Original Article

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Improved virus inhibition by microencapsulated monoclonal antibody against porcine epidemic diarrhea virus

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) has been affecting the swine industry, especially in suckling pigs with a high mortality rate. Among all the strategies to overcome PEDV, boosting mucosal immunity in the pig intestines through oral administration appears to be more efficient than other routes. However, there are biological obstacles such as an acidic environment that could damage biologics, a product from organisms often used for PEDV treatment. The plant-derived 2C10 monoclonal antibody (mAb) from *Nicotiana benthamiana* produced by transient expression was revealed as one of the potential candidates against PEDV through oral delivery. Herein, we demonstrated the calcium-alginate microencapsulation system to protect the 2C10 mAb from the harsh condition in the stomach and to be released the 2C10 mAb when arriving in the intestine. The pH-responsive encapsulated 2C10 mAb microbeads were constructed from the calcium-alginate system. The microbeads were well-tolerated under the acidic environment of simulated gastric fluid and were digested under the alkaline condition of simulated intestinal fluid. The encapsulated 2C10 mAb in the simulated physiological fluids (SPF)-treated microbeads exhibited high virus neutralization efficiency in Vero cells when compared to the unencapsulated 2C10 mAb treated by SPF that cannot neutralize the virus. For these reasons, the calcium-alginate microencapsulation system is an attractive platform to be considered as a candidate for the next generation of oral vaccine development.

Keywords: 2C10 monoclonal antibody, calcium alginate microencapsulation, microencapsulation, porcine epidemic diarrhea virus

INTRODUCTION

Porcine epidemic diarrhea (PED) is a highly infectious and enteric disease in pigs caused by PED virus (PEDV). The virus is an enteropathogenic disease that mainly infects epithelial cells in the small intestine.[1,2] Because of its severity, this disease has been threatening the swine industry in many regions worldwide.[3,4] PED was initially discovered in Belgium in 1978, a lot of PED outbreak dispersed and has been reported in Europe, America, and Asia. As a widespread of PEDV, the swine industry has been lost in many regions around the world.[5] For instance, PED outbreaks in Japan during September 1993 and June 1994 found a mortality rate of 30%–100% in suckling pigs.[6] The highly virulent strains have also been revealed in Canada, Mexico, Ukraine, Japan, Taiwan, and South Korea.[4,7,8] Normally, the PEDV can transmit to pigs in many ways, for example, the oral-fecal route through ingestion of PEDV-contaminated vomit, contaminated feed trunks, service vehicles, vehicles for pig transportation, other fomites, animals, and people.[4,9-12] The mortality rate of PED in suckling piglets was as high as 95%.[13,14]

To prevent the economic loss caused by PED disease in swine, an effective strategy to disinfect PEDV must be developed. The critical point is the biologics administer route into the body as it needs to protect and disinfect PEDV. The biologics are vaccines composite obtained from organism's product including animals, microorganism, and plants product such as recombinant protein, blood factor, and monoclonal antibodies.[15] Biologics are used mostly for treating and safeguarding diseases by oral route administration.[16] Consequently, the 2C10 mAb has been proposed as prophylaxis by oral route delivery. Despite their ability to fight the virus, biologics were often destroyed by the acidic environment including the enzyme. One of the challenges that need to be overcome especially for oral administration is protecting the biologics from degradation and denaturation in the delivery process through extreme conditions in the gastrointestinal system.[17] In our previous work, we developed microencapsulation technology, an attractive alternative to protect active molecules from the acidic environments in the stomach, and found that the system is appropriately infected at enteric.[18]

In 2009, the recombinant 2C10 single-chain antibody previously expressed in *Escherichia coli* exhibited the ability to neutralize PEDV.[19] However, there are limitations to the productions of recombinant protein in bacteria such as high manufacturing cost from plasmid loss, antibiotic-based maintenance, high difficulty to control quality and one of the important reasons is lack of post-translational modifications.[20,21] Yet, the plant production of recombinant protein is a promising method that yields high protein expression with a low risk of pathogenic contamination at low cost and scalable production. The available plants used to produce 2C10 mAb through transient expression are *N. benthamiana* Domin (Solanaceae) and *Lactuca sativa* L. var. *longifolia* (Asteraceae).[22-24] The 2C10 mAb produced from the plant has been proven to neutralize the PEDV. Over the last decade, natural biopolymers such as alginate have widely been utilized as effective materials for gastric protection and intestinal release in many applications including pharmaceutics and food research.[25-30] Therefore, it is possible to use calcium-alginate microencapsulation system to transport the 2C10 antibody to the pig intestine as well.

This study intended to develop the microbead as the potential carrier of 2C10 monoclonal antibody (mAb), to improve protection, guarantee endurance, and survival in the stomach with the rapid release in the small intestine. To illustrate the efficacy, the majority of this study focused on evaluating the calcium-alginate microbead platform and the encapsulated 2C10 mAb's ability to neutralize PEDV after microencapsulation and gastrointestinal testing as schematically shown in Figure 1. We are expecting this research to partake in the next generation of oral vaccination development through the application of the calcium-alginate microencapsulation system.

MATERIALS AND METHODS

Materials

The 2C10 mAb from *N. benthamiana* was produced by transient expression using a geminiviral vector according to the previous study.[23] Sodium alginate, pepsin, pancreatin, and calcium chloride were purchased from Sigma (Missouri, USA). Alexa Fluor™ 488 Phalloidin (AF-488P) was purchased from ThermoFisher Scientific Inc. (Massachusetts, USA). Deionized water was produced from Thermo Scientific™ Barnstead™ LabTower™ EDI Water Purification System (Massachusetts, USA). American green monkey kidney (Vero) cells were obtained from American Type Culture Collection (ATCC). RNeasy mini kit was purchased from QIAGEN Inc. (Germantown, MD, USA). PED viruses were obtained from Vet Products Research and Innovation Center Co., Ltd.

Plant Inoculation and Protein Production

The *N. benthamiana* seeds were received from Julian Ma's Laboratory (London, UK). The plants were percolated with Agrobacterium CV3101 strains composing of pBY2C10-gamma, pBY2C10-kappa, and p19 which includes gene silencing of transient protein fabrication in plant as described in the previous publication.[23] The target protein was extracted from the plants after maintaining in the growth chamber for 5 days.

Protein purification was performed by homogenization using a blender with 1X phosphate buffered saline (1X PBS: 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4 at pH 7.4), pH 7.5. Crude extract of *N. benthamiana* was infiltrated through Miracloth and centrifuged at 26,000 g for 20 min, followed by eliminating residue using 0.2 μm filter. The filtered solution was loaded into the protein A bead column and washed with 1X PBS, pH 7.5 for 10 bed volumes. The protein was subsequently eluted by 100 mM glycine, pH 2.5, and neutralized with 1 M tris base solution to reach a final pH of 7.5.[23]

Calcium-alginate Microbead Preparation and Characterization

To construct pH-responsive microbeads, 900 μL of a solution of 2% (w/v) of sodium alginate was mixed with 100 μL of

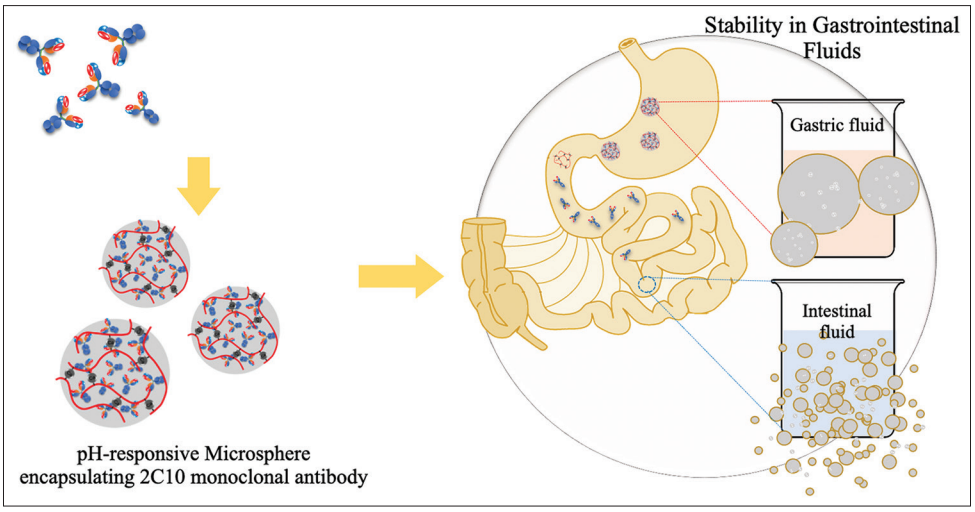

Figure 1: The major of this study to evaluate of efficacy the calcium-alginate microbead platform and the encapsulated 2C10 monoclonal antibody's ability to neutralize porcine epidemic diarrhea virus after microencapsulation and gastrointestinal testing

the 2C10 mAb protein (9:1 v/v, ratio). The mixture was then introduced drop wise from a syringe into 100 mL of a 3% (w/v) $CaCl_2$ under mechanical stirring at 400 rpm for 10–15 min. The calcium-alginate microbeads were then collected from the $CaCl_2$ solution by filtration and washed with deionized (DI) water. To dry the constructed microbeads, tissue paper was used to absorb excess water and oil on the microbead surface. After that, the microbeads were kept at 4°C. The size of the microbeads was examined by direct observation. The average size was also performed on 30 microbeads and recorded using digital Vernier calipers (Absolute Digimatic, Mitutoyo Corp., Japan). The morphology of the microbeads surface 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.

Release Behavior in Simulated Physiological Fluids

To study the release behavior of the targeted particles from the microbeads, the 2C10 mAb was labeled with Alexa Fluor™ 488 Phalloidin. SGF and simulated intestinal fluid (SIF) were prepared as explained in the previous protocol. In this study, SGF comprised 3.2 mg/mL pepsin in 0.2% (w/v) NaCl at pH 3.5, and SIF comprised 1 mg/mL pancreatin in 0.2% (w/v) NaCl at pH 8.0.[31] For all experiments, the calcium-alginate microbeads were maintained at 37°C in a shaking incubator at 250 rpm. The microbeads were initially performed in SGF for 3 h and then SIF for 2 h, respectively. Representatives of microbeads were collected at 1, 2, and 3 h for SGF and 1 and 2 h for SIF. Encapsulation of remaining Alexa Fluor™ 488 Phalloidin-labeled 2C10 mAb microbeads was determined under the fluorescence microscope.

Vero Cells, PEDV Preparation, and Virus Neutralization Assays

In this study, African green monkey kidney (Vero) cells (ATCC, CCL-81) were cultured in minimum essential (ME) media (MEM, antibiotic-antimycotic, and Na_2CO_3) consisting of 5% fetal bovine serum at 37°C in a humidified 5% CO_2. The PEDV strain CBR (GenBank number: KJ960179) was manifolded in Vero cells treated in MM medium containing 2 µg/mL of trypsin.

Vero cells were seeded into the 48-well plats and cultured until approximately 70–80% coverage of the plate. The 200 µL of 50% tissue culture infectious dose (TCID50) of the PEDV in MM medium was incubated with the simulated physiological fluids (SPF)-treated microbeads (1:1 (v/v), ratio) at 37°C for 1 h. The mixed solutions were subsequently transferred to the Vero cell plates and incubated at 37°C for 1 h. After that, the solution was removed and was filled with MM+2% fetal bovine serum. The plates were then incubated at 37°C in a humidified 5% CO_2 for 7 days. For this study, colostrum with and without neutralization was used as positive and negative control, respectively. The cytopathic effect was evaluated and compared with both positive and negative control.

RNA Extraction and Real-time Polymerase Chain Reaction (RT-PCR) Analysis

Total RNAs from the 7-day cultured cells were extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany following the manufacturer's instruction. The extracted RNAs were eluted with sterile DI water. cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA). RT-PCR reaction consisted of 1x PowerUp SYBR Green Master Mix (7500 Applied Biosystems, Carlsbad, CA), 900 nM forward and reverse primers [Table 1], 2 µL of cDNA template and DI water to adjust a total final volume as 20 µL was performed through the Standard PCR protocol. The RT-PCR experiments were operated following the cycling profile: 50°C for 2 min, 95°C for 2 min to dual-lock DNA polymerase, subsequently 40 cycles of denaturation at 95°C for 15 sec, and a combined annealing/extension step at 60°C for 60 s. All results were examined by the 7500 software version 2.0.5 (Applied Biosystems, USA) to express as the quantification cycle (Cq) value, in which the lower Cq value was defined as the higher virus.

Statistical Analysis

The paired *t*-test was used to analyze data generated by quantity of RNA of PED and TBP detected after SPF treatment by the RT-PCR method. The PED/TBP ratio value of the unencapsulated 2C10 mAb group was compared to the PED/TBP ratio value of encapsulated 2C10 mAb group by calcium alginate.

RESULTS

Calcium-alginate Microbead Fabrication and Characterization

To construct a pH-responsive microbead to deliver 2C10 mAb through the gastrointestinal tract, calcium and alginate were selected as the major materials to encapsulate these biomolecules. As indicated in Figure 2a and b, the PEDV attenuated strain or the 2C10 mAb was entrapped in calcium-alginate microbeads through gelation method using calcium ions as cross-linking agent. The mixture of alginate and the 2C10 mAb was dropped using syringe pump to control the average size and volume of the microbeads. Size measurement using digital Vernier calipers gave the average value of the calcium-alginate microbeads as 1.57 ± 0.08 mm in diameter. We found that size of the constructed microbeads was distributed between 1.5 and 1.6 mm in diameter with more than 75% of the microbead population. The morphology of the microbead was exhibited under SEM, which found that the surface of the microbeads involved a network structure and spherical shape, the microbead has a distribution comprising many small and compact pores as shown in Figure 2b.

Release Behavior in Gastric and Intestinal Fluids

The 2C10 mAb microbeads were examined for the acid resistance and enteric solubility after encapsulation process. In this study, fluorescent signal was used for monitoring the release profile of the 2C10 mAb (labeled by Alexa Fluor™ 488 Phalloidin dye). The microbeads were initially treated in SGF for 3 h, followed by SIF for 2 h, respectively. Based on in Figure 3, calcium-alginate microbeads showed a tolerance property when were treated in acidic conditions with SGF (pH 3.5). The calcium-alginate microbeads contained their spherical shape after soaking in SGF for 3 h. Notably, this means that the 2C10 mAb were still trapped in the microbeads and not released into the SGF. However, it is intriguing that after these microbeads were assessed in the SIF (SIF, pH 8.0), the microbeads were digested and the entrapped 2C10 mAbs were released from the microbeads to SIF, as shown in Figure 3.

Virus Neutralization and Neutralizing Efficiency of the pH Responsive Microbeads by RT-PCR

The SPF-treated microbeads were collected for studying virus neutralization assay. To evaluate neutralizing efficiency of the 2C10 mAb against PEDV in Vero cells, we collected the SPF-treated microbeads and incubated with the PEDV-infected Vero cells for 7 days. In comparison with the microencapsulated 2C10 mAbs, the unencapsulated 2C10 mAbs which were treated with simulated physiological fluids were also performed in the same way. The methodology designed for this study is shown in Figure 4a. RT-PCR was used to determine the number of

Table 1: Forward and reverse primer sequences and their targeted region

Assay name	Primer name	Oligonucleotide sequence (5' to 3')	Target	Reference
PEDV	PED_N_F	CGCAAAGACTGAACCCACTAATTT	Nucleocapsid	[32]
	PED_N_R	TTGCCTCTGTTGTTACTTGGAGAT		
TBP	GAPDH_F	GAAATCCCATCACCATCTTCCAGG	Glyceraldehyde 3-phosphate dehydrogenase	[33]
	GAPDH_R	GAGCCCCAGCCTTCTCCATG		

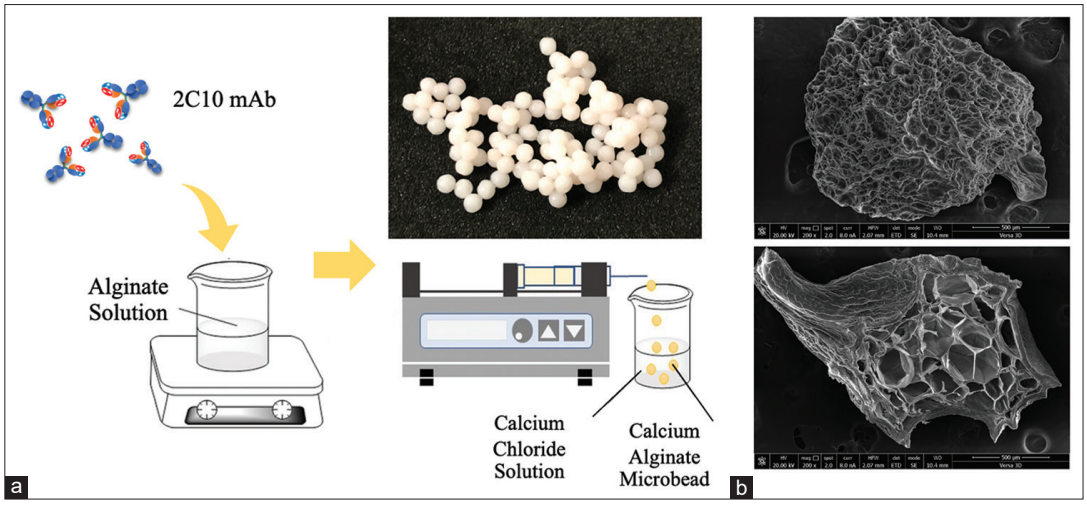

Figure 2: Schematic representation of the calcium-alginate microencapsulation. In this method, 2C10 monoclonal antibody was encapsulated by calcium-alginate microbead construction using calcium chloride as crosslinking reagent (a). The scanning electron microscope showing the characteristics of the surface of calcium-alginate microbeads (b). Scale bar = 500 μm

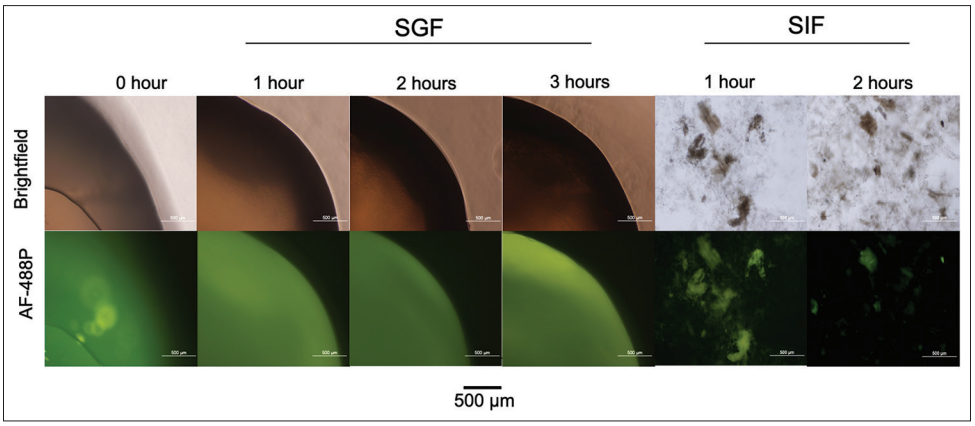

Figure 3: Acid resistance and release of calcium-alginate microbeads using an *in vitro* gastrointestinal digestion model. Fluorescent signal using Alexa Fluor™ 488 Phalloidin (AF-488P) dye was used to detect encapsulated 2C10 monoclonal antibody in constructed microbeads. Scale bar = 500 µm.

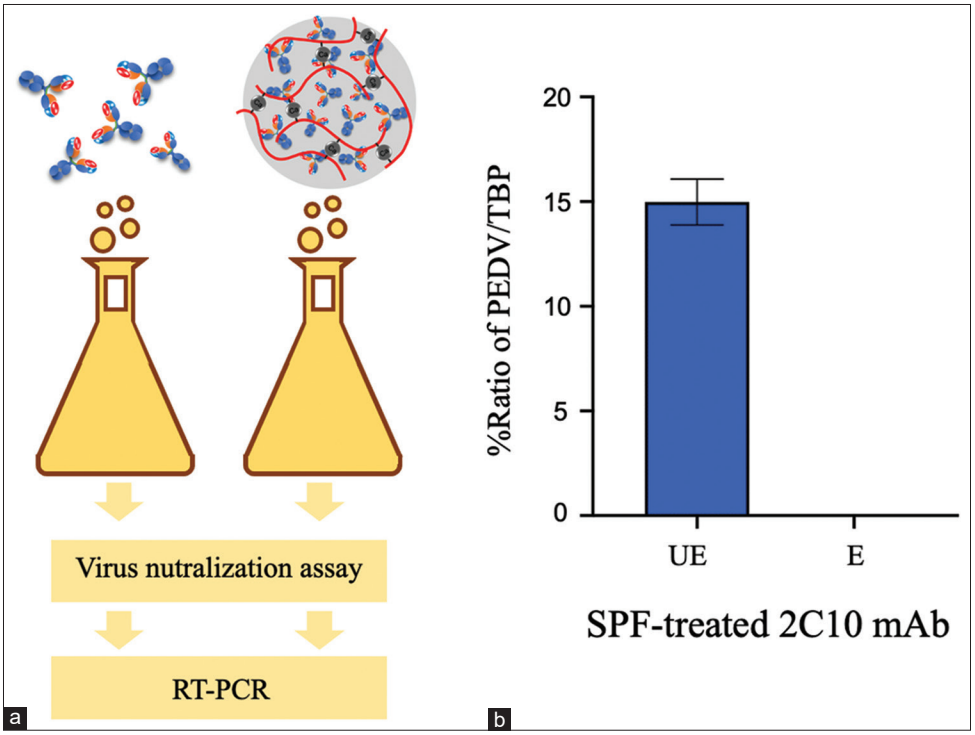

Figure 4: Virus neutralization efficacy determined by real-time polymerase chain reaction (RT-PCR). The encapsulated 2C10 monoclonal antibodys (mAbs) (abbreviated as E) from simulated physiological fluids (SPF)-treated microbeads was investigated with Vero cells and compared with unencapsulated 2C10 mAbs (abbreviated as UE) (a). The RT-PCR was used to detect the quantity of RNA after treated with SPF (b). The experiments were performed for three replicates. Data are represented as mean ± SD, $n = 3$

RNA products from virus neutralization. The percentage ratio of PEDV and TATA-box binding protein (TBP) primers was calculated and demonstrated in Figure 4b. From this result, there is obvious that the specific primers could detect RNA of PEDV from the SPF-treated unencapsulated 2C10 mAb sample with the % ratio as approximately 15. In contrast, the primers cannot detect PEDV's RNA from the SPF-treated 2C10 mAb microbeads as shown in Figure 4b.

DISCUSSION

The PED outbreak in many regions of the world showed a destructive effect on the swine industry.[5] Since the PED virus can annihilate the villus enterocytes of the jejunum and ileum of the infected pigs, several studies suggested that the attractive approach to protecting pigs from PEDV is to boost the intestinal mucosal immunity through oral administration.[34-36] Unfortunately, the oral vaccine available for commercial use is limited because of the harsh condition of the GI tracts which increases the likelihood of the active antigens, antibodies, and protein degradation and denaturation before arrival in the intestine.[37] We see the opportunity to apply the microencapsulation platform as the delivery cargo to effectively transport the active biomolecules to the specific area of the intestine and protect those biomolecules from the gastrointestinal environment. The proposed microbeads are composed of calcium-alginate microencapsulation. In this

study, the neutralizing anti-PED mAb from *N. benthamiana* was selected as a representative of biomolecules. Since PEDV belongs to the Coronaviridae family, PEDV enters the cell by fusing with the Spike (S) protein on the host cell membrane. Thus, the most optimal mechanism to prevent PEDV infection is to neutralize epitopes on S-protein. The 2C10 mAb recognizes the peptide on carboxy-terminal of the PEDV S-protein which inhibits the cell entry.[23] Because of these reasons, 2C10 mAb was encapsulated to deliver to the target deposit site in the gastrointestinal tract through the microbead, which constructed by calcium-alginate gelation method. The gelation process involves guluronic acid residues with the Ca^{2+} chelation to form "egg-box" structure[38] and entrapping the 2C10 mAbs in the spherical microbeads with small particle size and low porosity. The calcium-alginate microbeads are suitable agents to construct pH-responsive carriers for delivering active biomolecules to the intestine. The encapsulated 2C10 mAb microbeads fabricated from calcium-alginate gelation could be well-tolerated in the acidic environment of SGF but could be degraded in the alkaline condition of SIF and release the encapsulated mAbs into the environment. This phenomenon was also demonstrated in the previous reports.[25,26,39]

The structure of the calcium-alginate microbead came from specific interactions including intermolecular hydrogen bonding of mannuronate and glucosamine units.[40] Gelling property in low pH because of negative charges of mannuronate units and positive charges of glucosamine units under acidic condition. As the pH value increases, the condition neutralizes or becomes more basic, both mannuronate and glucosamine units are losing their charges which then breaks the gelling formation interactions.[40] The absence of the gelling property allows the calcium alginate microbead to degrade and release the active biomolecules. Because of the charges of the encapsulation molecules, the microbead shows the property of maintaining its stability in SGF (acidic condition) and becomes easily degradable in SIF (alkaline condition).

Our microbead shows promising performance in protecting the plant-produced 2C10 mAb from the SGF. Moreover, the 2C10 mAbs were able to PEDV neutralization efficiency in an alkaline environment. Contrary to the unencapsulated 2C10 mAb, it was not able to be neutralized PEDV before infecting Vero cells as shown in Figure 4b. Based on the neutralization results, the calcium-alginate crosslinked interaction from encapsulating molecules inside the microbead can be considered as one of the potent and interesting carriers to protect biomolecules (antigens, antibodies, and proteins) from harsh physiological conditions. Therefore, using microbead to deliver active biomolecules was demonstrated to be an effective platform that can be later applied to other active agents in the future.

CONCLUSIONS

The calcium-alginate microbead is proven to be effective in delivering the active biomolecules to the target site and protect the molecules through the major barriers including degradation and denaturation of active molecules when passing in harsh environments. The oral delivery formulation of the microbead containing 2C10 mAb from *N. benthamiana* has been demonstrated in this study. The gelling system of the calcium-alginate encapsulation in the microbead shows pH-responsive effect to protect the encapsulated 2C10 mAbs from the acidic conditions and release the 2C10 mAbs under neutral or alkaline conditions. This illustrates that the calcium-alginate microbead can be used for the oral administration of the 2C10 mAb to the intestine without acidic degradation and denaturation. Although this current study only focuses on delivering 2C10 mAb, the calcium-alginate microbead is considered as a promising carrier to apply with other biomolecules through an oral delivering application.

Limitation and Caution

This research focuses mainly on the development of biomolecule delivery system through oral administration. To maximize the encapsulation efficiency, the formulated microbeads should be kept at 4°C simultaneously since the content inside is protein (mAb).

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DISCLOSURE

The authors report no conflicts of interest in this work.

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