxis for the control of PEDV infection as schematically shown in Figure 3.

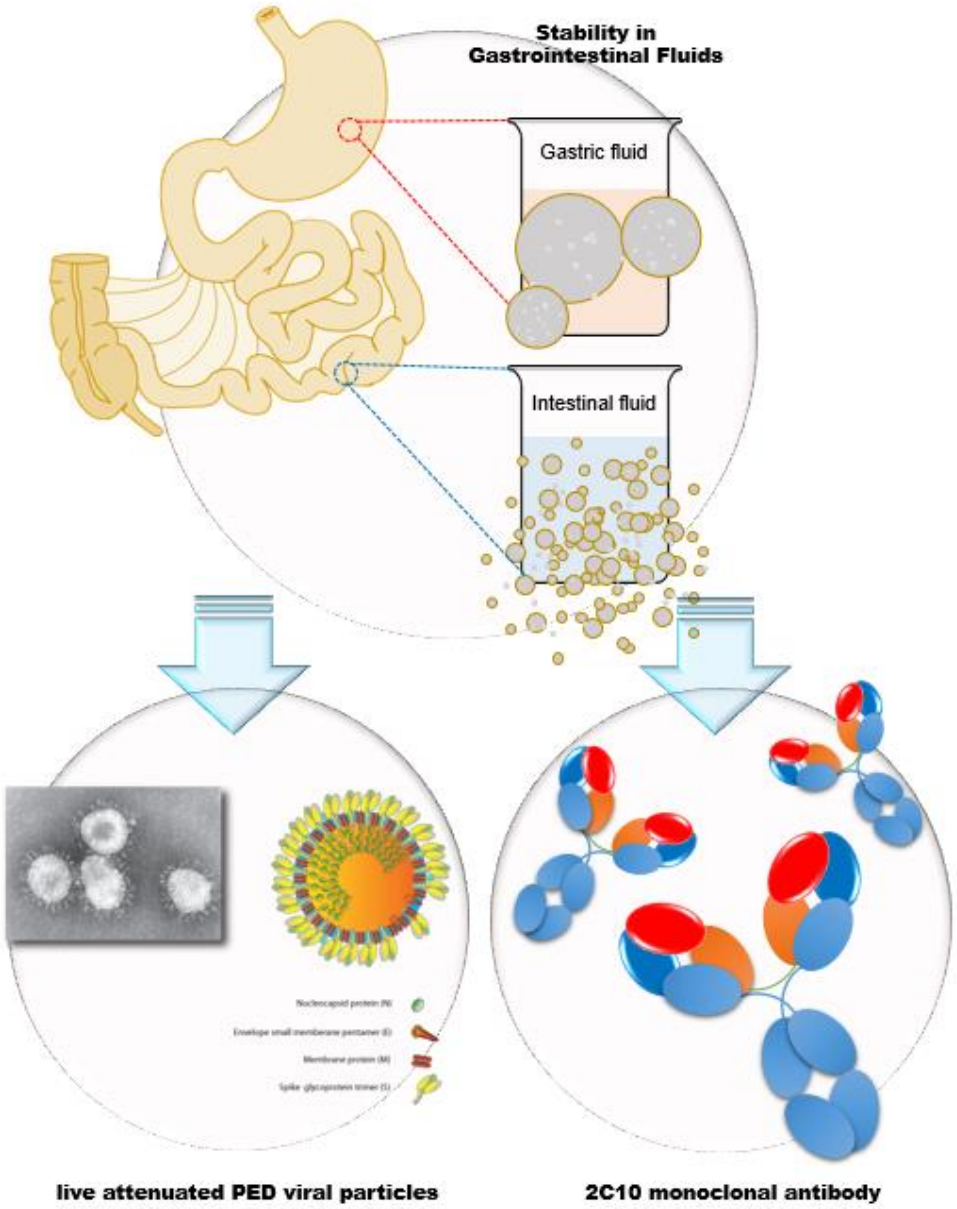


Figure 3; Two potential applications of the pH-responsive microsphere proposed in this study.

The pH-responsive microspheres will be exploited to improve the stability and efficacy of two different biopharmaceutical agents.

Research Questions

1. Does the pH-responsive microparticle maintain their structure in acidic environment and stimulate the release of encapsulated cargos in alkaline condition**?**
2. Can this microparticle system be used for oral delivery of biopharmaceutical agents to intestine?
3. Can the pH-responsive microsphere prevent encapsulated biopharmaceutical agents from low acidic environment and enzymatic degradation in the stomach?
4. Will encapsulated PED vaccines remain infectible after long incubation in simulated gastric fluids and release in simulated intestinal fluids?
5. Will encapsulated 2C10 monoclonal antibody remain effective against PED virus after long incubation in simulated gastrointestinal fluids?

Objectives

The main objective of this study is to investigate the application of pH-Responsive Microsphere as a smart carrier system for precision delivery of biopharmaceutical agents to the intestine for control of PED infections.

Objective I: To use a combination of pH sensitive polymers and a timed-release approach to optimize the formulation of a smart carrier for encapsulation of biopharmaceutical agents and release in the intestine

Objective II: To improve the stability and efficacy of live attenuated vaccines against PEDV by the pH-Responsive Microsphere when in contact with physiological fluids

Objective III: To improve the stability and efficacy of 2C10 monoclonal antibody by the pH-Responsive Microsphere when in contact with physiological fluids

Hypothesis

We propose in this study that the stability of biopharmaceutical agents could be protected by the pH-Responsive microsphere when in contact with physiological fluids. Therefore, we hypothesize that the encapsulated live attenuated PEDV is still infectious after long incubation in simulated gastrointestinal fluids, and encapsulated 2C10 monoclonal antibody is able to neutralize remain effective against PED virus after long incubation in simulated gastrointestinal fluids.

Expected benefits

- The success of this study potentially provides a significant extension to the development of innovative products by using this platform for efficient and targeted delivery of a broad spectrum of orally administered substances (drugs, biopharmaceutical agents and natural bioactive compounds) in order to improve the effectiveness for prevention and treatment of human and animal diseases.
- It is also expected that this platform would be used for the development of oral vaccines for veterinary use. In collaboration with Vet Products Research & Innovation Center Co.,Ltd. which focuses the development of an oral vaccine against the Porcine Epidemic Diarrhea Virus (PEDV), which causes a serious and high contagious swine disease, especially in neonatal piglets, leading to a significant loss in the swine industry.

CHAPTER II

LITERATURE REVIEWS

Presently, biologics is a product obtained from living systems include microorganisms, plants, and animal cells. The well-known biologics example recombinant protein, antibodies, peptide, and vaccines. Nearly a century ago, the biologics often used to treatment such as insulin which mostly it will use in oral drug delivery. It has many advantages such as low toxic, high specific but the important one of a weak point is often destroyed by the acidic environment and proteolytic enzyme (Vllasaliu, Thanou et al. 2018). Hence, the development of oral drug delivery is very important for high effectiveness. Nanotechnology is used in several industries especially in pharmaceutical science. There are several nanoparticles that can be applied to use in the drug delivery process such as liposome, albumin-bound, nanoemulsion, gold particle. There are two nanoparticles that will be used in this study, nanoparticle-in-microsphere and, microcapsules of alginate cross-linked with divalent ions.

1. Nanoparticle-in-Microsphere (NiMos)

Nanoparticles-in-microsphere (NiMOS) is an encapsulated gene, drug, or biologics of interest with a nanoparticle polymer as shown in Figure 4. Which NiMOS can specifically design for various routes of delivery depending on the users (Kriegel and Amiji 2011).

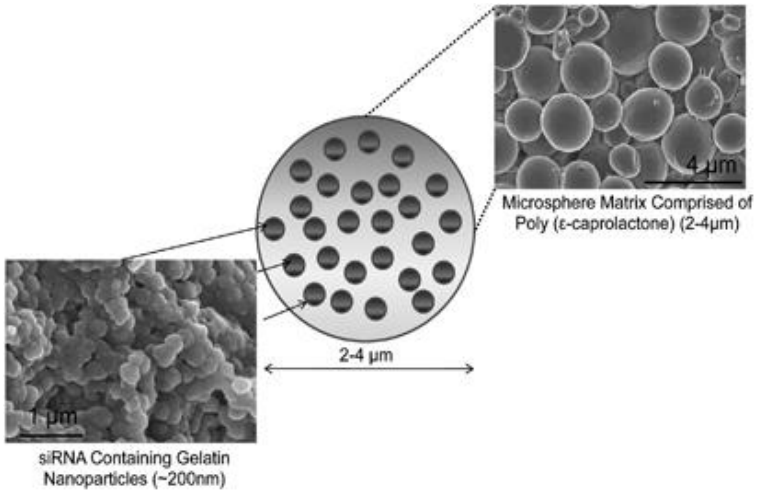


Figure 4; Nanoparticles-in-microsphere oral system (NiMOS) for short interfering RNA (siRNA) delivery by Gelatin nanoparticle and poly(e-caprolactone) microsphere.

Previous studies reported the plasmid DNA can be encapsulated in gelatin nanoparticles. These systems can protect the plasmid DNA during delivery to the target and increase the transforming efficiency (Kaul and Amiji 2005). Previous reports showed that Nanoparticles-in-microsphere oral system (NiMOS) can apply several drugs and polymers (Kaul and Amiji 2005).

2. Microcapsules of alginate cross-linked with divalent ions

Microencapsulation is a technology that can control the release of nutrients in specific places. It is a new technology that was firstly used in carbonless copy paper (Wilson and Shah 2007). Over the past few decades, the use of natural polymers for the development of drug delivery systems has received considerable attention due to their easy availability, cost effectiveness, biodegradability, and biocompatibility (Hua, Ma et al. 2010, Chen, Fang et al. 2011, Balaure, Andronescu et al. 2013, Wischke, Schneider et al. 2013). Microencapsulation is a barrier that protects and helps to avoid material in a capsule to direct contact with the environment (Poshadri and Aparna 2010). Among several natural polymers, alginate is one of the most abundant polymers found in brown marine algae (Nayak and Pal 2011). Alginate is a linear co-polymer consisting of guluronic (G) and mannuronic (M) acid-forming regions of M- and G-blocks and alternating structure (MG-blocks) as shown in Figure 5 (De'Nobili, Curto et al. 2013). Doubly charged calcium ions (Ca^{2+}) or barium ions (Ba^{2+}) can bind two different alginate strands simultaneously, and therefore crosslinking and solidifying the solution (Mørch, Donati et al. 2006, Lee and Mooney 2012). Frequently used in various applications, alginate is so far mainly processed into capsules, beads, and fibers (Degen, Leick et al. 2012) and has been commonly used for encapsulation of bioactive agents (Capretto, Mazzitelli et al. 2010).

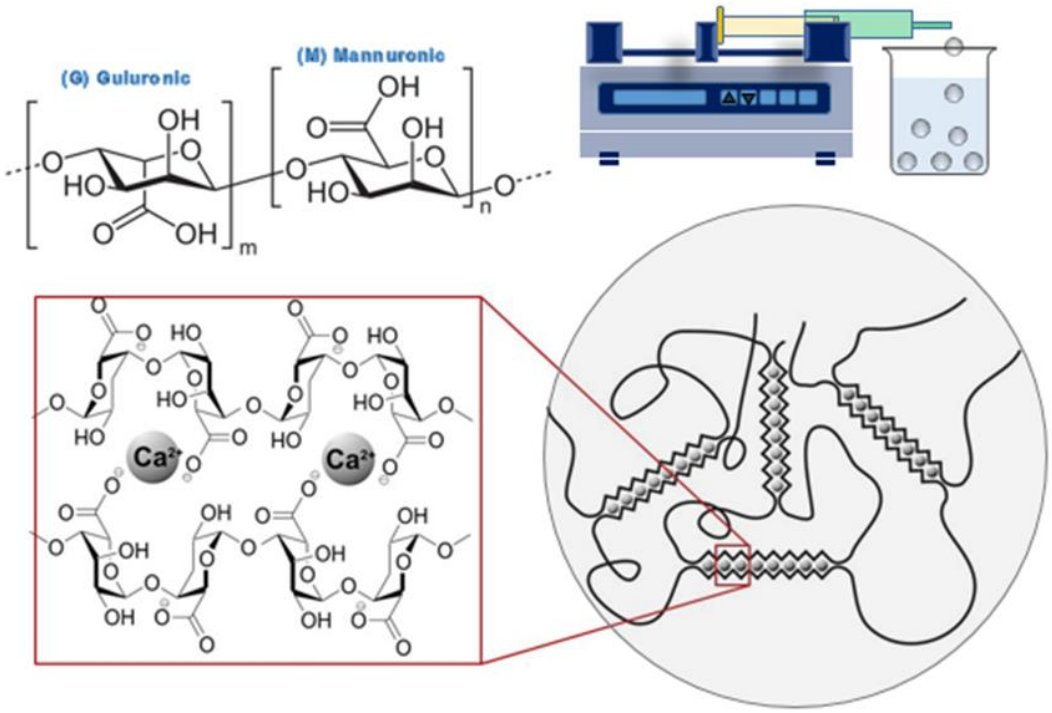


Figure 5; Microcapsules of alginate cross-linked with divalent ions.

Divalent cations such as Ca^{2+} and Ba^{2+} can bind to the G-blocks of alginate in a highly cooperative manner, such that a gel is formed. In most cases, spherical alginate particles are available by use of a typical droplet formation approach.

Chitosan is a polysaccharide that is a derivative of chitin. Chitosan is extracted from the shells of crab and shrimp. Mostly It is used in medicine because it can be prepared in the form of gel or film (Rinaudo 2006). Previously study, it was reported that the pore size of alginate gel was approximately 5 nm (Boontheekul, Kong et al. 2005, Lee and Mooney 2012).

3. Porcine Epidemic Diarrhea (PED) Virus

Porcine epidemic diarrhea (PED) is an enteric disease by Porcine Endemic Diarrhea (PED) Virus. PED virus is an enveloped virus with positive-sense single-stranded RNA genome of about 28 kb. It is gram-positive bacteria and belongs to the *Alphacoronavirus* genus in the *Coronaviridae* family. The PEDV consisted of the spike [S], envelope, membrane and nucleocapsid [N] proteins, which the S protein is a type I glycoprotein present on the surface of virus, it was used for entry to host cell and it is target site for neutralizing antibodies (Li, van Kuppeveld et al. 2016).

The PED virus was called Coronavirus-like particles (Pensaert and De Bouck 1978) because when PED virus infected swine, the swine have symptoms including intense diarrhea, vomiting, weight loss, and dehydration (Chasey and Cartwright 1978). These symptoms are like the symptom of Transmissible Gastroenteritis (TGE) and rotavirus infection. Therefore, the scientists misunderstand that the infection is occurred from rotavirus. PED virus is different from TGE because it has severely affected newborn piglets. The diarrhea cause PED virus spreads among the swine at a slower rate and diarrhea happens in most of the piglet. *Yang et al,* shown the structure of the PED virus by EM images, found that a diameter size about 80 to 120 nm as figure 6 (Yang, Kim et al. 2018).

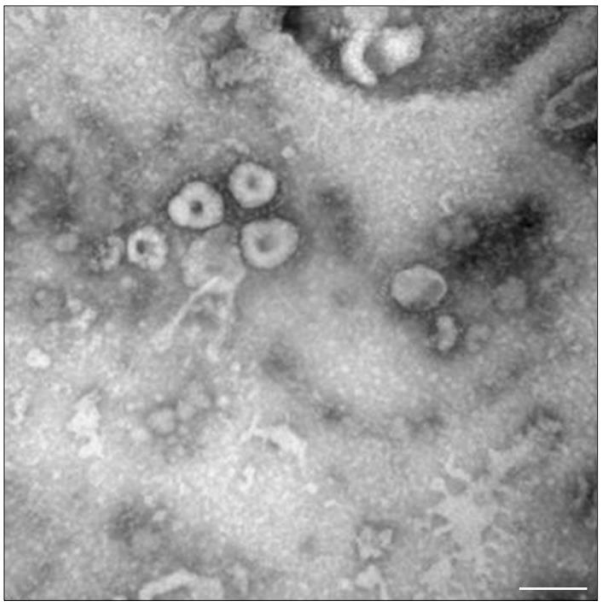


Figure 6; The morphology of Porcine Endemic Diarrhea (PED) Virus (Yang, Kim et al. 2018). Scale bar = 100 nm.

PED virus was found in the early 1970s in Europe and the outbreak was spread to Asia. The virus can infect swine by oral route and replicates in the small intestine cells (Vlasova, Marthaler et al. 2014). Although the PED virus is non-severe to sow, whereas it is severe in piglet or newborn piglet. When the piglets were infected by the PED virus, they have symptoms as mentioned above, and finally, die from severe water shortage. Therefore, this problem adversely affects the growth performance of piglets (Chasey and Cartwright 1978). Now, there is no specific treatment for PED, however the swine with diarrhea should have free access to water and separate out of the group for 4-5 days to outbreak decrease (Harris 2012).

Over the years, several types of orally administrating vaccines have been established and some of these vaccines have sufficient potential to inactivate PEDV, for example, live attenuated vaccines (Song et al., 2007) and recombinant protein vaccines from bacteria (Hou et al., 2018). Has been reported that the traditional vaccination induces low immunogenic efficacy in sow. Moreover, the transfer of immunity from sow to newborn piglet by transfer the antibodies in milk cannot forward to neonatal piglet because destroyed by strong pH and enzyme in GI tract (Wang, Huang et al. 2019). Therefore, the oral vaccination for inducing specific antibodies through intestinal mucosal immune system is probably more efficient strategy than parenteral route to protect PEDV mucosal infection (Mutwiri, Watts et al. 1999, Chattha, Roth et al. 2015).

Currently, there are many platforms available for recombinant protein production, including bacteria, insect cells, yeast, mammalian cells, and plants (Cereghino, Cereghino et al. 2002) (Demain and Vaishnav 2009). However, the protein production in bacterial have weak point such as endotoxin contamination. The benefit of the recombinant protein production from the plant is low toxicity and high specificity (Oo and Kalbag 2016) especially there are post-translational modifications

which it helping more complete protein. Among all of these systems, plants were developed lately to use as bioreactors to recombinant proteins (HASHEMI, Jourabchi et al. 2005). Recombinant protein production in plants can be done by two methods including transgenic and transient expression. The transgenic plant is a plant that contained the recombinant gene in the plant chromosome. This method takes a long time to develop. Transient expression is one alternative way to produce protein in plants without inserting the recombinant gene into the plant chromosome. It takes a short time for protein production. This can be an alternative method for recombinant protein production in a plant. Previous studies showed that there are several proteins successfully produced in plant by transient expression technique, such as influenza vaccine (Shoji, Chichester et al. 2008), rabies monoclonal antibody (Yusibov, Hooper et al. 2002), Ebola monoclonal antibody (Olinger, Pettitt et al. 2012), and PEDV monoclonal antibody (Rattanapisit, Srijangwad et al. 2017).

In 2009, the 2C10 monoclonal antibody was expressed in *Escherichia coli* and also found its potential to neutralize PEDV (Pyo, Kim et al. 2009). In recent years later, one of our researchers was found that the 2C10 mAb can be produced from plants such as *Nicotiana benthamiana* Domin (Solanaceae) by transient expression. The 2C10 mAb against PED virus is IgG antibody and have a diameter size approximately 6.9 nm (Steinbock, Krishnan et al. 2014). In addition, it is one more candidate for prophylaxis of PED because it has been proven to neutralize the virus (Rattanapisit, Srijangwad et al. 2017).

CHAPTER III

METERIALS AND METHODS

MATERIALS

Sodium alginate, chitosan, pepsin, pancreatin, calcium chloride, and Nile Red dye were purchased from Sigma (Missouri, USA). Deionized water was produced from Thermo Scientific™ Barnstead™ LabTower™ EDI Water Purification System (Massachusetts, USA). The antibody labelled with Alexa FluorTM 488 Phalloidin was purchased from Thermo Scientific™ Barnstead™ LabTower™. Vero cell line derived (African green monkey kidney) was obtained from American Type Culture Collection (ATCC). RNeasy mini kit was purchased from QIAGEN Inc. (Germantown, MD, USA). The live attenuate PED viruses were growing by serial passage and obtained from Vet Products Research & Innovation Center Co.,Ltd.

METHODS

1. Preparation of pH-responsive microbeads

For the formation of pH-responsive microbeads, 900 µL of various concentrations of pH sensitive polymers (2% alginate and 1% chitosan) is mixed with 100 µL of biopharmaceutical agents (9:1 v/v, ratio). The mixture is then introduced dropwise from a glass syringe with a size-30 needle into 50 mL of an aqueous $Cacl_2$ solution being stirred at 400 rpm. The concentration of $CaCl_2$ in the solution is fixed at 3% w/v. The stirring is continued for 10 mins and the beads are harvested from the $CaCl_2$ solution by filtration, washed with distilled water. Tissue paper is used to absorb the surface excessive water and oil onto the wet microbeads.

2. Physical characterization of pH-responsive microbeads

The size and morphology of the obtained microbeads are assessed by direct observation, and images are recorded digitally. The measurements are carried out using digital Vernier calipers (ABSOLUTE DIGIMATIC, Mitutoyo Corp., Japan).

Individually, 30 beads are subjected to size measurement, and the average was calculated. The morphology of surface of microbead was investigated under a scanning electron microscope (SEM, JEOL- 2100 Japan), coated with a gold layer under vacuum for 20 min operated at 20 kV. For Transmission Electron Microscope analysis, the samples are fixed in 4% v/v glutaraldehyde in 0.1 M PIPES buffer at 4°C for 1 hour and post fixed in 1% w/v osmium tetroxide in 0.1 M PIPES buffer at room temperature for 1 hour. The samples are dehydrated using graded ethanol series from 50%, 70%, 90% and 100% v/v. The samples are then progressively infiltrated with Epon 812 resin (Electron Microscopy Science Ltd.) and polymerized at 60°C for 24 hours. The ultrathin sections are cut via ultramicrotomy using a 45° diamond knife. Bright-field TEM is performed on a JEOL 2100, operated at 120 kV.

3. Release behavior in Simulated Physiological Fluids (SPF).

In order to investigate the release behavior of biopharmaceutical agents from the matrix, the liposome represented by the PED virus is labelled with Nile Red, a fluorescent lipophilic dye was entrapped in the pH-responsive microbeads. After synthesis, the liposomes are separated from unencapsulated Nile Red by centrifugation at 650,000*g* for 30 min. The Simulated Physiological Fluids (SPF) separated two type including Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) are prepared as previously described (Nobrega, Costa et al. (2016). For this, SGF comprised of 3.2 mg/mL pepsin in 0.2% (w/v) NaCl at pH 3.5, and SIF comprised of 1 mg/mL pancreatin in 0.2% (w/v) NaCl at pH 8.0 are prepared. Samples are added to the pre-warmed solutions at 37 °C and incubated at 37 °C. At specified time intervals, samples of microbeads are collected. Entrapment of remaining Nile Red-labelled the liposomes in pH-responsive microbeads is visualized under the fluorescence microscope.

At the same time, the antibody labelled with Alexa Fluor™ 488 Phalloidin is represented as 2C10 was entrapped in the pH-responsive microbeads and tested the same above step.

4. Quantification of encapsulated the liposomes labelled with fluorescent dye used in this study

After microbead dissolution with 5 mL of MBS buffer contained 50 mM sodium citrate, 0.2 M sodium bicarbonate, and 50 mM Tris-HCl, pH 7.5, for 10 min with shaking at room temperature. The dissolved solution was quantified for Nile Red by fluorescence measurement with a fluorescence plate reader at 552 nm/636 nm. Vero cell infection of the PED virus was analyzed by Real-Time Polymerase Chain Reaction (Real-Time PCR) assay.

5. Production of 2C10 monoclonal antibody

The 2C10 monoclonal antibody against PED virus is produced from *Nicotiana benthamiana* as previously described (Rattanapisit, Srijangwad et al. 2017). The 2C10 monoclonal antibody-encoding genes are inserted into the geminiviral vector and transduced into Agrobacterium. Transformation of transgenes into plants is mediated by Agrobacterium co-infiltration. Proteins are extracted and purified from leaves and used for microencapsulation in further experiments.

6. Neutralization assay

Vero cells are seeded at a density of 5×10^4 cells/well in 48-well plates and allowed to grow until 70-80% confluent followed by infection with PED viruses in the presence dissolved 2C10 monoclonal antibody-loaded pH-responsive microbeads after incubation in simulated physiological fluids at specific time points as indicated by the experiment.

7. RNA extraction and reverse transcription PCR

The total RNAs were extracted according to the manufacturer's protocol RNeasy Mini Kit (Qiagen, Hilden, Germany). The extracted RNA was eluted in sterile water. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit

(Applied Biosystem, USA). The manufacturer's protocol was followed. cDNA was diluted before performing real-time PCR reactions.

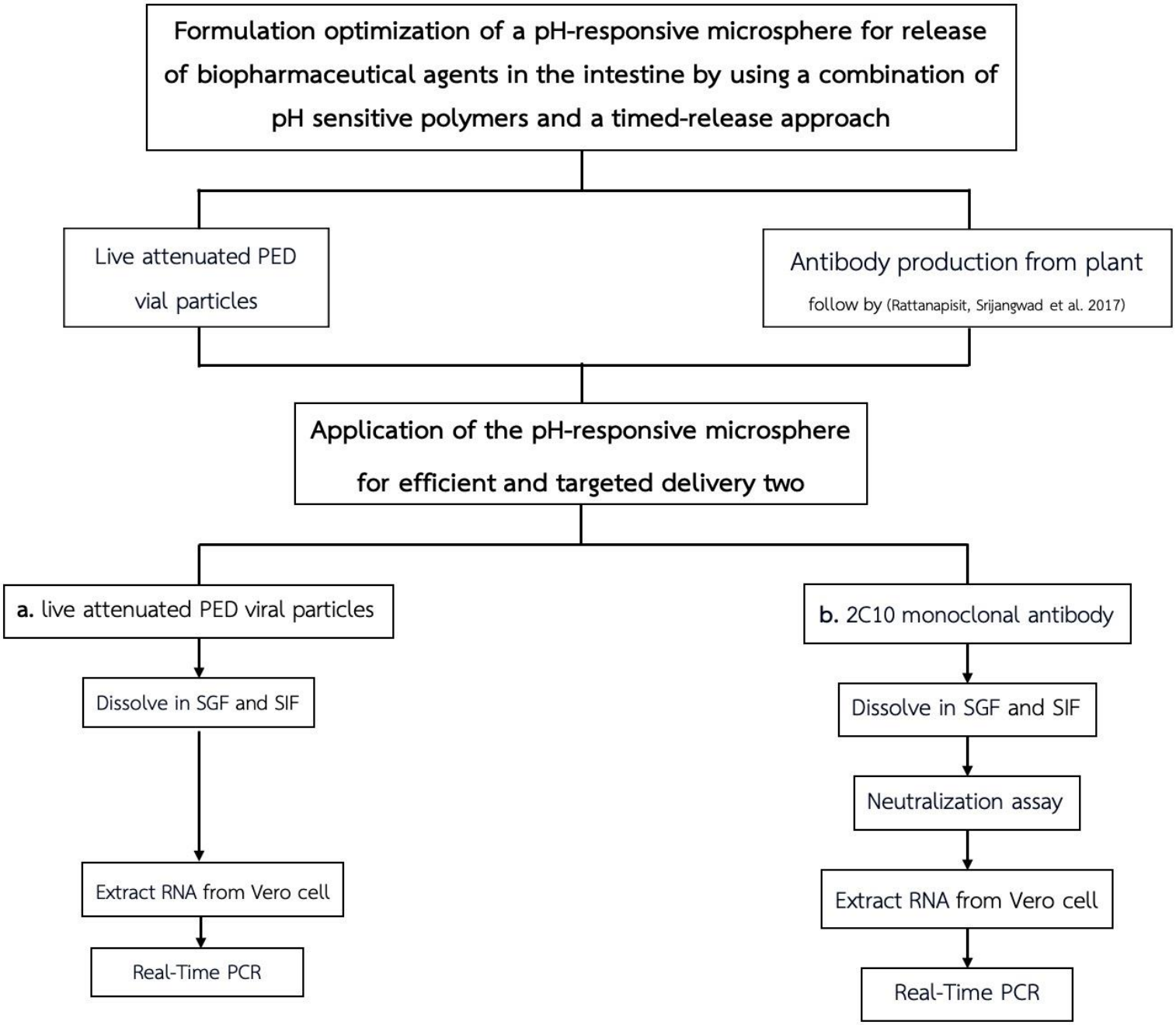
8. Real-time PCR

Real-time PCR reaction contained 1x PowerUp SYBR Green Master Mix (7500 Applied Biosystems, Carlsbad, CA), 900nM forward and reverse primers (Table 1), 2 µL of cDNA template and water to a final volume of 20 µL. The Standard PCR protocol is performed following the cycling profile: 50 °C for 2 min, 95 °C for 2 min to dual-lock DNA polymerase, then 40 cycles of denaturation at 95°C for 15 sec and a combined annealing/extension step at 60°C for 60 sec. Real-time PCR results are analyzed by the 7500 software version 2.0.5 (Applied Biosystems) to express as the quantification cycle (Cq) value, in which the lower Cq value is defined as the higher virus.

Table 1; Primer sequences and target region

Assay name	Primer name	Oligonucleotide sequence (5' to 3')	Target	Reference
PED	PED_N_F	CGCAAAGACTGAACCCACTAATTT	Nucleocapsid	Yu *et al.* (2015)
	PED_N_R	TTGCCTCTGTTGTTACTTGGAGAT		
TBP	GAPDH_F	GAAATCCCATCACCATCTTCCAGG	Glyceraldehyde 3-phosphate dehydrogenase	Ahn *et al.* (2008)
	GAPDH_R	GAGCCCCAGCCTTCTCCATG		

EXPERIMENTAL DESIGN



Experimental Design

1. **Formulation optimization of a pH-responsive microsphere for the release of biopharmaceutical agents in the intestine by using a combination of pH sensitive polymers and a timed-release approach.**

This could be achieved by varying types and concentrations of precursors (polymers and lipid components) based on transit time through the small intestine. The stability and release profile of biopharmaceutical agents (labelled with fluorescent dye) from the different formulation of microbeads will be determined in simulated physiological fluids. Samples of microbeads will be collected and analyzed at specified time intervals associated with gastrointestinal transit time.

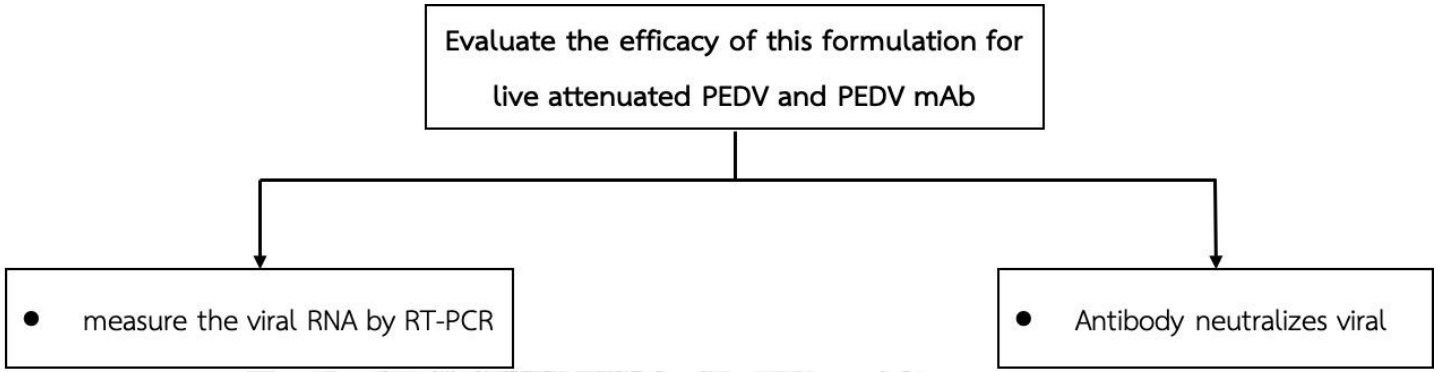
2. **Application of the pH-responsive microsphere for efficient and targeted delivery of two**

biopharmaceutical agents. We propose to use a pH-responsive microsphere system for two biopharmaceutical agents in order to control PEDV infections.

These include

- **a.** live attenuated PED viral particles as a potent vaccine for the prevention of PED
- **b.** 2C10 monoclonal antibody as PEDV prophylaxis for the control of PEDV infection

CONCEPTUAL FRAMEWORK



CHAPTER IV
RESULTS

1. Formulation of Sodium Alginate-Calcium Chloride-chitosan to be pH-responsive microbeads

In this study, there were two types of nanoparticles including live attenuated PED virus, represented as viral particles, and the 2C10 monoclonal antibodies. For the formulation of the microbeads, one bead will encapsulate a single type of nanoparticle. Thus, the microbeads were prepared two times, starting with the microbeads encapsulating PEDV.

First of all, we constructed the microbeads using a mix of Sodium with the nanoparticle on the stirrer shown in Figure 7A. Then, the sample was added into a syringe, as in Figure 7B, after which a syringe pump was used to control the size and volume of microbeads and for dropping in Calcium Chloride-Chitosan, as in Figure 7C. After getting the microbeads, it was washed by DI water three times. Finally, the microbeads were dried on filter paper and stored at 4 °C. The colours of the microbeads depended on the colour of the nanoparticle, as seen in Figure 7D. Simultaneously, the microbeads encapsulating 2C10 monoclonal antibody were produced by following the process production for microbeads, as described previously.

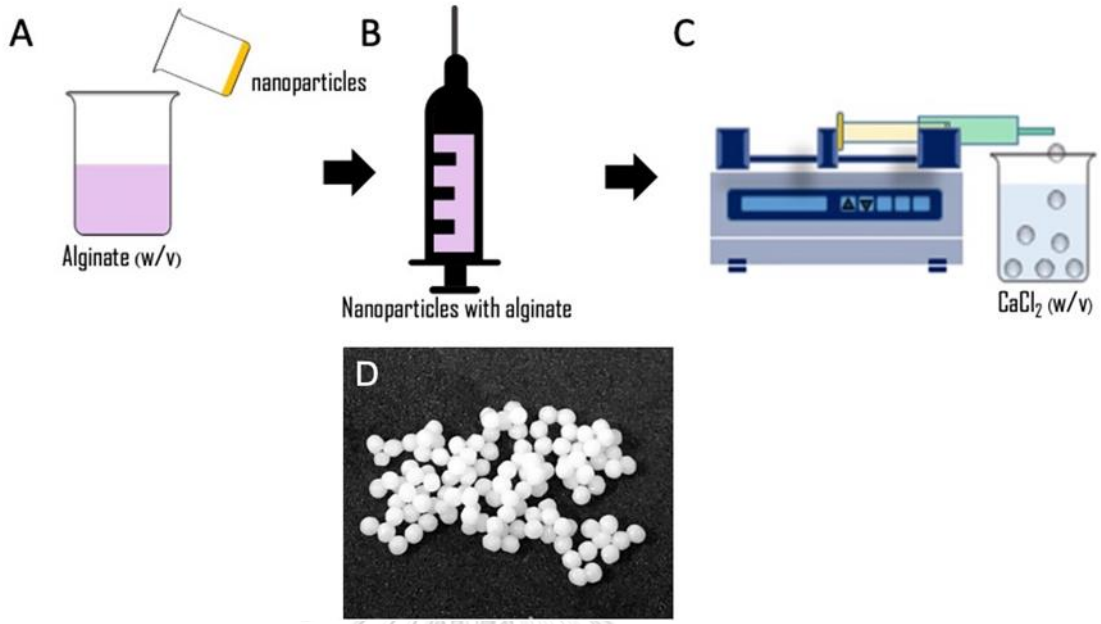


Figure 7; Formulation of Sodium Alginate-Calcium Chloride-chitosan microbeads.

Sodium alginate a mix of the nanoparticles on the stirrer **(A)**, the solution was added into a syringe and put on a size-30 needle **(B)**. The syringe was brought on the syringe pump to drop into Calcium Chloride-Chitosan **(C).** The colour of the microbeads was turbid **(D)**.

2. Physical characterisation of Sodium Alginate-Calcium Chloride-Chitosan microbeads

The environment around the microbeads, such as pH value, has effects on metamorphism. Therefore, the microbeads were measured by Digital Vernier to observe the diameter size of the alginate-chitosan microbeads; the sample of microbeads was prepared using 30 beads. It was found that the diameter size distribution of the microbeads was mostly between 1.5 - 1.6 mm, which is more than 70% and there is an average size of about 1.57 ± 0.078 mm, as shown in Figure 8.

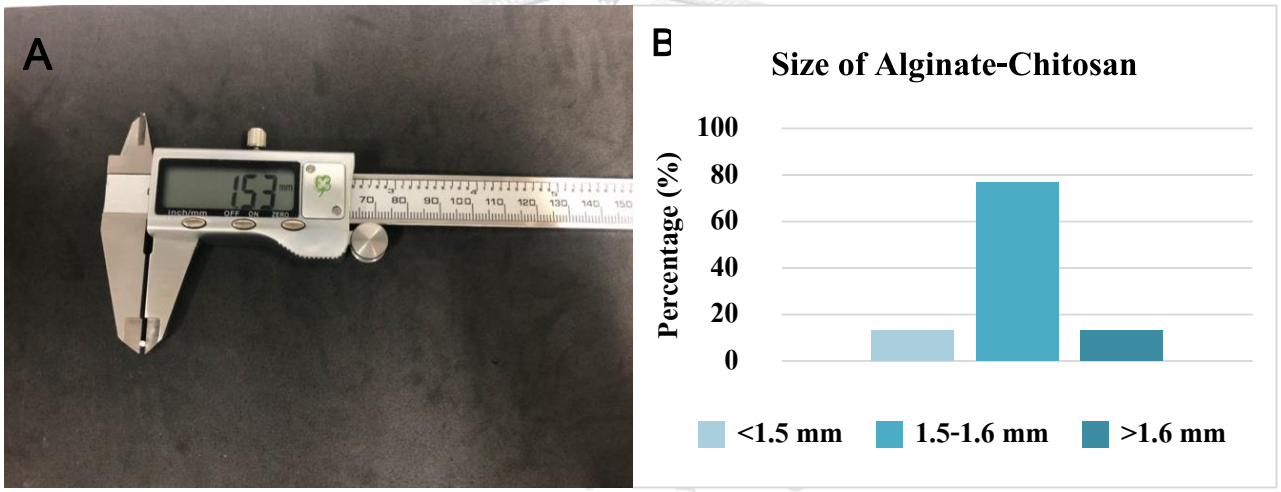


Figure 8; The diameter size distribution of microbeads mostly between 1.5-1.6 mm as measured in size by Vernier.

After the alginate-chitosan microbeads were checked for diameter size, the morphology of the surface for alginate-chitosan microbeads was investigated under SEM at different magnifications. The SEM exhibited images of the morphology of the microbeads under a magnification of x200 and x800, which found that the surface of the microbeads involved a network structure and spherical shape (Fig. 9A); the microbead has a distribution comprising many small and compact pores (Fig. 9B).

Furthermore, we observed inside the pores at 50,000x magnification (Fig. 9C) and found the nanoparticles were kept in microbeads.

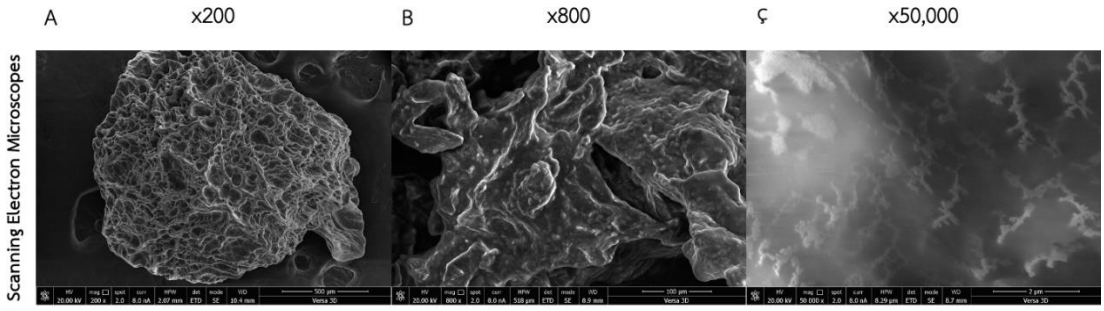


Figure 9; Scanning electron microscopy showing the characteristics for the surface of alginate-chitosan microbeads at different magnifications.

(A) x200, (B) x800, and (C) x50,000.

Subsequently, the aspect of nanoparticles within microbeads was checked and images recorded inside of microbeads by TEM. We found that nanoparticles were contained in the microbeads, as shown in Figure 10. From observation, the nanoparticles showed both a single-particle form (Fig. 10A) and a multiparticle form (Fig. 10B) in one microbead.

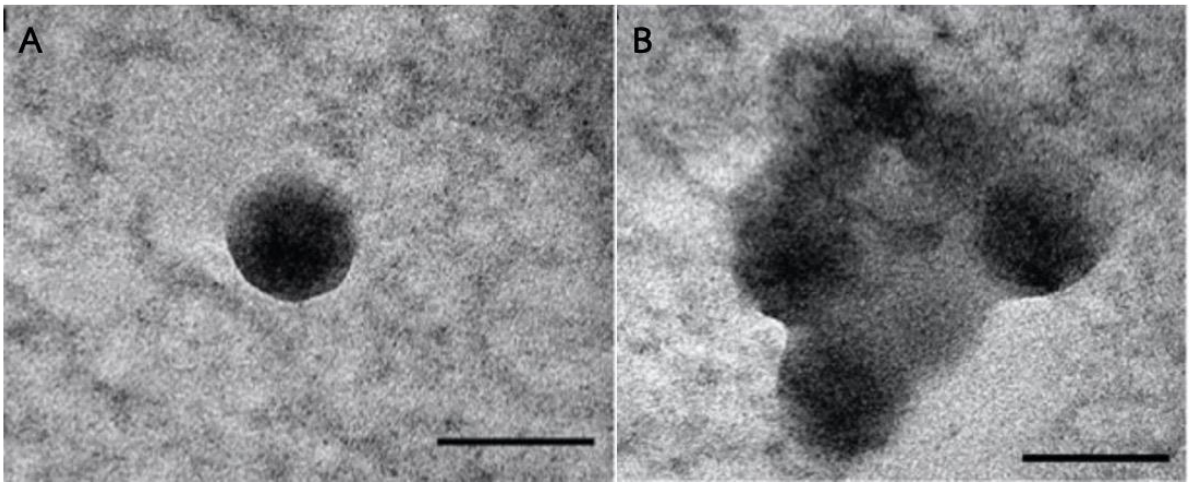


Figure 10; The nanoparticles were investigated under TEM.

The nanoparticles in **(A)** single-particles form and **(B)** multiparticle form

Finally, to investigate the morphology of the alginate-chitosan microbeads using testing by SPF, the samples were soaked in SGF for 1, 2, 3, and 24 hours and the images were recorded with a miniscope and were measured by Digital Vernier. The result of the diameter size change as shown in Figure 11 and from Figure 12, the size of the microbeads soaked for 24 hours were smaller compared to those soaked for 1 hour. Subsequently, the samples were tested with SIF for 1 and 2 hours. It was found that the microbeads had clear swelling, and the outside surfaces of the microbeads were more fragile.

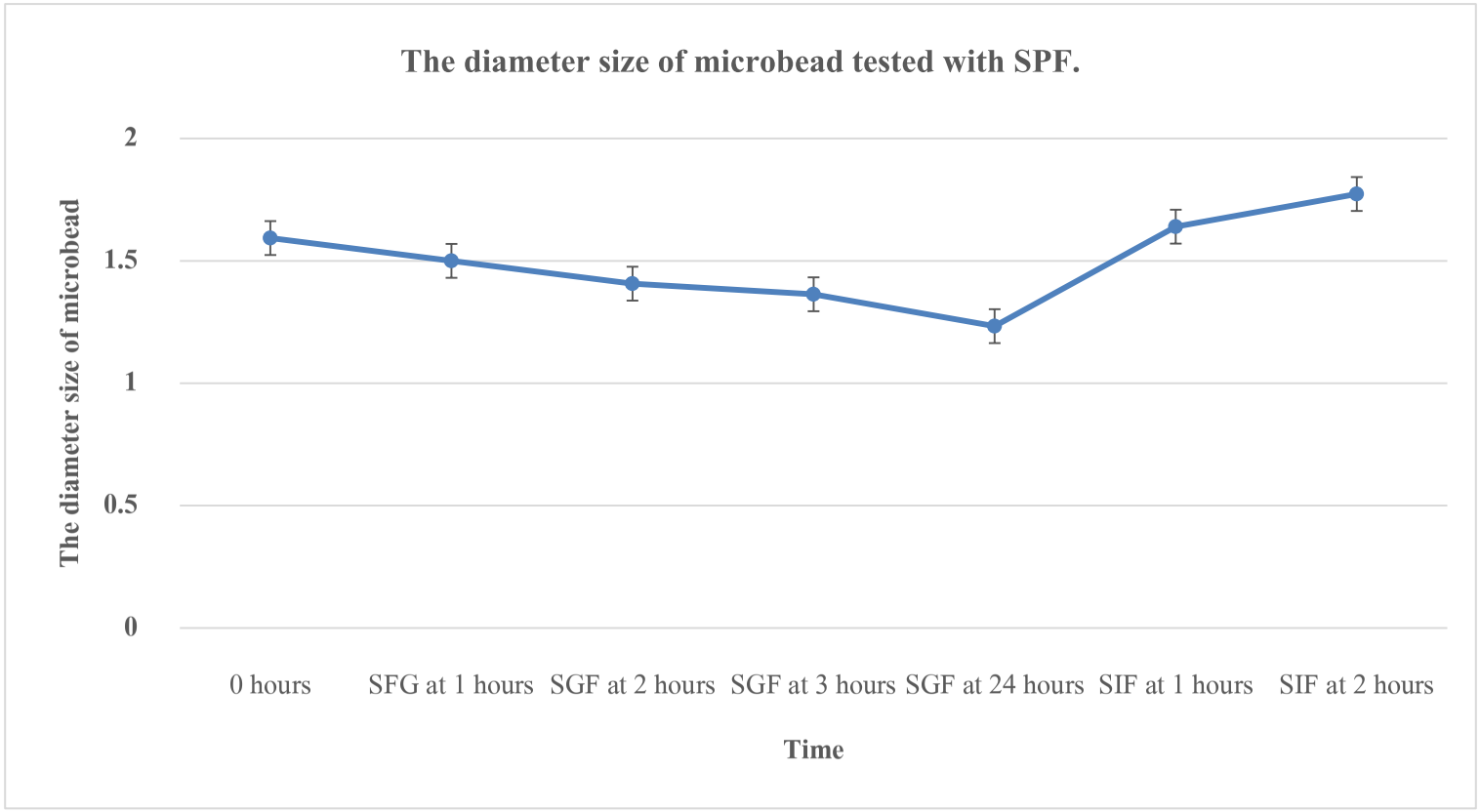


Figure 11; The diameter size change when testing with SPF.

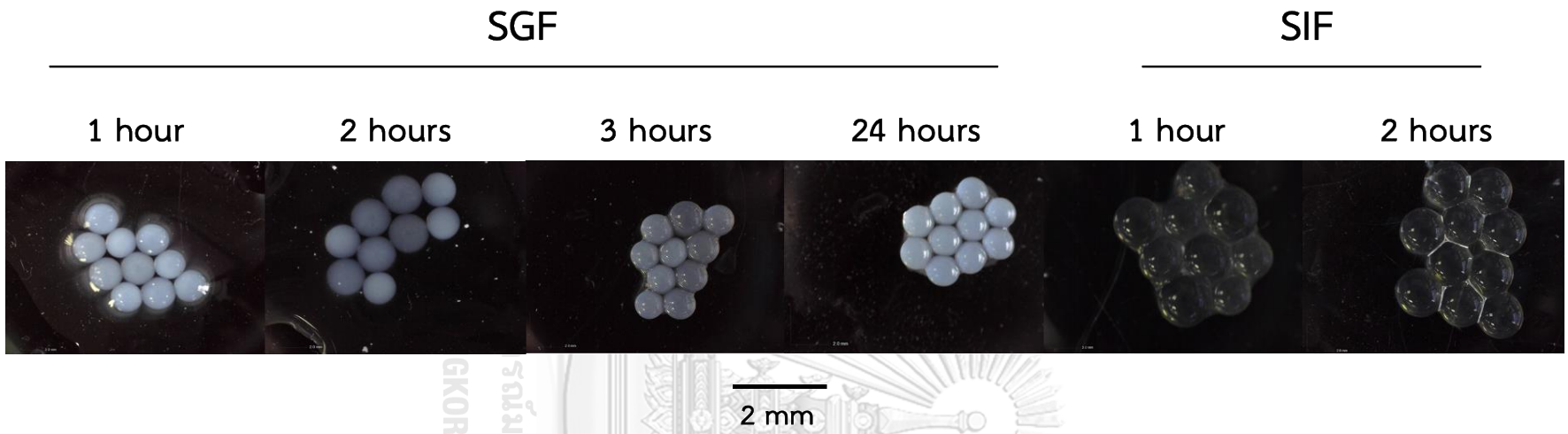


Figure 12; The metamorphism of the alginate-chitosan microbeads tested with SGF and SIF at different times. Scale bar = 2 mm.

3. Study of nanoparticles release behaviour for Alginate-Chitosan microbeads in Simulated Physiological Fluids (SPF)

Preliminarily, we investigated the nanoparticles labelled with Nile red, which were encapsulated by alginate and soaked in SGF. It was found that the alginate microbead could retain nanoparticles, but not enough for a long duration (figure 19 in appendixtrtion). Therefore, the alginate-chitosan in this study was tested for the encapsulation of the microbeads. To observe the nanoparticles release behaviour of the microbead when the nanoparticles encapsulation labelled with Nile red, which represented viral particles, the microbead was brought for testing with SGF and SIF, respectively.

The nanoparticles labelled with Nile red were encapsulated by microbeads and observed as well as recorded images under a fluorescent microscope at 0 hours. The microbeads were then soaked in SGF at 1 hour, 2 hours, and 3 hours to observe and record images each hour. We found that the size of the microbead each hour was smaller when compared with at 0 hours, but still glowing. Then, it was soaked in SIF for 1 hour, 2 hours, and found that almost the entire microbead was dissolved into a solution; some nanoparticles were entrapped in alginate-chitosan debris, as shown in Figure 13. Moreover, we found the nanoparticles that were entrapped could still glow.

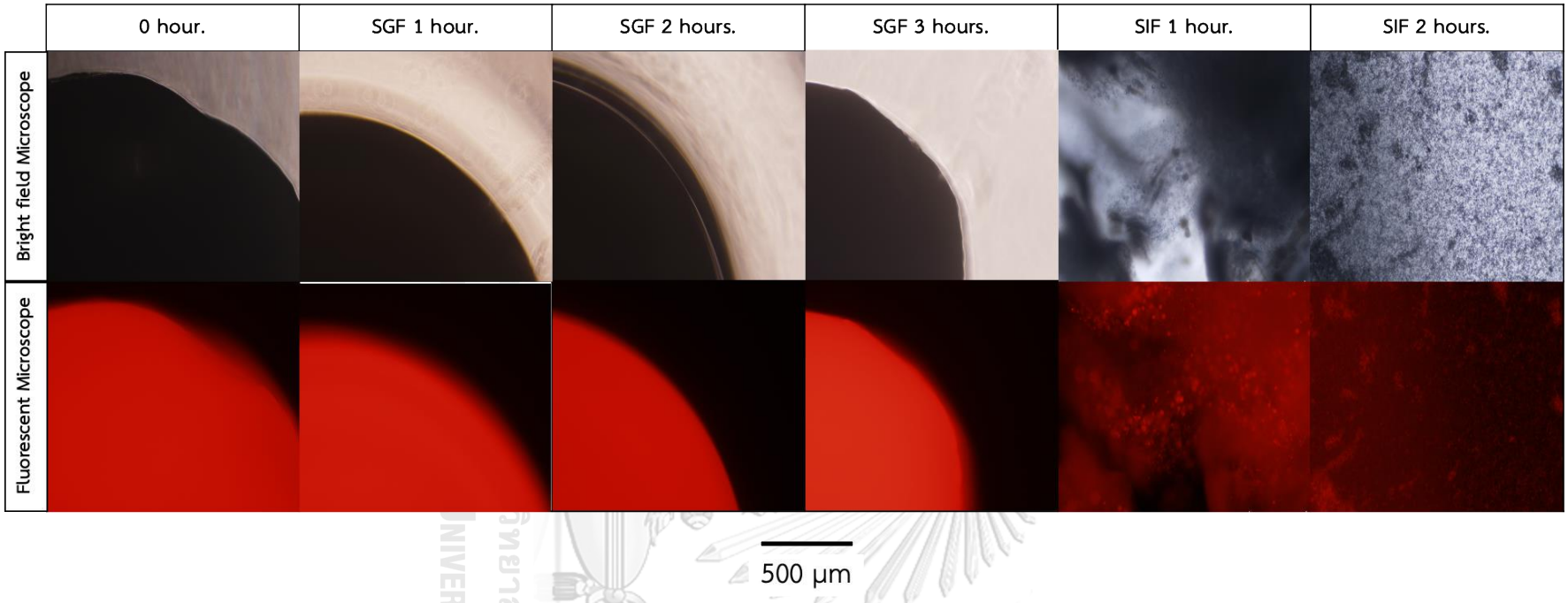


Figure 13; The microbead was observed to release behaviour under a fluorescent microscope when tested with simulated physiological fluids.

The microbead was recorded as an image at 0 hours and when it was soaked in SGF at 1 hour, 2 hours, and 3 hours, after which it was dissolved in SIF at 1 hour, 2 hours. Scale bar = 500 µm.

Meanwhile, we encapsulated antibodies labelled with fluorescent (Alexa Fluor™ 488 Phalloidin) to represent 2C10 monoclonal antibodies against PEDV, after which the microbead was tested in SGF and SIF, respectively. Before the microbead was tested with a solution, it was recorded as an image both under Bright-field and a fluorescent microscope at 0 hours. After that, it was taken to soak in SGF for 1 hour, 2 hours, and 3 hours to observe and record images each hour. We found that the size of the microbead decreased when compared with 0 hours. Next, the microbead was dissolved in SIF for 1 hour and 2 hours then observed under a fluorescent microscope, which found that the microbead was dissolved; most of it had become a solution. Some parts could not be dissolved and had nanoparticles trapped inside but were able to glow, as in Figure 14.

The method and result of the experiments mentioned above cannot, however, confirm the efficacy of the nanoparticles. Hence, the live attenuated PEDV and 2C10 monoclonal antibodies were tested by real-time RT-PCR in the next step.

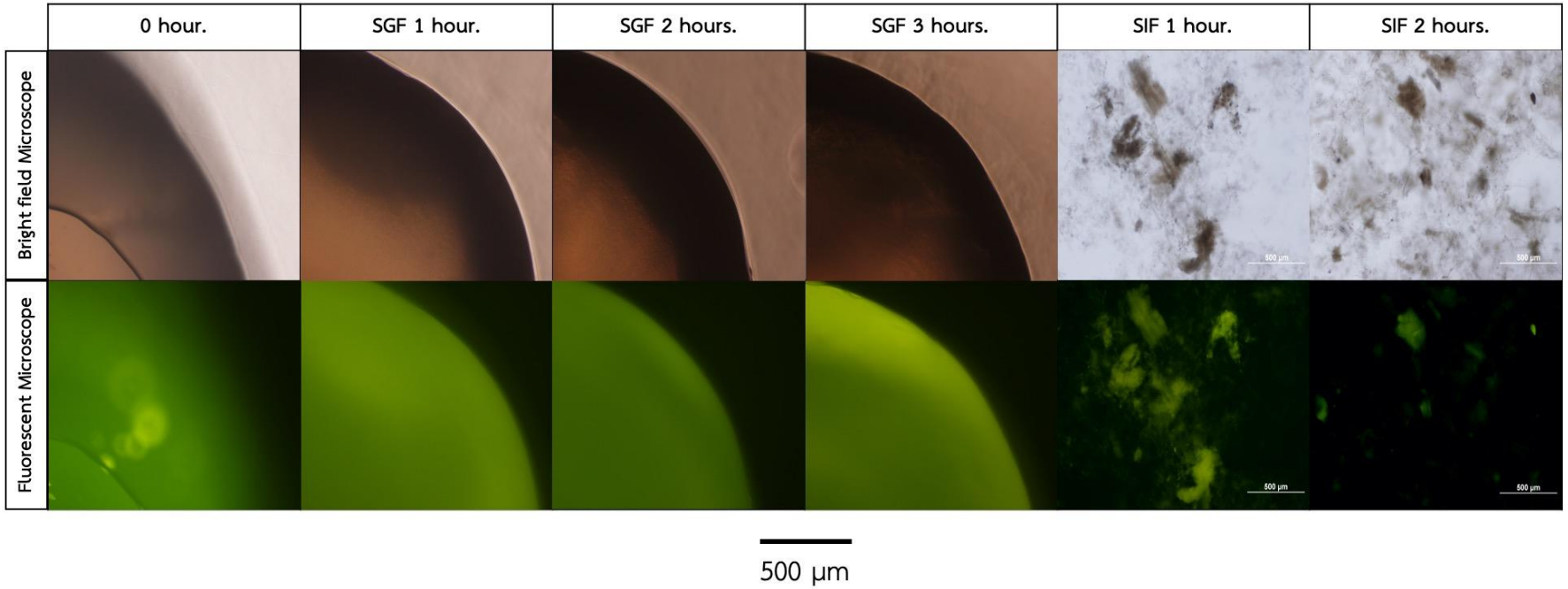


Figure 14; The microbead was observed release behaviour under a fluorescent microscope when tested with simulated physiological fluids.

The microbead was recorded as an image at 0 hours. Then, the microbead was observed and recorded as an image under a microscope when soaked in SGF at 1 hour, 2 hours, and 3 hours. After that, the microbead was dissolved in SIF for 1 hour and 2 hours. Scale bar = 500 µm.

4. Quantification of encapsulated the liposomes labelled with fluorescent dye used in this study

To predict the number of the liposomes in microbeads, the liposomes labelled with fluorescent dye were encapsulated by sodium alginate. Then, the microbeads (5 beads) were brought to dissolve with a Microbead Broken Solution (MBS) and then analysed with a microplate reader under fluorescent intensity mode. It was found that the experimental group, after testing 3 times, had particles approximately 0.0005306 μg or 0.5306 ng when compared with the stand curve of Nile Red (Figure 18 in appendixtrrion).

5. The efficiency of nanoparticles encapsulated by pH-responsive microsphere with Real-Time PCR after testing with simulated physiological fluids

To determine the efficiency of nanoparticles encapsulated by pH-responsive microsphere, the microbeads were tested with SPF then RNA extraction and reverse transcription PCR. Both cDNA of live attenuated PED virus and 2C10 mAb were analysed by real-time RT-PCR, for which the PED and TBP primer were used for finding the amount of RNA viral after testing and calculating the %ratio of PEDV: TBP.

The results of the live attenuated PED virus found that, when the unencapsulated group was tested with SGF, the cDNA of viral cannot be detected by the primer of PED virus. Therefore, it cannot calculate the quantity of cDNA after testing with SGF. Meanwhile, the encapsulated group that can be detected, and the quantity of cDNA can be calculated, as shown in Figure 15.

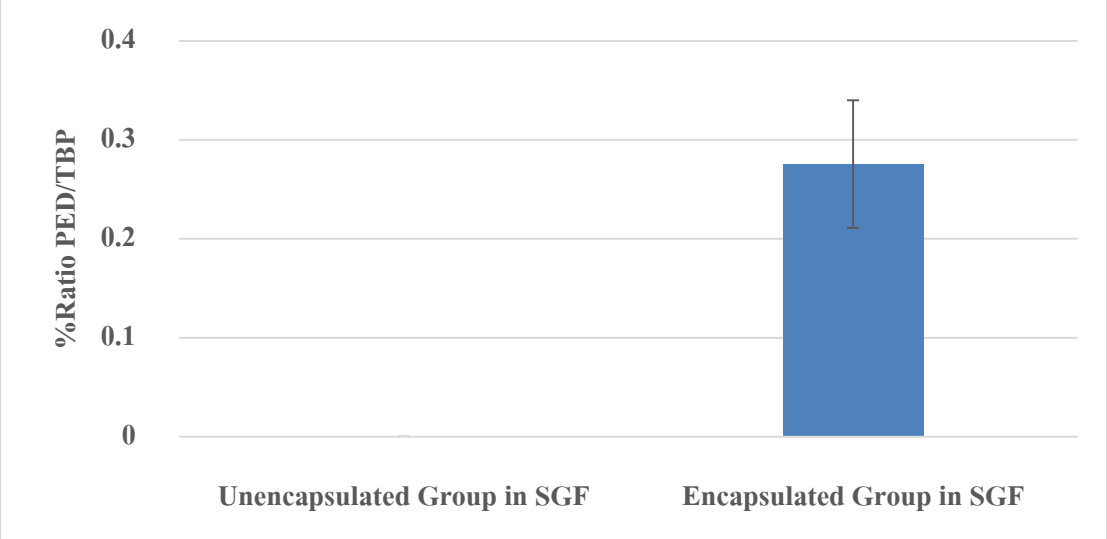


Figure 15; The viral particles were tested with SGF and evaluated by Real-Time PCR.

The unencapsulated group is viral particle delivery by not using encapsulation. Thus, it cannot detect the cDNA of PEDV. The encapsulated group is viral particle delivery by using encapsulation, which can be detected for RNA.

Simultaneously, for the tests with SIF of both unencapsulated group and encapsulated group, the primer of PEDV can be detected cDNA of viral. While it can be calculated for %ratio between PEDV: TBP and found that the primer can be detected for RNA of the encapsulated group more than cDNA of the unencapsulated group, as shown in Figure 16.

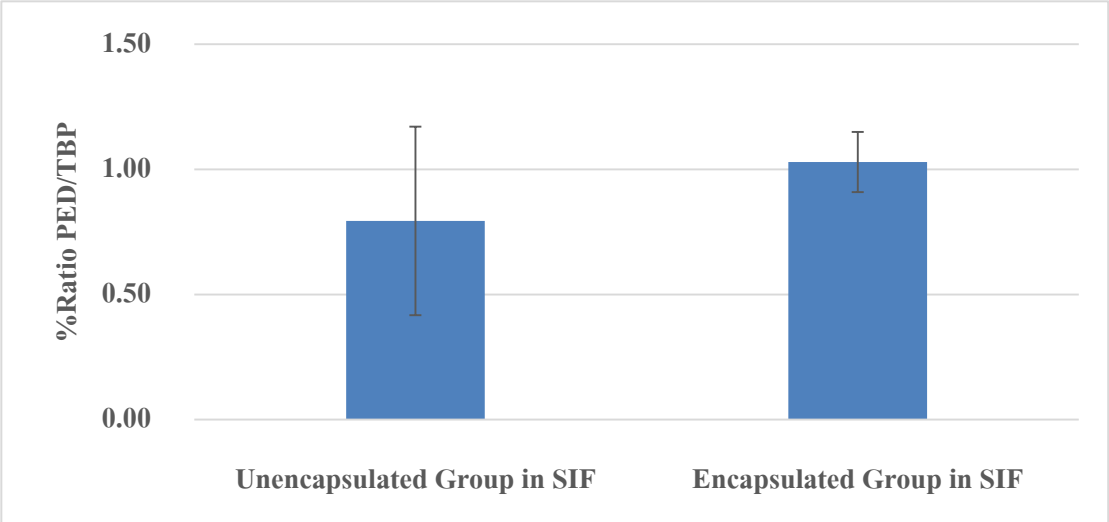


Figure 16; The viral particle was tested with SIF and evaluated by Real-Time PCR.

The cDNA of both the unencapsulated group and the encapsulated group were detected by the primer of PEDV and it can be calculated for %ratio between PEDV: TBP found that the viral of the encapsulated group has more than the unencapsulated group.

In addition to the experiment between viral particles and simulated physiological fluids, the 2C10 mAb were tested with SGF to confirm the efficacy of nanoparticle delivery. The 2C10 monoclonal antibodies, both the unencapsulated group and the encapsulated group, were tested by soaking SGF and then taking dissolved samples for incubation with PEDV in the Vero cell.

Efficacy was tested by the primer of the PEDV. The result of the 2C10 monoclonal antibodies found that the primer could detect cDNA of PEDV in the unencapsulated group. Thus, it can be calculated for %ratio between PEDV: TBP, but it cannot detect PEDV in the encapsulated group. Because the 2C10 antibody of the unencapsulated group touches directly with SGF, the 2C10 antibody was destroyed by the low pH of SGF. Meanwhile, the 2C10 of the encapsulated group used encapsulation for delivery. Therefore, the 2C10 antibody can be neutralized for PEDV. Accordingly, the primer cannot be detected for PEDV (Figure 17).

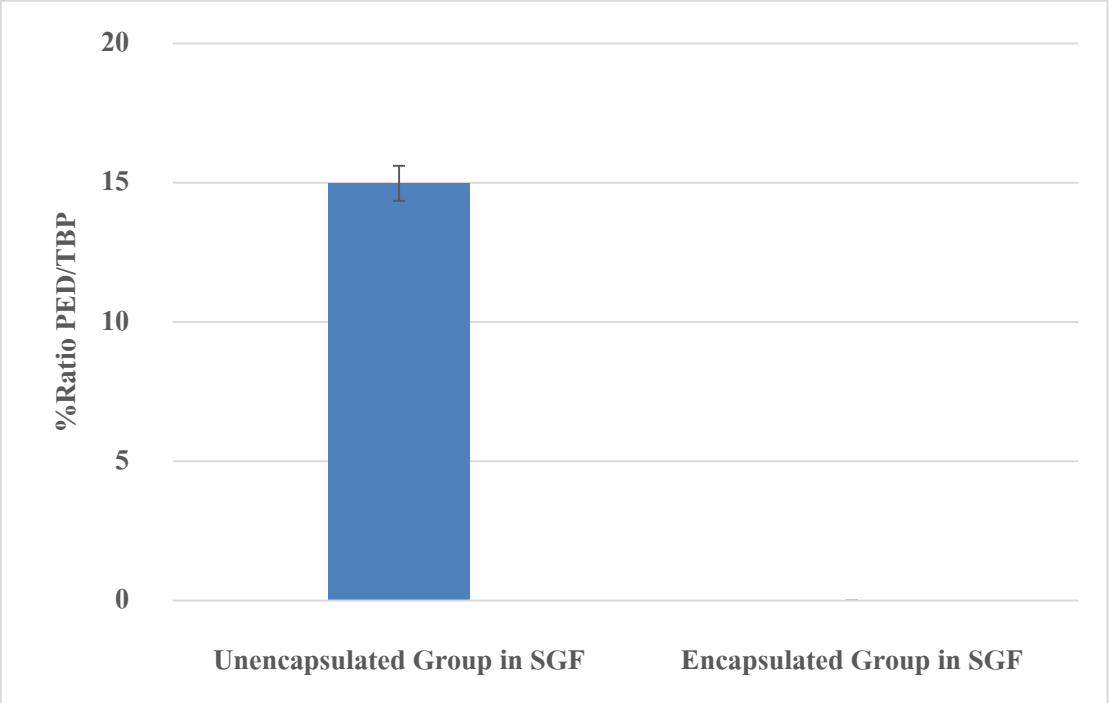


Figure 17; The 2C10 monoclonal antibody were tested with SPF and evaluated by Real-Time PCR.

The unencapsulated group of 2C10 monoclonal antibody cannot be neutralized for PEDV. Therefore, the primer of PEDV can be detected for PEDV. Meanwhile, the encapsulated group of 2C10 antibody can be neutralized for PEDV. Therefore, it cannot be detected for PEDV.

CHAPTER V
DISCUSSION AND CONCLUSION

Over several years, biologics have continued to develop owing to there being more interesting properties for conventional drugs such as low toxicity and high specificity (Oo and Kalbag 2016). Nevertheless, biologics are macromolecular. Hence, there are still weak points including sensitivity to heat, easy destruction by strong pH, and degradation by enzymes.

Oral drug delivery is a widespread platform for drug delivery due to there being several advantages including being low-cost, pain-free, non-invasive and having a modified surface of particle to enhance a specific target. The gastrointestinal tract involves various factors that influence oral drug delivery, especially a strong acid environment, and digestive enzymes are an important barrier for biologics due to the strong acidity, which has a serious impact on absorption. Therefore, a major property of oral drug delivery is stability in an acidic environment to enhance absorption in the intestine (Sahoo, Bandaru et al. 2021).

Microencapsulation has been used in various industries such as food processing, which can protect the oxidation of oil (Carneiro, Tonon et al. 2013), nutrients preservation, and transport (Choi and Kwak 2014). In the pharmaceutical industry, insulin is one of the drugs used for oral drug delivery, which has been reported to use microencapsulation to protect the drug from exposure to the enzymes in the gastrointestinal tract (Cárdenas-Bailón, Osorio-Revilla et al. 2013).

We are interested in microencapsulation techniques for oral drug delivery and would like to apply them in this work, which uses nanoparticles-in-microspheres with a process similar to microencapsulation; it is a mild condition for sensitive biologics ((Ma, Pacan et al. 2008, Chávarri, Marañón et al. 2010). Biologics were encapsulated via microspheres, in which the material of microspheres should have good biocompatibility and biodegradability, similar to Alginate-Chitosan (Murano 1998). Actually, alginate is prominent in food processing and oral drug delivery; it can

protect biologics from digestive enzymes and releases to the target site (Ma, Pacan et al. 2008). Meanwhile, alginate is a chelating agent that is rather sensitive with an acidic agent. When coating alginate by chitosan, however, it can decrease the porosity of alginate (Chávarri, Marañón et al. 2010).

Preliminarily, the nanoparticles use cinnamon oil and encapsulate it by alginate microbeads. The microbeads were prepared using various concentrations. We found that size and entrapment efficacy was increased when the concentration was increased. In this study, we would like to formulate microbeads for the PEDV and monoclonal antibodies, both kinds have a nm level size. We choose a concentration of 2% sodium alginate plus 1% chitosan because the formula as mentioned can be mixed homogeneously with nanoparticles better than 2.5% sodium alginate and it is a suitable qualification. Further, it provides the distribution of diameter size of microbeads between 1.5-1.6 mm, which is more than 70%. This size can protect the core of the microbeads better than small size (Tang, Huang et al. 2015). Moreover, it has been reported that they used 2% sodium alginate and 1% chitosan for the formulation of microbeads because it is a formula appropriate for oral delivery (Li, Jin et al. 2007).

When observing the surface of alginate-chitosan microbeads under SEM, the SEM showed the structure of alginate-chitosan was a network with varying porosity. In the report (Ma, Su et al. 2020), the role of porosity is to exchange nutrients and oxygen between nanoparticles in the hydrogel with capillaries. Previously in this study, it was reported that the pore size of alginate gel was approximately 5 nm (Boontheekul, Kong et al. 2005, Lee and Mooney 2012). The 2C10 monoclonal antibody (Steinbock, Krishnan et al. 2014) and viral particles PED virus (Pensaert and De Bouck 1978, Yang, Kim et al. 2018) have bigger sizes than the pores of alginate-chitosan gel. In summary, the alginate-chitosan microbeads were tested with simulated physiological fluids (SPF), which found that the strong pH and enzymes influenced the contraction and swelling of the microbeads (Ling, Wu et al. 2019). Hence, the structure of alginate-chitosan shrinks and the pore size of the microbead is reduced, leading to nanoparticle protection from the strong pH and enzymes.

Therefore, those can be trapped in microbeads. Meanwhile, the TEM demonstrated the ability to detect nanoparticles in two forms, both single-nanoparticles and multi-nanoparticles. This was examined and confirmed that our microbeads have a nanoparticle (Figure 10).

The above experiment cannot, however, predict the number of nanoparticles. Thus, we used nanoparticles labelled with Nile red to predict the number by fluorescence intensity. The result of taking the microbeads (5 beads) to dissolve with the MBS solution and compare with the Nile Red standard curve found that the number of nanoparticles that could be predicted was approximately 0.5306 ng (Table 5).

As mentioned above, we would like to observe the nanoparticle release behaviour of microbeads when exposed to simulated physiological fluids, which simulated gastric fluids and simulated intestinal fluids as popular for the gastrointestinal tract model. As shown in Figures 5 and 6, the microbeads encapsulated nanoparticles labelled and colour. When soaking in SGF, it was found that both types of labelled and coloured nanoparticles were retained by the microbeads because it still glows, even after being incubated in SGF for three hours. Further, the shape of the microbeads remained spherical, but shrunk when compared at zero hour by the protonation of chitosan (Ling, Wu et al. 2019). Subsequently, the microbead was soaked in SIF to discover that the microbead swelled up. Further, the positive charge of chitosan changed to a negative charge ((Li, Jin et al. 2007, Ling, Wu et al. 2019) and mostly dissolved into a solution. It also releases the nanoparticles to SIF, but some nanoparticles stay trapped with the remains of alginate. This result confirmed that Sodium Alginate-chitosan is an appropriate material and can be a carrier for oral drug delivery (Li, Jin et al. 2007).

It is well known that PEDV can destroy villi enterocytes within the intestinal tract (Cárdenas-Bailón, Osorio-Revilla et al. 2013) and 2C10 monoclonal antibody is used against PEDV; both of them have the same target area. From the above results, we obtained the microbeads for use to deliver our nanoparticles. Meanwhile, the methods mentioned above cannot confirm the efficacy of nanoparticles. Therefore,

real-time RT-PCR was used to analyse the nanoparticles after RNA extraction. We used two types of nanoparticles including viral particles and the 2C10 mAb against PED virus. The results of viral particles showed we can confirm that microbeads retain the efficacy of viral particles from SGF. At the same time, for SIF, we can detect cDNA both two groups, but the encapsulated group can detect more non-encapsulated groups. Moreover, the 2C10 monoclonal antibody was delivered when tested with SGF. We can confirm its efficacy and ability to neutralize PEDV. Therefore, the primer cannot detect cDNA of PEDV, but unencapsulated group can detect RNA. This means the 2C10 antibody was destroyed by SGF. However, we can increase the %ratio of PED/TBP by adding several microbeads in each group.

In conclusion, the gastrointestinal environment will often decrease the efficacy of oral drug delivery, in which the main factors include the enzymes and acidity in the stomach. In this study, the development of oral drug delivery will assist in more effective treatment. We used PEDV to represent oral vaccine delivery and 2C10 monoclonal antibodies from plants to represent oral drug delivery. From all the results, we can summarize that alginate alone is not efficient enough to be used in the oral drug delivery system (figure 19 in appendixtrtion). At the same time, alginate-chitosan is efficient enough to retain the nanoparticles and can thus protect the nanoparticles from contact with strong acid. More importantly, alginate-chitosan has good biocompatibility. Furthermore, alginate-chitosan could release nanoparticles at the target area when tested with simulated intestinal fluids. In the future, we hope that this model for oral drug delivery system development will be able to be used as an alternative for the most effective treatment.

REFERENCES

Balaure, P. C., et al. (2013). "Fabrication, characterization and in vitro profile based interaction with eukaryotic and prokaryotic cells of alginate–chitosan–silica biocomposite." International journal of pharmaceutics **441**(1-2): 555-561.

Boontheekul, T., et al. (2005). "Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution." Biomaterials **26**(15): 2455-2465.

Capretto, L., et al. (2010). "Preparation and characterization of polysaccharidic microbeads by a microfluidic technique: application to the encapsulation of Sertoli cells." Acta biomaterialia **6**(2): 429-435.

Cárdenas-Bailón, F., et al. (2013). "Microencapsulation techniques to develop formulations of insulin for oral delivery: A review." Journal of microencapsulation **30**(5): 409-424.

Cárdenas-Bailón, F., et al. (2013). "Microencapsulation techniques to develop formulations of insulin for oral delivery: a review." J Microencapsul **30**(5): 409-424.

Carneiro, H. C., et al. (2013). "Encapsulation efficiency and oxidative stability of flaxseed oil microencapsulated by spray drying using different combinations of wall materials." Journal of Food Engineering **115**(4): 443-451.

Cereghino, G. P. L., et al. (2002). "Production of recombinant proteins in fermenter cultures of the yeast Pichia pastoris." Current opinion in biotechnology **13**(4): 329-332.

Chasey, D. and S. Cartwright (1978). "Virus-like particles associated with porcine epidemic diarrhoea." Research in veterinary science **25**(2): 255-256.

Chattha, K. S., et al. (2015). "Strategies for design and application of enteric viral vaccines." Annu Rev Anim Biosci **3**: 375-395.

Chávarri, M., et al. (2010). "Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions." International journal of food microbiology **142**(1-2): 185-189.

Chen, C.-C., et al. (2011). "Transdermal delivery of selegiline from alginate–Pluronic composite thermogels." International journal of pharmaceutics **415**(1-2): 119-128.

Choi, M.-J. and H.-S. Kwak (2014). Advanced Approaches of Nano- and Microencapsulation for Food Ingredients**:** 95-116.

Correia, B. E., et al. (2014). "Proof of principle for epitope-focused vaccine design." Nature **507**(7491): 201.

De'Nobili, M. D., et al. (2013). "Performance of alginate films for retention of L-(+)-ascorbic acid." International journal of pharmaceutics **450**(1-2): 95-103.

Degen, P., et al. (2012). "Magnetic switchable alginate beads." Colloid and Polymer Science **290**(2): 97-106.

Demain, A. L. and P. Vaishnav (2009). "Production of recombinant proteins by microbes

and higher organisms." Biotechnology advances **27**(3): 297-306.

Ferraro, B., et al. (2011). "Clinical applications of DNA vaccines: current progress." Clin Infect Dis **53**(3): 296-302.

Harris, H. (2012). "Porcine epidemic diarrhea." The Merck Manual. Merck, Sharpe and Dohme Corp.

HASHEMI, S. H., et al. (2005). "Transient expression of human growth hormone in potato (Solanum tuberosum), tobacco (Nicotiana tobacum) and lettuce (Lactuca sativa) leaves by agroinfiltration."

Hua, S., et al. (2010). "pH-sensitive sodium alginate/poly (vinyl alcohol) hydrogel beads prepared by combined Ca2+ crosslinking and freeze-thawing cycles for controlled release of diclofenac sodium." International journal of biological macromolecules **46**(5): 517-523.

Hua, S., et al. (2015). "Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue." Nanomedicine: Nanotechnology, Biology and Medicine **11**(5): 1117-1132.

Jorge, S. and O. A. Dellagostin (2017). "The development of veterinary vaccines: a review of traditional methods and modern biotechnology approaches." Biotechnology Research and Innovation.

Kaul, G. and M. Amiji (2005). "Cellular interactions and in vitro DNA transfection studies with poly (ethylene glycol)-modified gelatin nanoparticles." Journal of pharmaceutical

sciences **94**(1): 184-198.

Kriegel, C. and M. M. Amiji (2011). "Dual TNF-α/Cyclin D1 gene silencing with an oral polymeric microparticle system as a novel strategy for the treatment of inflammatory bowel disease." Clinical and translational gastroenterology **2**(3): e2.

LaFrentz, B. R., et al. (2008). "Isolation of rifampicin resistant Flavobacterium psychrophilum strains and their potential as live attenuated vaccine candidates." Vaccine **26**(44): 5582-5589.

Lee, K. Y. and D. J. Mooney (2012). "Alginate: properties and biomedical applications." Progress in polymer science **37**(1): 106-126.

Li, W., et al. (2016). "Cellular entry of the porcine epidemic diarrhea virus." Virus research **226**: 117-127.

Li, X.-Y., et al. (2007). "Chitosan– alginate microcapsules for oral delivery of egg yolk immunoglobulin (IgY)." Journal of agricultural and food chemistry **55**(8): 2911-2917.

Ling, K., et al. (2019). "Alginate/chitosan microparticles for gastric passage and intestinal release of therapeutic protein nanoparticles." Journal of controlled release **295**: 174-186.

Ma, L., et al. (2020). "Synthesis and characterization of injectable self-healing hydrogels based on oxidized alginate-hybrid-hydroxyapatite nanoparticles and carboxymethyl chitosan." International journal of biological macromolecules.

Ma, Y., et al. (2008). "Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery." Appl. Environ. Microbiol. **74**(15): 4799-4805.

Ma, Y., et al. (2008). "Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery." Applied and environmental microbiology **74**(15): 4799-4805.

McVey, S. and J. Shi (2010). "Vaccines in veterinary medicine: a brief review of history and technology." Veterinary Clinics: Small Animal Practice **40**(3): 381-392.

Mørch, Ý. A., et al. (2006). "Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads." Biomacromolecules **7**(5): 1471-1480.

Murano, E. (1998). "Use of natural polysaccharides in the microencapsulation techniques." Journal of Applied Ichthyology **14**(3‐4): 245-249.

Mutwiri, G., et al. (1999). "Ileal and jejunal Peyer's patches play distinct roles in mucosal immunity of sheep " Immunology **97**: 455-461.

Nayak, A. K. and D. Pal (2011). "Development of pH-sensitive tamarind seed polysaccharide–alginate composite beads for controlled diclofenac sodium delivery using response surface methodology." International journal of biological macromolecules **49**(4): 784-793.

Nobrega, F. L., et al. (2016). "Genetically manipulated phages with improved pH resistance for oral administration in veterinary medicine." Sci Rep **6**: 39235.

Olinger, G. G., et al. (2012). "Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques." Proceedings of the National Academy of Sciences **109**(44): 18030-18035.

Oo, C. and S. S. Kalbag (2016). Leveraging the attributes of biologics and small molecules, and releasing the bottlenecks: a new wave of revolution in drug development, Taylor & Francis.

Pensaert, M. and P. De Bouck (1978). "A new coronavirus-like particle associated with diarrhea in swine." Archives of virology **58**(3): 243-247.

Poshadri, A. and K. Aparna (2010). "Microencapsulation technology: a review." Journal of Research ANGRAU **38**(1): 86-102.

Pyo, H. M., et al. (2009). "Escherichia coli expressing single-chain Fv on the cell surface as a potential prophylactic of porcine epidemic diarrhea virus." Vaccine **27**(14): 2030-2036.

Rattanapisit, K., et al. (2017). "Rapid Transient Production of a Monoclonal Antibody Neutralizing the Porcine Epidemic Diarrhea Virus (PEDV) in Nicotiana benthamiana and Lactuca sativa." Planta medica **83**(18): 1412-1419.

Rinaudo, M. (2006). "Chitin and chitosan: properties and applications." Progress in polymer science **31**(7): 603-632.

Sahoo, D., et al. (2021). Chapter 9 - Oral drug delivery of nanomedicine. Theory and Applications of Nonparenteral Nanomedicines. P. Kesharwani, S. Taurin and K. Greish,

Academic Press**:** 181-207.

Shoemaker, C. A., et al. (2011). "Efficacy of a modified live Flavobacterium columnare vaccine in fish." Fish & shellfish immunology **30**(1): 304-308.

Shoemaker, C. A., et al. (2009). "Use of modified live vaccines in aquaculture." Journal of the World Aquaculture Society **40**(5): 573-585.

Shoji, Y., et al. (2008). "Plant-expressed HA as a seasonal influenza vaccine candidate." Vaccine **26**(23): 2930-2934.

Silin, D. S., et al. (2007). "Oral vaccination: where we are?" Expert Opin Drug Deliv **4**(4): 323-340.

Steinbock, L., et al. (2014). "Probing the size of proteins with glass nanopores." Nanoscale **6**(23): 14380-14387.

Tang, Z., et al. (2015). "Preparation and characterization of dry powder bacteriophage K for intestinal delivery through oral administration." LWT-Food Science and Technology **60**(1): 263-270.

Unnikrishnan, M., et al. (2012). "Recombinant bacterial vaccines." Current opinion in immunology **24**(3): 337-342.

Vlasova, A. N., et al. (2014). "Distinct characteristics and complex evolution of PEDV strains, North America, May 2013–February 2014." Emerging infectious diseases **20**(10):

1620.

Vllasaliu, D., et al. (2018). "Recent advances in oral delivery of biologics: Nanomedicine and physical modes of delivery." Expert Opin Drug Deliv **15**(8): 759-770.

Wang, J., et al. (2019). "Mucosal immune responses induced by oral administration recombinant Bacillus subtilis expressing the COE antigen of PEDV in newborn piglets." Bioscience reports **39**(3).

Wilson, N. and N. Shah (2007). "Microencapsulation of vitamins." ASEAN Food Journal **14**(1): 1.

Wischke, C., et al. (2013). "Polyalkylcyanoacrylates as in situ formed diffusion barriers in multimaterial drug carriers." Journal of controlled release **169**(3): 321-328.

Yang, D.-K., et al. (2018). "Isolation and characterization of a new porcine epidemic diarrhea virus variant that occurred in Korea in 2014." Journal of veterinary science **19**(1): 71-78.

Yusibov, V., et al. (2002). "Expression in plants and immunogenicity of plant virus-based experimental rabies vaccine." Vaccine **20**(25-26): 3155-3164.

APPENDIX

TABLES AND FIGURESS OF EXPERIMENTAL RESULTS

Table 2; The mane size and the entrapment efficacy of microbeads in different concentrations.

% Alginate Solution	Mean Size (mm.) (±S.D.)	Entrapment efficacy (%EE)
1.0	1.66±0.115	86.15
1.5	1.83±0.086	89.48
2.0	1.98±0.039	95.07
2.5	2.15±0.007	96.13

Values are means of the triplicate experiments ± SD.

Table 3; The size of pH-responsive microbeads (30 beads) was measured by Digital Vernier.

Number microbeads				mean	S.D.
1	1.67	1.64	1.61	1.64	0.0783
2	1.72	1.71	1.69	1.70666667	0.0783
3	1.55	1.52	1.54	1.53666667	0.0783

4	1.73	1.75	1.71	1.73	0.0783
5	1.55	1.54	1.54	1.54333333	0.0783
6	1.55	1.57	1.58	1.56666667	0.0783
7	1.61	1.63	1.6	1.61333333	0.0783
8	1.6	1.58	1.59	1.59	0.0783
9	1.56	1.51	1.55	1.54	0.0783
10	1.55	1.54	1.53	1.54	0.0783
11	1.67	1.65	1.67	1.66333333	0.0783
12	1.72	1.73	1.66	1.70333333	0.0783
13	1.55	1.52	1.54	1.53666667	0.0783
14	1.54	1.5	1.55	1.53	0.0783
15	1.42	1.46	1.49	1.45666667	0.0783
16	1.55	1.57	1.58	1.56666667	0.0783
17	1.61	1.6	1.61	1.60666667	0.0783
18	1.53	1.55	1.52	1.53333333	0.0783
19	1.56	1.61	1.58	1.58333333	0.0783
20	1.45	1.44	1.44	1.44333333	0.0783
21	1.67	1.61	1.65	1.64333333	0.0783

22	1.72	1.73	1.69	1.71333333	0.0783
23	1.55	1.52	1.54	1.53666667	0.0783
24	1.61	1.59	1.63	1.61	0.0783
25	1.45	1.42	1.49	1.45333333	0.0783
26	1.55	1.57	1.58	1.56666667	0.0783
27	1.51	1.53	1.51	1.51666667	0.0783
28	1.57	1.55	1.52	1.54666667	0.0783
29	1.44	1.45	1.44	1.44333333	0.0783
30	1.56	1.54	1.53	1.54333333	0.0783

Values are means of the triplicate experiments ± SD.

Table 4; The concentration of Nile Red was tested under microplate reader.

Content	Blank corrected based on Raw Data (Em spectrum)
wavelength(nm)	636
Blank B	0
0.0001 ug	1482
0.0002 ug	4313

0.0004 ug	6261
0.0006 ug	7845
0.0008 ug	10183
0.0010 ug	12297

Table 5; The nanoparticles labelled with Nile Red encapsulated was soaked and measured under microplate reader.

Content	Blank corrected based on Raw Data (Em spectrum)	Concentration of Nile Red (µg) (Compare with Stand curve)
wavelength(nm)	636	
Blank B	0	0
Alginate Nanoparticles Nile Red (1)	6596	0.0004214
Alginate Nanoparticles Nile Red (2)	7878	0.00060252
Alginate Nanoparticles Nile Red (3)	7425	0.00056788
mean		0.0005306

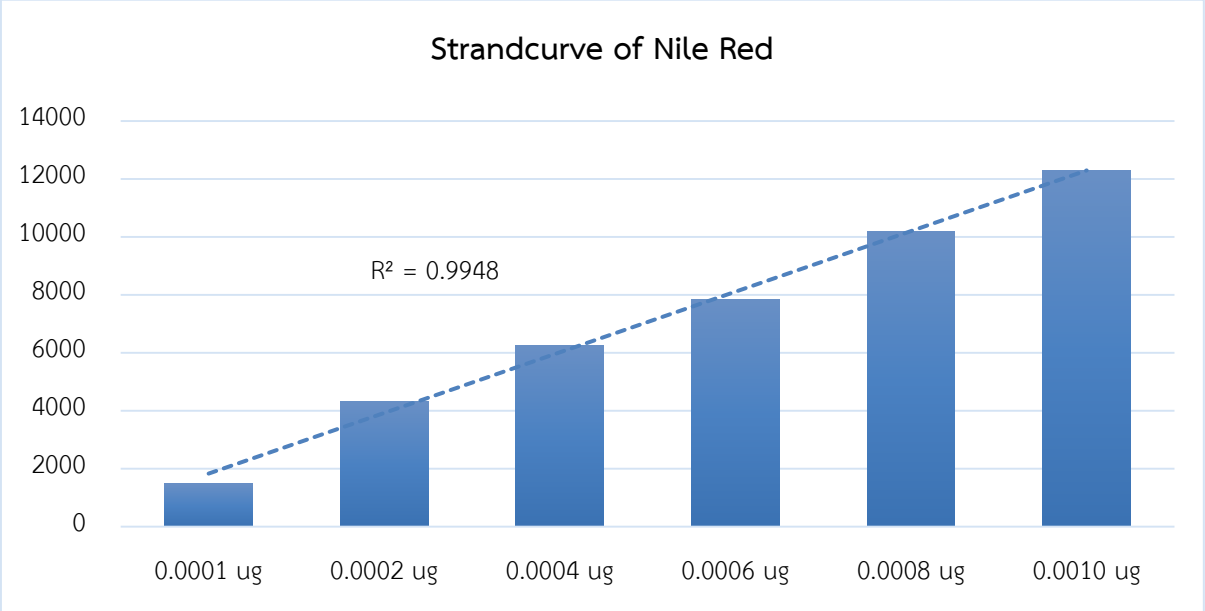


Figure 18;The standard curve of concentration of Nile Red was evaluated under microplate reader

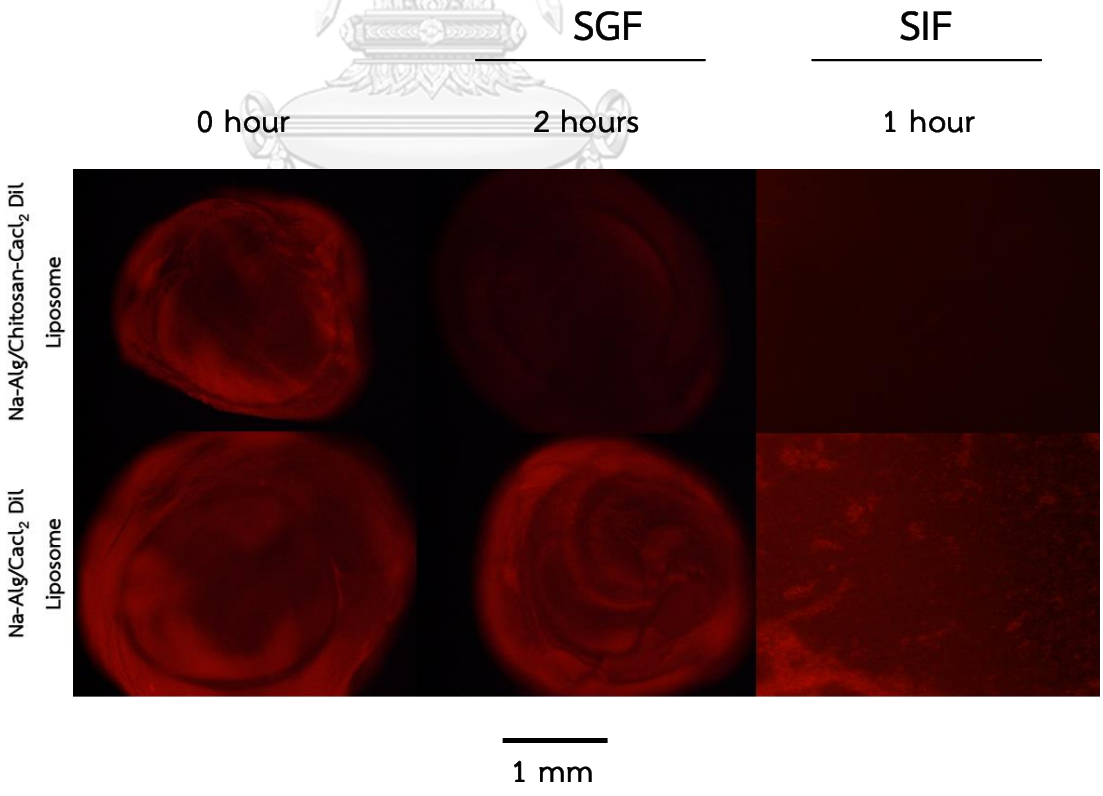


Figure 19; The test liposome labelled Nile Red encapsulated by alginate microbeads and alginate-chitosan microbeads.

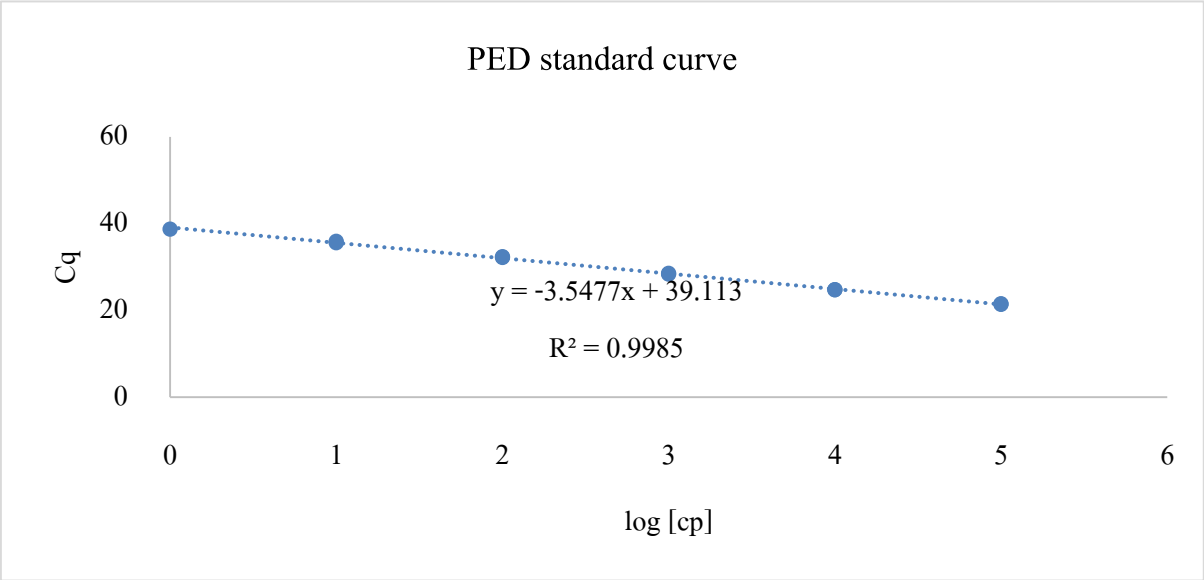


Figure 20; The PED standard curve for Real-Time RT-PCR.

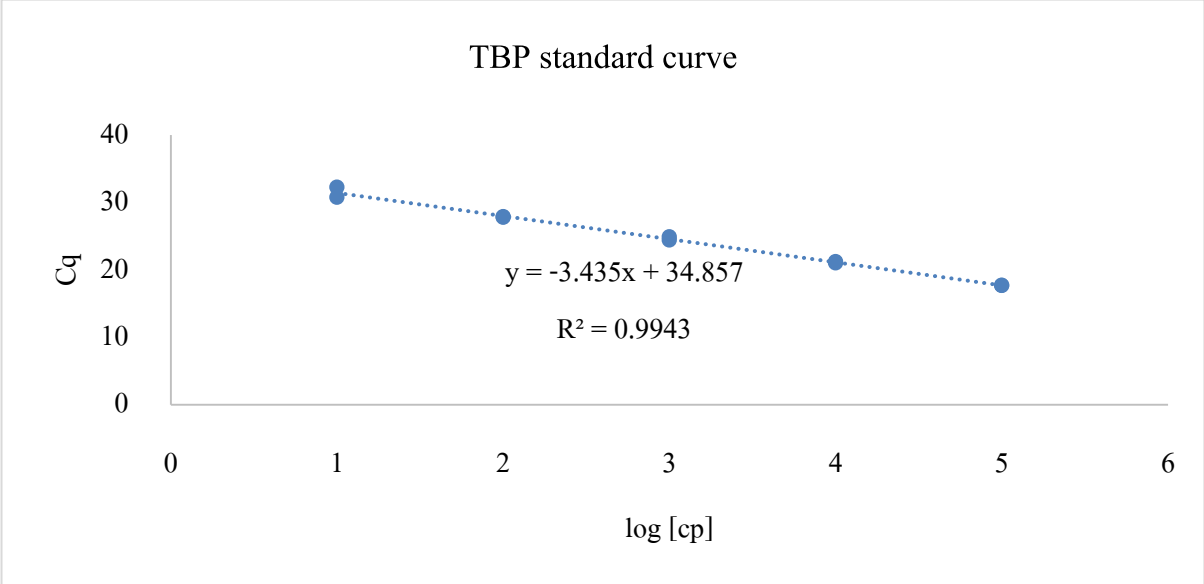


Figure 21; The PED standard curve for Real-Time RT-PCR.

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