Designing an extracorporeal plasma filtration column with specific binding to dengue virions

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การติดเชื้อไวรัสเดงกีเป็นปัญหาด้านสาธารณสุขที่สำคัญอย่างหนึ่งและยังไม่มีการ
รักษาที่เฉพาะเจาะจงในปัจจุบัน ระบบไหลเวียนเลือดนอกร่างกายเป็นทางเลือกหนึ่งในการ
รักษาผู้ป่วยไข้เลือดออกขั้นรุนแรงที่กำลังจะเกิดอาการช็อก อย่างไรก็ตามอุปกรณ์ดูดซับ
ภูมิคุ้มกันในเชิงพาณิชย์มีลักษณะเป็นแบบขนาดจำเพาะ ไม่ได้ออกแบบมาสำหรับใช้งาน
อเนกประสงค์ ดังนั้นจึงไม่เฉพาะเจาะจงสำหรับกลุ่มอาการไข้เลือดออก การศึกษานี้มุ่งเน้นไปที่
การสร้างแอนติบอดีจำเพาะของ flavivirus (4G2) ที่ติดอยู่ในเม็ดอัลจิเนต ผลการทดลองแสดง
ให้เห็นว่าปริมาณไวรัสลดลงมากกว่า 90% หลังจากการทำงานบนวงจรจำลอง 3 ชั่วโมง โดยวัด
ปริมาณด้วยวิธี reverse transcription qPCR และ plaque titration assay การทดลองซ้ำด้วย
การจำลองแบบทางชีวภาพสองครั้งและผลลัพธ์สอดคล้องกัน ยิ่งไปกว่านั้นคอลัมน์นี้ไม่พบการ
ดูดซับที่เฉพาะเจาะจงกับเชื้อโรคอื่น ๆ ที่ทำให้เกิดการติดเชื้อแบบแพร่กระจาย เช่น
Escherichia coli และ Enterovirus A71 งานนี้เป็นการพิสูจน์แนวคิดของการดูดซับไวรัสที่
จำเพาะ โดยใช้โมโนโคลนอลแอนติบอดีต่ออนุภาคของไวรัส ระบบนี้สามารถปรับปรุงและปรับ
ให้เหมาะสมเพื่อขยายการประยุกต์ใช้ทางคลินิกกับโรคทางระบบอื่น ๆ โดยการแทนที่
แอนติบอดีที่จำเพาะหรือแอนติบอดีค็อกเทล

สาขาวิชา	จุลชีววิทยาทางการแพทย์	ลายมือชื่อนิสิต
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Dengue infection is one of the major public health concerns and no specific treatment is currently available. An extracorporeal system is one of the alternative treatment in severe dengue patients with impending shock. However, the commercial immunoadsorption devices were size-exclusive, non-selective designed for multipurpose thus not specific for dengue syndrome. This study focuses on establishing the flavivirus-specific (4G2) antibody entrapped into the alginate bead. Results showed that the viral titer reduced more than 90 % after 3 h running on a simulated circuit quantified by reverse transcription qPCR and plaque titration assay. The experiments were repeated with two biological replicates and results were consistent. Moreover, the column showed no specific adsorption to other pathogens inducing systemic infection such as *Escherichia coli* and Enterovirus A71. This work is the proof-of-concept of a specific viral adsorption using a monoclonal antibody against the viral particle. The system could be improved and optimized to broaden the clinical application to other systemic diseases by replacing the specific antibody or antibodies cocktail.

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

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Sasiwimon Thonghong

TABLE OF CONTENTS

จุฬาลงกรณ์มหาวิทยาลัย

CHULALONGKORN UNIVERSITY

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The dengue virus infection is a major public health problem with outbreaks in more than 100 tropical countries. Four serotypes of the dengue virus infect humans via mosquito vectors, *Aedes aegypti and Aedes albopictus* (1) . Most infections are asymptomatic or mild (undifferentiated febrile illness, UFI). Classic terms of severe manifestations were described as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (2); whereas, severe plasma leakage, massive hemorrhage, and multiple organ failure (3) were used in WHO 2009. The progress of the dengue illness is characterized into 3 phases: febrile phase, critical phase, and recovery phase. During the febrile phase, factors indicating the progression to severity include signs of bleeding, deranged liver function test, presence of urinary red blood cells, and urinary protein (4, 5). The secondary heterotypic infection triggers immunopathological cell-mediated and humoral responses resulting in excessive cytokine production, increase viral titer by opsonization, coagulation defect from impaired hepatocytes, production of platelet and plasmin autoantibodies, etc. At present, the treatment does not include any specific antiviral chemotherapy. Supportive care still plays an important role as a primary treatment for severe patients. Severe complications such as liver failure, renal failure, and neurological symptoms (1, 5, 6) could occasionally occur despite intensive monitoring.

Severe dengue manifestation is caused by the immunopathological responses increasing the viral titer during the febrile phase, leading to life-threatening conditions during the critical phase. Stabilizing hematological and biochemical profiles during a febrile phase could be key for prospective treatment in severe cases. In fact, a few case reports mentioned the plasmapheresis in severe dengue with encephalopathy (7-9). Moreover, a similar platform has been developed in Ebola hemorrhagic fever (10), MERS-CoV (pseudovirus), and MARV soluble glycoprotein (1 1) . In principle, the patient's whole blood ran through a hemodialysis system coupled with an extracorporeal lectin affinity plasmapheresis device (Hemopurifier®) (Figure 1). The column was

capable of reducing 1-1.5 log copies/ml of circulating Ebola viruses in the patient. The column consisted of layers of lectin-binding material arranged in a wafer-like pattern (Figure 2a, 2b).
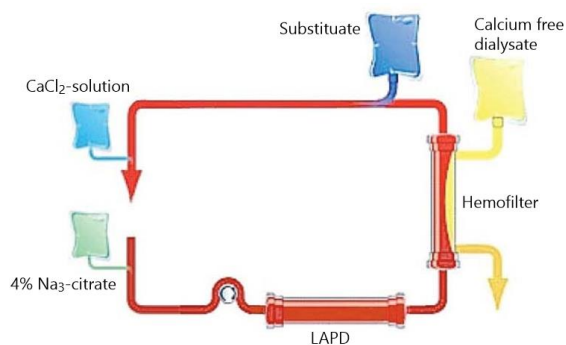
Figure 1 Extracorporeal circuit sketch displaying the hemofilter's upstream lectin affinity plasmapheresis (retrieved from Buttner et al. (10)).
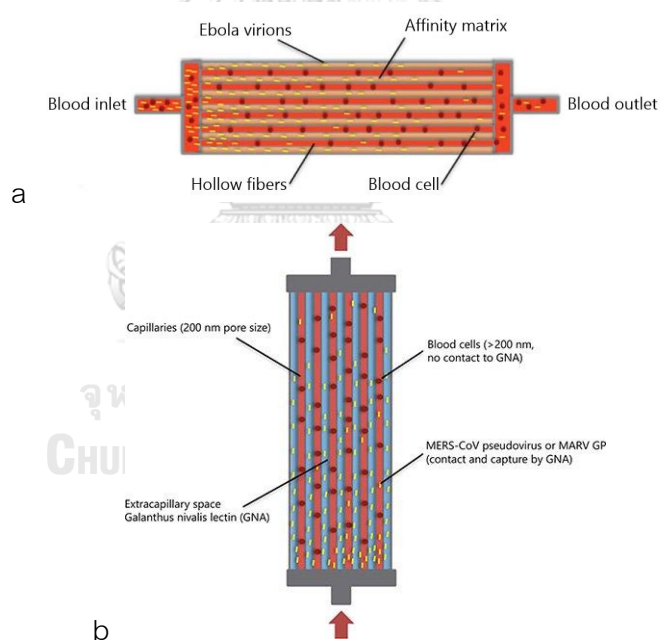
Figure 2 Lectin affinity column used in (a) Ebola hemorrhagic fever (retrieved from Buttner et al. (10)) and (b) MERS and Marburg virus (retrieved from Koch et al. (11)).

Moreover, there is a commercially available cytokine absorber (CytoSorb®) targeting inflammatory cytokines such as TNF-alpha, interleukins, and IFN-gamma. The column utilized a size-exclusion method that targets the 10-50 kDa proteins (12).

Therefore, it is likely to incorporate the extracorporeal blood purification filtration column into plasmapheresis or hemodialysis treatment of selected dengue patients with predicted indicators towards severity. This novel procedure is expected to reverse the immunopathological process by removing viral pathogen and excessive inflammatory cytokines before the disease progresses to a critical phase. From the above statement, we aimed to prove this concept by developing a filtration column capable of binding to the dengue virus.

Objectives

1. To verify the adsorption efficacy of DENV-specific antibody integrated to alginate bead.

2. To quantify the viral load reduction in DENV-spiked fetal bovine serum in extracorporeal circulation *in vitro* using DENV-specific antibody integrated to alginate bead.
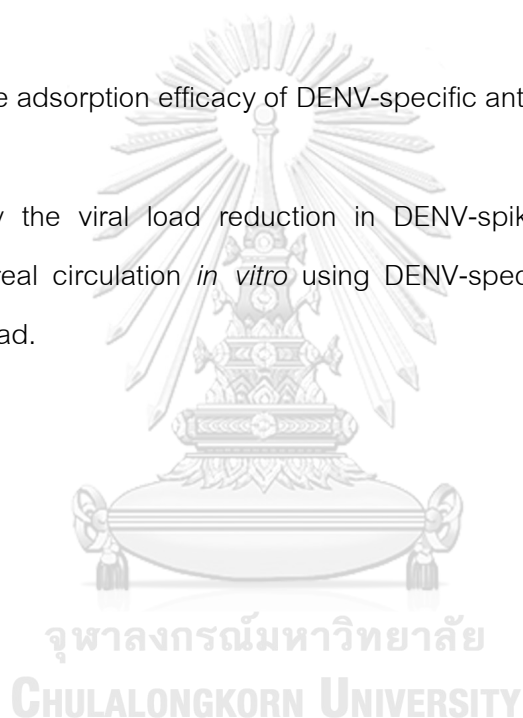
CHAPTER 2
LITERATURE REVIEWS

1. Dengue diseases

1.1. Dengue virus

Dengue virus is a member of the family *Flaviviridae* (13). There are four serotypes, including DENV1, DENV2, DENV3, and DENV4, with 60% similarities. The viral particle is 50-60 nm in diameter (14). The viral genome consists of a positive-sense RNA of ～11 kb. This RNA encodes three structural proteins (C, prM, and E) and seven non-structural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5). The life cycle of the dengue virus in host cells begins with the virus binding to a specific receptor on the host cell. The virus resides in an endosome containing proton pumps, which lowering the pH and causing structural changes of the E proteins. This process allows the viral RNA to be released to the cytoplasm. Then, the RNA binds to ribosomes on the rough endoplasmic reticulum (14) and starts the translation. Replication occurs in the invaginated RER membrane directed by viral and host proteins. Assembly occurs at the ER-Golgi complex, and the virion travels through the protein transport system. Maturation occurs when the viral prM protein is catalyzed, causing rearrangement of the M-E heterodimer to E-E homodimer. Finally, the virus is released from the original cell and ready to infect other cells (15, 16). The *Aedes* vector can be infected with multiple dengue serotypes, as well as Zika and Chikungunya viruses. After taking a blood meal from infected individuals, the mosquito is capable of transmitting the virus within 7-10 days until the rest of its life. On the contrary, human becomes infected with the virus after bitten by mosquitoes for 3-15 days, most often between 5-6 days. The virus remains detectable in bloodstream for 3-5 days after fever onset (5, 17, 18).

1.2. Pathogenesis

The virus enters the human body via a mosquito bite and infects immature dendritic cells (D.C.) in the skin. The infected cell travels to the regional lymph node and releases the virion progenies to other immune cells. Then, the infected cells carry the virus into the blood circulation or a primary viremia. This event causes systemic viral

infection, and the virus can infect multiple organs such as the liver, spleen, and bone marrow. In primary dengue infection, the pathogenesis resembles other viral infections in that the viremia occurs for a short period (1-3 days). Both humoral and cell-mediated responses effectively eliminate the viral infection. IgM and IgG can be seen in the bloodstream in 5-7 and 7-10 days after fever onset, respectively (19).

The secondary heterotypic infection causes the most severe manifestations due to the misleading of immunological responses towards the previous infection (20). The pathogenesis occurs when the infected D.C. activates CD4-positive T cells, and the memory cells of the previous dengue infection are chosen. This misled clonal selection creates a sequence of robust but incompetent responses. Antibodies cannot completely neutralize the virus but can enhance opsonization by macrophages, which is a primary target of the dengue virus. This "antibody-dependent enhancement" amplifies the viral infection, thus increases and prolongs the virus propagation in the body. Also, these antibodies can cross-react to plasmin and platelet and deplete them from blood stopping process. Moreover, excessive proinflammatory cytokines from T cells and mast cells led to a "cytokine storm" event (21, 22).
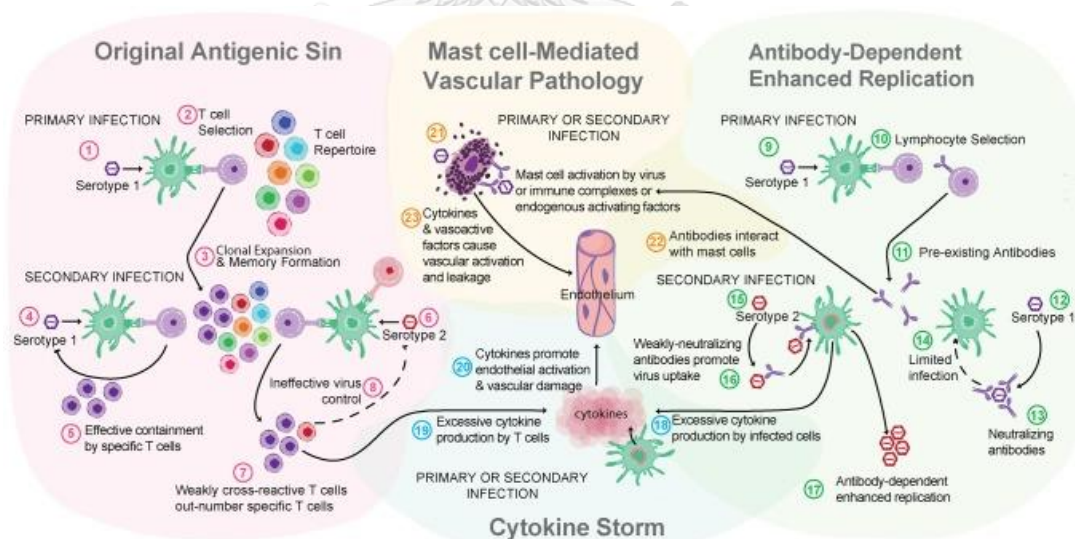
Figure 3 Dengue immune pathogenesis (retrieved from St John et al. (21))
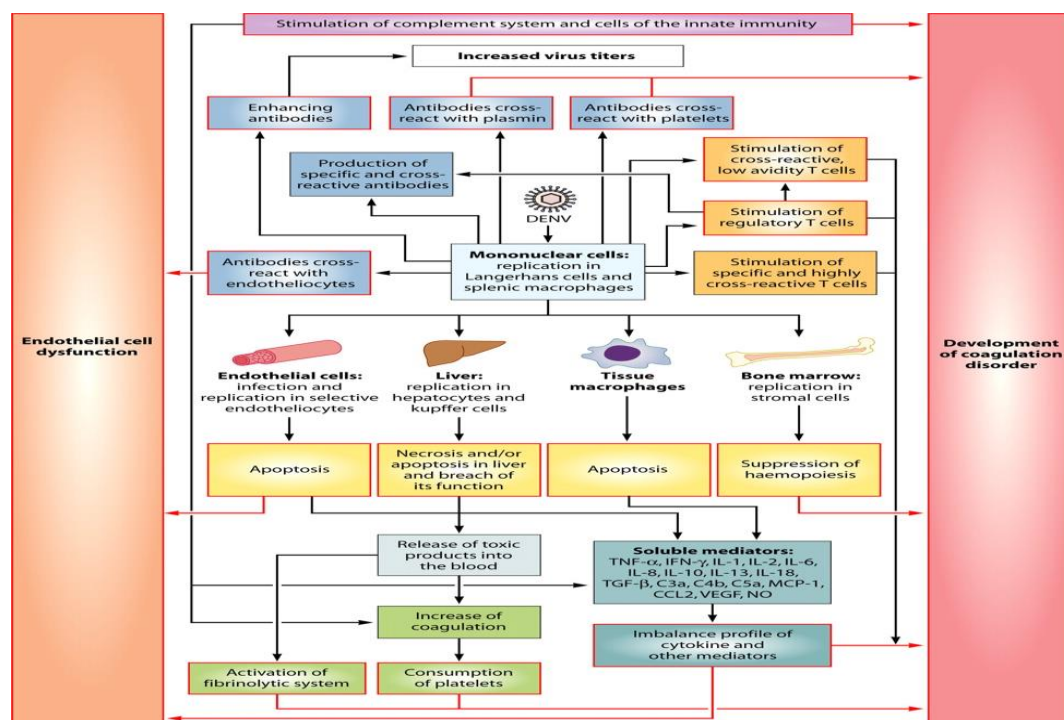
Figure 4 Proposed model for D.F., DHF and DSS pathogenesis (retrieved from Martina et al. (23))

Plasma leakage is a consequence of endothelial dysfunction and coagulation defect (Figure 4). Capillary endothelium is an important pathological location of the dengue virus. The endothelial cells can be infected and undergo apoptosis or can be impaired by nonspecific cross-reaction of antibodies. Moreover, the cells can be induced by an imbalanced profile of proinflammatory cytokines creating endothelial dysfunction. Coagulation defect is a combined consequence of multiple causes such as excessive complement stimulation, impaired plasmin and platelets, suppression of hematopoiesis, imbalanced cytokines, and release of toxic substances from liver necrosis (23).

1.3. Clinical presentation

Most dengue virus infections are asymptomatic or mild, called undifferentiated febrile illness (UFI). However, 1-2% of infections develop plasma leakage, hemorrhage, and shock (2). Noted that plasma leakage is often seen in children, whereas thrombocytopenia (low platelets) is more common in adults (24, 25). About 0.1% of

cases develop to mortality, and the major cause of death are shock, severe bleeding, liver failure, renal failure, and unconsciousness (5, 6, 26, 27).

The clinical presentation is characterized into three phases as follows; febrile, critical, and recovery (Figure 5). The febrile phase is described mainly by high fever (>38.5 oC) for 2-7 days. High viral load and severe clinical manifestation are often related to progression to shock and bleeding in a critical phase. Other symptoms in the febrile phase are fever, myalgia, headache, which are usually found in most viral infections. Evidently, a decrease in body temperature and platelets suggest that the patients enter the critical phase. This phase lasts 24-48 hours. Since shock, bleeding, and multiple organ failure can occur in this phase, the patients should be closely monitored. The recovery phase is a reverse of clinical and laboratory profiles by fluid resorption, platelet and hematocrit recovery, and detection of specific IgM/IgG. Fluid overload is a common complication of this phase causing circulatory failure and pleural effusion (5, 26).
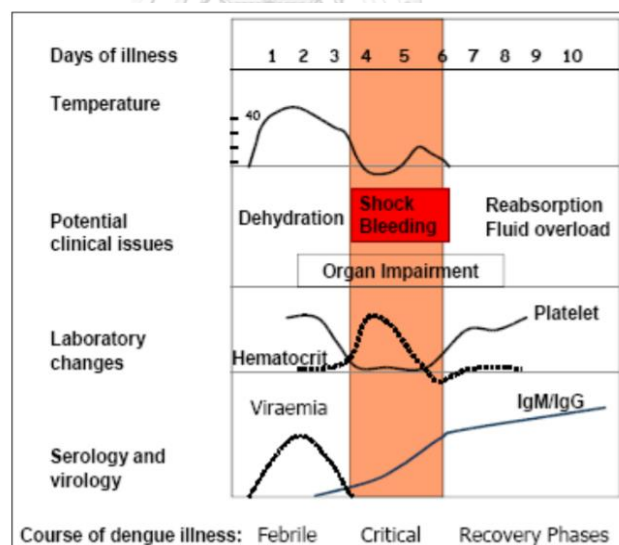
Figure 5 The scheme of dengue disease (retrieved from WHO et al. (26))

1.4. Treatment

Currently, no specific treatment is available for dengue patients. Supportive care still plays an important role as a primary treatment for severe patients, such as providing fluid substitution in patients with dehydration. The appropriate management of this

disease depends on early recognition of warning signs and continuous monitoring of the patients (1, 5, 28).

2. Extracorporeal blood purification therapies

Severe dengue manifestation is caused by the immunopathological responses increasing the viral titer, leading to endothelial cell dysfunction impairing capillary permeability and life-threatening plasma leakage. Selective plasma exchange is an extracorporeal treatment that removes targeted abnormal substances from the patient's circulation and replaces the lost volume with supplementary fluid (Figure 6) (29). Selective plasmapheresis techniques include the following three modalities. First, the plasma is separated of the whole blood by centrifugation or membrane filtration. This volume is replaced by saline, albumin, or fresh frozen plasma. However, replacement fluid, such as albumin, could be expensive and may cause allergic reactions. Moreover, fresh frozen plasma could cause an allergic reaction, mismatch reactions, transfusion-related acute lung injury (TRALI), transfusion-associated circulatory overload (TACO), etc. (30)
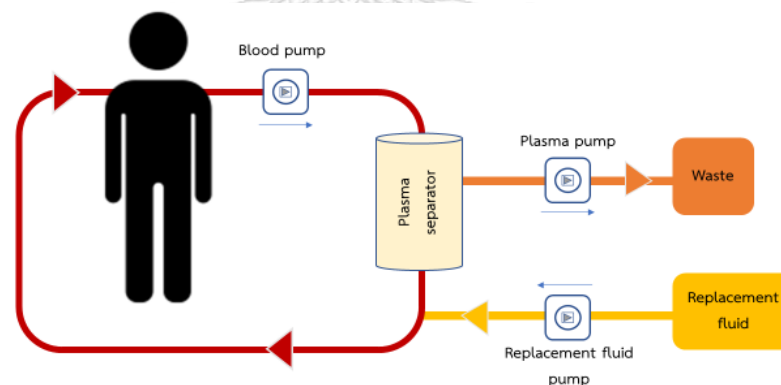
Figure 6 Plasma exchange

The second modality is an adsorbent column called "coupled plasma filtration adsorption" (CPFA) running in a serial circuit after the plasma filtration or centrifugal separation (Figure 7). The plasma is circulated through sorbent columns and then returned to blood within the extracorporeal circuit, where it passes through a hemodialyzer before returning to the patient. CPFA allows regeneration of plasma,

hence avoiding the complications of plasma volume replacement fluids that may occur in plasma exchange (31).
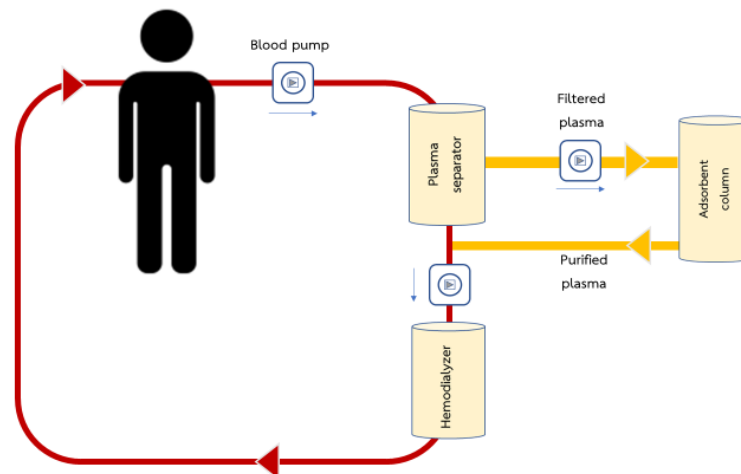
Figure 7 Coupled plasma filtration adsorption

Third, a coupled plasma filtration immunoadsorption (CPFIA) or immunoadsorption (I.A.) is a CPFA technique with an additional feature. The sorbent is an immobilized antigen or specific monoclonal antibody attached to beads by covalent linkage (31). The immunoadsorption (I.A.) device removes immunoglobulins by binding them to select ligands, including staphylococcal or recombinant protein A, sheep polyclonal anti-human antibodies, tryptophan, synthetic oligopeptides, monoclonal camel antibody fragments on the supporting matrix membranes or beads. Most I.A. columns are capable of retaining all major immunoglobulin subclasses. Applications of the columns include allotransplantation and xenotransplantation (ABO blood group and related antigens, natural antibodies and transplantation; Immunobiological barriers to xenotransplantation) (32). In recent years, various selective plasma separations and I.A. becomes more prevalent in reducing the circulating autoantibodies (33, 34). There are examples of commercial immunoadsorption devices in extracorporeal therapies, as shown in **Table 1**.

Table 1 A commercial immunoadsorption device in extracorporeal therapies

Type of immunoadsorption	Trade name	Company	Adsorber column		Application	Reference
			Ligand	Matrix		
Protein-A immunoadsorption	Prosorba®	Cypress Bioscience, USA	Protein-A	Silica	Remove all subclasses of human IgG (except subclass 3) by binding to the Fc portion of IgG to treat autoimmune diseases.	Felson et al. (1999) (35)
	Immunosorba®	Fresenius, Germany		Agarose beads (Sepharose)		Kuhn et al. (2006) (36)
Anti-IgG immunoadsorption	TheraSorb® – Ig adsorbers	Miltenyi Biotec, Germany	specific antihuman-Ig sheep antibodies	Sepharose CL 4B	Remove human IgG, IgA, IgM, IgE, and immune complexes to treat autoimmune diseases	Julius et al. (2009) (37)
Peptide immunoadsorption	Globaffin® adsorber	Affina, Germany	Peptide goat anti-mouse (GAM®)	Sepharose CL 4B	Remove human Ig to treat rheumatoid arthritis, SLE, and acute renal transplant rejection	Rönspeck et al. (2003) (38)
	Coraffin® adsorber		Peptide mimicking beta1-adrenergic receptor		Remove autoantibodies from idiopathic dilated cardiomyopathy patients	Rönspeck et al. (2003) (38)
Lectin immunoadsorption	Hemopurifier®	Aethlon Medical, USA	*Galanthus nivalis* lectin	Chromasorb resin	Remove broad-spectrum of viral pathogens such as MERS, MARV, Ebola virus and soluble glycoprotein	Buttner et al. (2014)(10) Koch et al. (2018)(11)
Antibody immunoadsorption	-	Patent in China	gp120 antibody	glass microsphere	Remove human immunodeficiency virus	CN102631 891A (2012)

Prosorba® and Immunosorba® are examples of immunoadsorption constructed from *Staphylococcus aureus* protein A (42 kDa) immobilized on silica or agarose bead, respectively (35, 36, 39). Both columns have been reported to effectively treat autoimmune neurological diseases, rheumatologic diseases, and hematological diseases by adsorbing free immunoglobulins and immune complexes by binding to the Fc portion to a maximum of 30–50% (40). The blood flow rate reported in the

Immunosorba® device is 50 ml/min. The Prosorba column is designed for a single-use only.

Moreover, a new technology called "regenerative double-column system" is claimed to increase the elimination capacity by reducing up to 75–90% of circulating immunoglobulins within a 2.5 total volume session. Examples of this column system are Immunosorba®, TheraSorb® – Ig adsorbers (Miltenyi Biotec, Germany), and Globaffin® (Affina, Germany). All columns are designed for multiple runs within the same in a single patient (40). The adsorber Coraffin® is the first adsorber for the specific removal of β1-adrenergic autoantibodies (β1-AAB) in idiopathic dilated cardiomyopathy (DCM), whereas Globaffin® is an adsorber for the removal of Ig in antibody-mediated disorders (38).

The idea to remove the circulating virus has been discussed. Many devices have been designed and tested on HIV, Ebolavirus, Marburg virus, and dengue virus. The first design was originated for HIV treatment. In the patent CN102631891A, the human immunodeficiency virus (HIV) affinity adsorption column has an affinity to a main receptor CD4 molecule (gp120 antibody). The affinity microsphere is a glass microsphere with a size of 1 mm, crosslinked to chitosan with a diameter of more than 500 microns. This invention is claimed to eliminate circulating HIV in the bloodstream and relieving the immunodeficiency syndrome of HIV patients.

The more recent technology was the *Galanthus nivalis* lectin affinity column, capable of adsorbing heavily glycosylated virions such as MERS, Marburg, and Ebola virus. Many commercialized products utilizing lectin have been launched. For example, chromasorb® is a lectin-binding resin column running at a flow rate of 1 ml/min and a Hemopurifier® claimed a reduction of 80% and 70% of MERS-CoV (pseudovirus) and MARV-soluble glycoproteins, respectively, within 3 hours *in vitro* (11). This lectin-affinity column is constructed into a wafer-like pattern that allows the whole blood to run through the system without a plasma filter or separator requirement. Examples of nonselective adsorption in extracorporeal therapies were show in the Table 2.

Table 2 Nonselective adsorption in extracorporeal therapies

Sorbent	Application	Target	Reference
Charcoal	Poisoning, chronic renal failure	Small toxic substances	Ronco et al. (2000) (41)
Macroporous resin	To modulate the immune response and prevent multiorgan dysfunction syndrome	TNF-α, Interleukins, IFN-γ (Size selectivity <55 kDa)	Khan (2016) (12)

In addition to specific binders, there are nonspecific binders currently available. The most popular sorbent is charcoal used for trapping toxic substances, especially for low molecular weight wastes accumulating in kidney or liver failure (41).

Moreover, a novel sorbent in a nonselective category is a macroporous resin used to adsorb 10-50 kDa proinflammatory cytokines such as TNF-alpha, interleukins, and IFN-gamma. This size-selective removal is critical in the treatment of cytokine storms of any cause. The objective is to prolong the clinical course and prevent multiorgan dysfunction (12). A commercially available cytokine absorber (CytoSorb®) targeting inflammatory cytokines (Figure 8) were used as an extracorporeal cytokine adsorption device to treat a severe dengue case with multiple organ dysfunction. The patient was administered with three cycles of plasma exchange along with renal replacement therapy on the 2nd, 4th, and 6th days after admission. The duration of each session was six hours, with a blood flow rate of 250 ml/min. The clinical improved as the patient was shifted out of intensive care unit on the 13th day after admission, and was subsequently discharged.
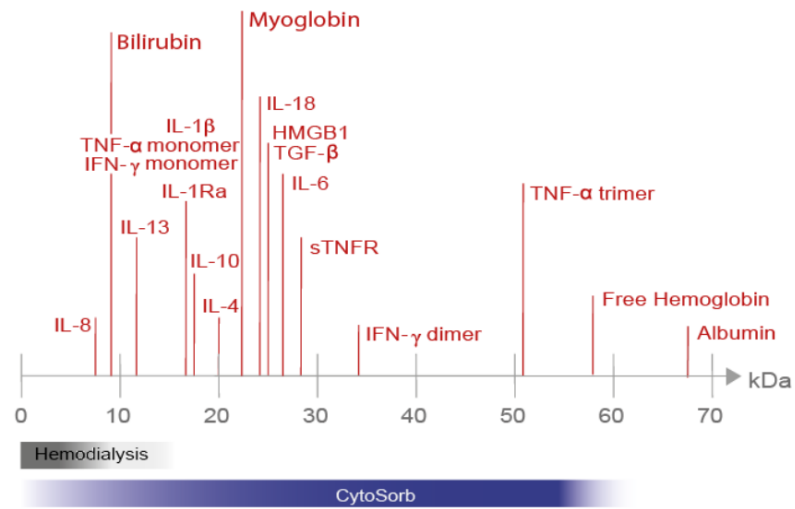
Figure 8 Spectrum of adsorption in CytoSorb®

Available from Internet, https://cytosorb-therapy.com/the-adsorber/,

accessed 21 April 2019.

CHAPTER 3

METHODS

1. Cell cultures

    1.1. D1-4G2-4-15 (ATCC®HB-112™)

        1.1.1. Cell culture D1-4G2-4-15 (ATCC®HB-112™) in Hybri-Care medium (ATCC® 46-X™) supplemented with 20% fetal bovine serum

The D1-4G2-4-15 cells were quickly thawed at 37 °C in a water bath and transferred to a 6-well plate containing Hybri-Care medium supplemented with 20% fetal bovine serum, 4 ml/well. The starting number was more than 5×10^5 cells/ml in 1 well. Then, cells were incubated at 37°C under 5% carbon dioxide (CO_2) for 3-5 days. The culture media was replaced continuously for at least 3-5 passage until the cell entered an exponential phase. Cells were harvested and counted to more than 2×10^5 cells/ml in 1 well of 6-well plate for producing monoclonal antibodies.

        1.1.2. Cell proliferation D1-4G2-4-15 (ATCC®HB-112™) in Hybri-Care medium (ATCC® 46-X™) supplemented with 20% fetal bovine serum

Cells from subsection 1.1.1 were placed in a T75 cell culture flask and incubated at 37°C under 5% carbon dioxide (CO_2) for 3 - 5 days until reaching the amount of 1×10^6 cells/ml verified by a Neubauer hemocytometer.

        1.1.3. Cell culture D1-4G2-4-15 (ATCC®HB-112™) in serum-free media with Hybridoma-SFM medium (Gibco, Catalog No. 12045084)

Cells were cultured in the Hybri-Care medium supplemented with 20 % fetal bovine serum until 1×10^6 cells/ml was reached. Cells were washed twice with the 10 ml Hybridoma-SFM medium and centrifugation at 1,500 rpm for 5 minutes. Then, at least 1×10^6 cells/ml of the harvested cells were placed in the T75 cell culture flask containing 50 ml Hybridoma-SFM medium and incubated at 37°C under 5% carbon dioxide (CO_2) until 50-80% of dead cells were reached. The supernatant was harvested by centrifugation at 1,500 rpm for 5 minutes, aliquoted, and collected at -20°C.

1.2. C6/36 cells

C6/36 cells, a continuous cell line derived from a larva of *Aedes albopictus* (ATCC®CRL-1660), were maintained in minimal essential medium (MEM) (Gibco, USA) medium supplemented with 10% FBS at 28°C.

1.3. LLC/MK2 cells

LLC/MK2 cells (ATCC®CCL-7), derived from renal epithelial cells of the adult rhesus monkey, were maintained in minimal essential medium (MEM) (Gibco, USA) supplemented with 10% FBS at 37°C under 5% carbon dioxide (CO_2).

1.4. R.D. cells

R.D. cells (ATCC®CRL-1573™), derived from human rhabdomyosarcoma cells, were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% FBS at 37°C under 5% carbon dioxide (CO_2).

2. Monoclonal antibody production

2.1. Purification of mouse monoclonal IgG_{2a} on HiTrap™ Protein A HP

The monoclonal antibody was purified from a D1-4G2-4-15 (ATCC®HB-112™) hybridoma cell culture supernatant following the manufacturer's protocols 71-7002-00 A.R. (HiTrap™, USA). Briefly, prepared collection tubes by adding 100 µl of 1 M Tris-HCl, pH 9.0 per ml of a fraction to be collected. Then, filled the syringe with binding buffer (1X PBS, pH 7.4) and connected the column using a syringe fitted to the luer connector. Washed the column with 10 column volumes of binding buffer at 5 ml/min. Applied the cell culture supernatant at 5 ml/min and washed with 10 column volumes of binding buffer. Lastly, eluted with 4 column volumes of elution buffer (0.1 M glycine-HCl, pH 2.7) and flow-dialyzed against phosphate-buffered saline (1X PBS, pH 7.4).

2.2. Validation of the monoclonal antibody purity by SDS-PAGE and Western blot assay

2.2.1. SDS-PAGE

The 10 µl sample from the subsection 2.1 was mixed with the 10 µl SDS loading buffer (2x Laemmli Sample Buffer, BIO-RAD, USA) and heated at 95°C for 5 minutes before loading onto a 10%SDS-polyacrylamide. The electrophoresis was run using Mini-

PROTEAN® Tetra system (BIO-RAD, USA) in the 1 x Tris/Glycine/SDS running buffer at 150 V. Then, the gel was stained for 30 min with gentle agitation and destained gel in destaining solution until the background was fully destained.

2.2.2. Western blot

The nitrocellulose membrane (BIO-RAD, USA) was presoaked in a blotting buffer for 5 minutes. The proteins in the SDS-PAGE gel were transferred to the presoaked membrane using a TRANS-BLOT® SD SEMI-DRY TRANSFER CELL (BIO-RAD, USA) at 16 V for 30 minutes. The membrane was blocked overnight with 1% bovine serum albumin (BSA; Sigma, USA) in 0.05% Tween-Tris-buffered saline (TTBS). The membrane was washed three times with TTBS and incubated for 1 hour with 1:5,000 HRP Goat anti-mouse IgG antibody (BioLegend, USA) diluted in 1% BSA in TTBS. The membrane was washed 3 times with TTBS. The target band was developed using Immobilon® Classico Western HRP substrate (Merck, USA) and signals were detected using ChemiDoc XRS+ Gel Imaging system (BIO-RAD, USA).

2.3. Quantification of the amount of monoclonal antibody by Bradford protein assay

The monoclonal antibody was quantified using Quick Start™ Bradford Protein Assay (BIO-RAD, USA) and prepared to 1 mg/ml concentration.

3. Covalently coupled monoclonal antibody with silica

3.1. Functionalized-silica (SiO_2-R-NH_2)

The 250 mg commercial-silica was dispersed in the 100 ml of 2-propanol before a dropwise addition of (3-Aminopropyl) triethoxysilane (APTES) to 2 ml. The mixture was continuously stirred for 24 h expecting for the SiO_2-R-NH_2 product. The functionalized-silica was washed twice with 30 ml 2-propanol, followed by twice washing with 30 ml dichloromethane.

3.2. Amide coupling, Silica coated with bovine serum albumin (BSA) or 4G2 antibodies (SiO_2-R-NHCO-BSA, SiO_2-R-NHCO-4G2Ab)

Amide coupling reactions of the functionalized-silica (SiO_2-R-NH_2) with a fraction of amine groups of APTES was prepared by mixing the functionalized-silica (SiO_2-R-NH_2)

(0.006 g) with 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (200 mM, 1 ml) and N-Hydroxysuccinimide (NHS) (500 mM, 1 ml). The solution was dissolved in phosphate-buffered saline (PBS, pH 6). The BSA or 4G2Ab was prepared by dissolving 1 mg into 1 ml PBS at room temperature under nitrogen atmosphere. The mixture was continuously stirred for 24 h. to got. The expected silica coated with particles SiO_2-R-NHCO-BSA, or SiO_2-R-NHCO-4G2Ab was washed with three times with 30 ml deionized water.

3.3. Validation of the covalent linkage by chemical method

A covalent linkage association between the monoclonal antibody and the functionalized silica was characterized using Fourier-transform infrared spectroscopy (NICOLET 6700 FT-IR).

4. Immobilization of the monoclonal antibody in alginate bead by entrapment technique

4.1. Preparation of alginate-monoclonal antibody mixture

Dried sodium alginate at 70°C for 48 h was used to prepare a 3% (w/v) sodium alginate in distilled water by pulsed blending until the mixture was smooth. The monoclonal antibody was added to the mixture and mixed thoroughly to a final mixture of 2% (w/v) sodium alginate. This mixture was stored at 4°C until use.

4.2. Preparation of monoclonal antibody integrated to alginate bead

The mixture from the subsection 4.1. was loaded into a 30 Gauge syringe and injected slowly into 1% (w/v) calcium chloride solution to uniform beads with the size of 2 mm in diameter. The nascent alginate beads were rested in the solution for 1 min to set before harvesting. The alginate beads were washed three times with distilled water and were stored at 4°C until use.
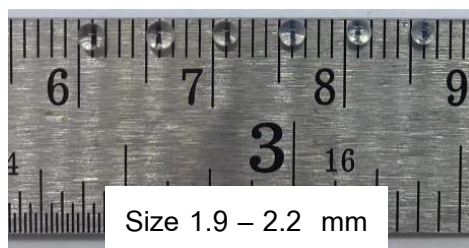
Figure 9 The alginate beads

5. Virus stock preparation

5.1. Dengue virus

C6/36 cells preseeded in T25 flasks were infected with DENV2 (New Guinea C, NGC) at the M.O.I of 0.01 by continuous rocking at room temperature for 1 h. Next, maintenance medium (Medium MEM supplemented with 1% FBS) was added into the flask and incubated at 28°C until more than 75% of the cells showed cytopathic effect. The supernatants were centrifuged at 1,500 rpm, 4°C for 5 minutes. The supernatant was aliquot and stored at -80°C until use. The titer of the DENV2 stock was determined by plaque titration assay.

5.2. Enterovirus A71

R.D. cell monolayer were infected with EV-A71 (strain BRCA) at the M.O.I. of 0.1 at 37°C with gentle rocking every 15 minutes for 1 h. Next, maintenance medium was added and incubated at 37°C until more than 75% of the cells showed cytopathic effects. Then, the culture flask underwent three rounds of freezing at -80°C for 30 minutes and thawing at 37°C in a water bath. The supernatant was collected and centrifuged to remove cell debris at 2,000 rpm, 4°C for 10 minutes. The supernatant was aliquot and stored at -80°C until use. The titer of EV-A71 stock was determined by plaque titration assay.

6. Plaque titration assay

A 96-well plaque titration assay determined the DENV supernatant or DENV virus stock on LLC/MK2 cells (42). Briefly, the supernatant or virus stock was 10-fold serially diluted in the maintenance medium and sampled out for 50 µl to mix with 50 µl of 2×10^5 LLC / MK2 cells/ml. The experiment was performed in quadruplicate. The maintenance medium was used as a mock infection. The plate was incubated at 37°C under 5% carbon dioxide (CO_2) for 3 h before adding 100 µl 0.8% gum tragacanth (Sigma Aldrich, USA) semisolid medium. The plate was incubated at 37°C under 5% CO_2 until the plaques were observed under light microscope. Cells were fixed and stained with 1% crystal violet in 10% formaldehyde. The formula calculated the number of plaque forming units (PFU/ml).

The EV-A71 supernatant or EV-A71 virus stock was determined by 96-well plaque titration assay on R.D. cells similar to that of the DENV2. Briefly, the supernatant or virus stock was 10-fold serially diluted in the maintenance medium, and 50 µl of each dilution were sampled out to mix with the 50 µl of 5×10^5 R.D. cells/ml. The experiment was done in quadruplicate and the maintenance medium was used as a mock infection. The mixture of R.D.-virus was incubated at $37°C$ under 5% carbon dioxide (CO_2) for 3 h. Then, the 100 µl of 1.2% gum tragacanth semisolid medium was added to the plate. The plate was incubated for 2 days or until the plaques were observed under microscope. Cells were fixed and stained with 1% crystal violet in 10% formaldehyde. The formula calculated the number of plaque forming units (PFU/ml).

PFU/ml = plaque no. per dilution x dilution factor x 20

7. Efficiency of dengue virus adsorption test

Before staring the experiment, the alginate beads incorporated with the 1 mg/ml 4G2 monoclonal antibody were brought out of $4°C$ storage to room temperature, and the 2.5 ml MEM medium or fetal bovine serum was premixed with the DENV2 stock (1.7×10^{11} PFU/ml). The alginate beads (300 beads/well) were transferred to each well of a 6-well plate and incubated with 2.5 ml DENV2-spiked MEM medium or fetal bovine serum for 3 h at $25°C$ with a continuous rocking. The DENV2-spiked MEM medium or fetal bovine serum before and after the incubation was sampled for quantification by RT-qPCR and plaque titration.

Similarly, the experiment was done under a simulated circuit using 50 ml of DENV-spiked MEM medium or fetal bovine serum in the presence of 4G2-integrated alginate beads (1000 beads) prepacked into a 5 ml syringe for 3 h (Figure 10). The alginate beads (1000 beads) without 4G2-integration was used as a control. The DENV2-spiked MEM medium or fetal bovine serum before and after the simulated run were sampled for quantification by RT-qPCR and plaque titration. Data was calculated as a percent viral load reduction before and after the simulated run.

8. Viral RNA extraction and reverse transcription qPCR

According to the manufacturer's protocol, the supernatant was extracted for viral RNA using QIAamp® Viral RNA Mini kit (Hilden, Germany). Total RNAs were stored at -70°C until used.

To determined the reduction of viral genome in experiments, total RNA was quantified by Nanodrop (Eppendorf Bio Photometer D30, New York, USA). The RT-qPCR were performed using 1x Power SYBR® Green RNA-to-CT™ 1-Step kit (Applied Biosystems™, California, USA), 400 nM C-protein primer with a Step-OnePlus Real-Time PCR System ABI 7500 (Applied Biosystems™, California, USA).

9. *Escherichia coli* DH5-Alpha adsorption specificity test

To performed the specificity test, the 4G2-integrated alginate beads (300 beads/well) were incubated with 2.5 ml of *Escherichia coli* DH5-Alpha -spiked L.B. broth adjusted to 0.5 McFarland standard (approximately 10^8 CFU/ml) in each well of a 6-well plate for 3 h by continuous rocking. The medium before and after the incubation was sampled out for quantification using a colony-forming units assay. Briefly, the 200 μl sampled broth was added into a 96-well plate for a 10-fold serial dilution to 10^{-7}. The sample from each dilution was spotted on L.B. agar plates at 10 μl/spot for 10 spots, and incubated at 37°C under 5% carbon dioxide (CO_2) for 24 h. The bacterial colonies were counted and calculated for a number of colony forming units (CFU/ml) using the formula.

CFU/ml = colony no. per dilution x dilution factor x 10

10. Enterovirus A71 (strain BRCA) adsorption specificity test

The EV-A71-spiked fetal bovine serum (1.14×10^7 PFU/ml) was run on a simulated circuit with the 4G2-integrated alginate beads column (1000 beads) for 3 h as previously described. In addition, the EV-A71-spiked fetal bovine serum before and after the simulated run was sampled for quantification by RT-qPCR and plaque titration. Data was calculated as a percent viral load reduction before and after the simulated run.

11. Description of a simulated circuit design

The simulated circuit was calculated to a 1:100 scale of the normal human total blood volume (five liters) and flow rate (100-120 ml/min). In brief, the total volume of 50 ml medium or fetal bovine serum was continuously run in the simulated circuit equipped with the 4G2-integrated alginate beads (1000 beads) packed in a 5 ml syringe (TERUMO®, Japan) at the rate of 1 ml/min for 3 h. A peristaltic pump controlled the circuit rate. The simulated circuit was designed and generated by Mr. Pat Sinananpat under supervision of Dr. Saran Salakij (Establishing a design process of components in *in vitro* antibody-linked resin plasma filtration system. Bachelor's Thesis, Department of Mechanical Engineering, Faculty of Engineering, Chulalongkorn University, 2020) using the CytoSorb® as a reference model (Figure 10).
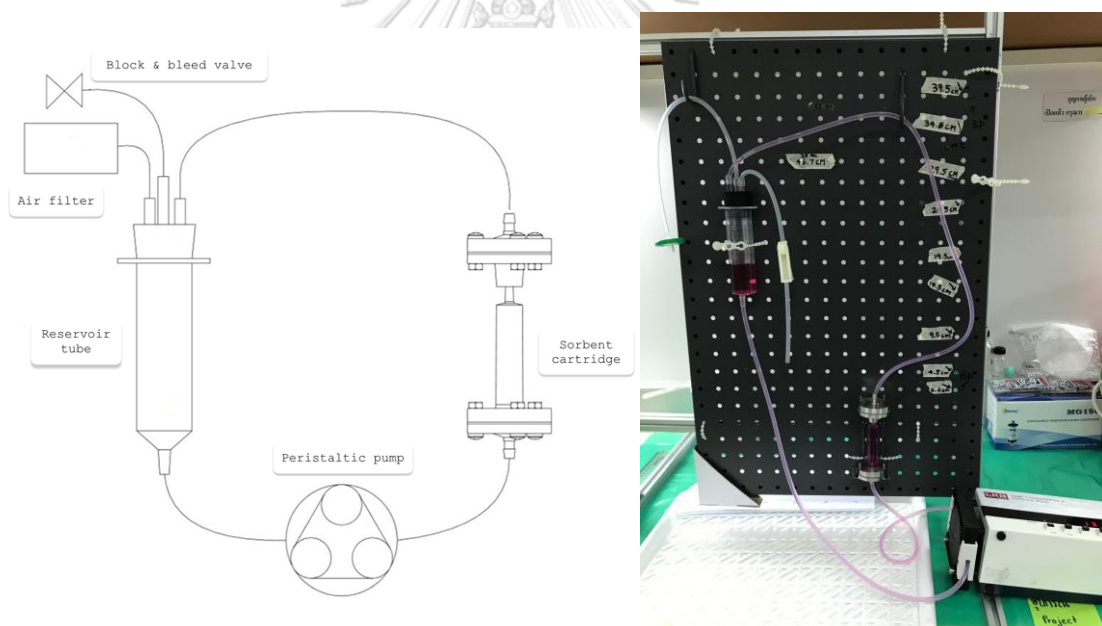
Figure 10 A simulated circuit design.

(a) Diagram and (b) actual workpieces.

CHAPTER 4

RESULTS

1. Monoclonal antibody production

The monoclonal antibody was produced and purified according to subsection 2.1. The SDS-PAGE of heavy and light chains was shown at ～50 and ～25 kDa (Figure 11). The 1 mg/ml monoclonal antibody was quantified by Bradford protein assay (BIO-RAD, USA).
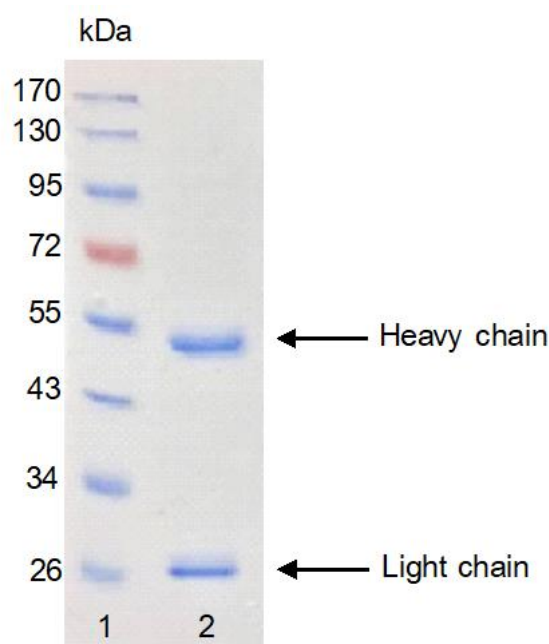
Figure 11 SDS-PAGE analysis of purified 4G2 monoclonal antibody.
Lane 1: molecular weight marker, Lane 2: purified monoclonal antibody. The monoclonal antibody was separated by SDS-PAGE and stained with Coomassie blue.

2. The optimization of a covalently coupled 4G2 monoclonal antibody with silica matrix

2.1. Immobilization of bovine serum albumin on functionalized silica matrix

The initial optimization was setup using a commercially available bovine serum albumin (BSA) as a representative protein. Chemical bonds or functional groups of SiO_2, SiO_2-NH_2 and SiO_2-NH-BSA were demonstrated by FT-IR spectroscopy (Figure 12). The spectrum of SiO_2 (Figure 12, (a)) showed a peak at 1630 cm^{-1} which was an O-H bending of H_2O molecules adsorbed by silica and a broad peak of O-H stretching at

$3300\text{-}3400 \text{ cm}^{-1}$ which could be assigned to the stretching vibrations of Si-OH groups in the structure of amorphous SiO_2. The successful functionalization was represented by the spectrum of functionalized silica $SiO_2\text{-}NH_2$ (Figure 12, (b)) with the two peaks at 2928 and 1562 cm^{-1} contributed by C-H stretching of sp^3 carbon on APTES silane, and N-H bending of amine group, respectively. Finally, three peaks were observed in the $SiO_2\text{-}NH\text{-}BSA$ spectrum (Figure 12, (c)) as follows. The two peaks corresponding to C-H stretching and N-H bending slightly shifted to 2963 and 1540 cm^{-1}, respectively. An additional peak at 1653 cm^{-1} corresponding to a C=O stretching characteristic indicates a successful formation of an amide bond ($1630\text{-}1690 \text{ cm}^{-1}$) between the BSA protein and the functionalized silica.
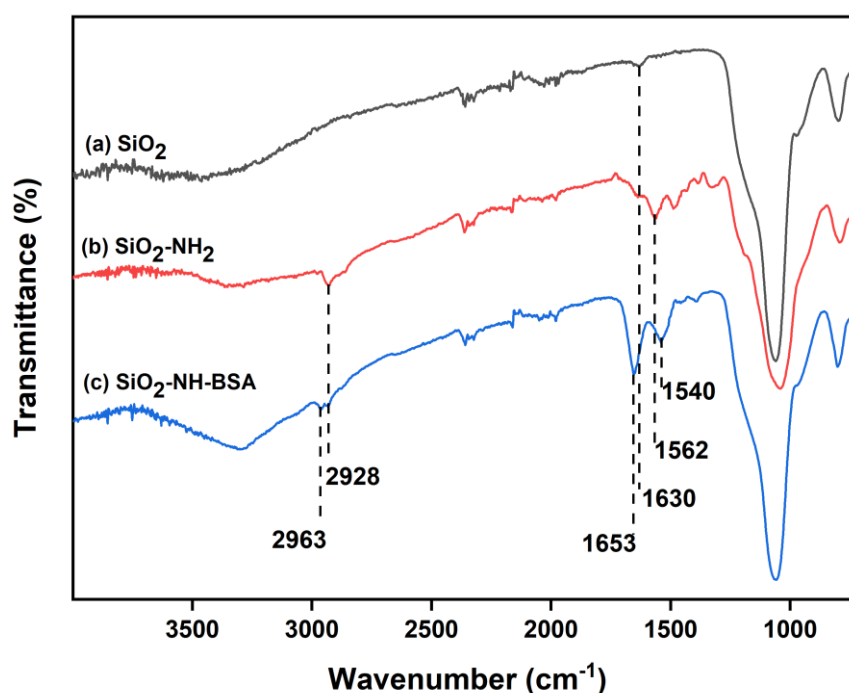
Figure 12 FT-IR spectra of SiO_2 (a), $SiO_2\text{-}NH_2$ (b) and $SiO_2\text{-}NH\text{-}BSA$ (c).

2.2. Immobilization of monoclonal antibody on functionalized silica matrix

The silica was functionalized and coupled with a 4G2 antibody under the BSA-optimized condition (Figure 13). However, the characteristic peak of C=O stretching of

amide group and C-H stretching of sp^3 carbon on APTES silane were not observed (Figure 13, (e)). The results indicated that the silane (i.e. the N.H. functional group) of SiO$_2$-NH-4G2Ab was detached from the particle. The reactions with and without cross-linker (EDC/NHS) (Figure 13, (c-d) in phosphate-buffered saline solution were performed to investigate which step is responsible for the silane fallout. Results showed that the fallout was observed early in the first step (shown in the schematic below) as the 1562 cm^{-1} peak was lost in all products (Figure 13, (c-e)). The other peaks corresponding to amide bond formation were also undetected, suggesting the failure of a covalent linkage formation of 4G2 antibody and the NH-functionalized silica. A similar result was observed in bovine immunoglobulin purified from fetal bovine serum (data not shown), indicating that the BSA-optimized condition was not suitable for globulin proteins.
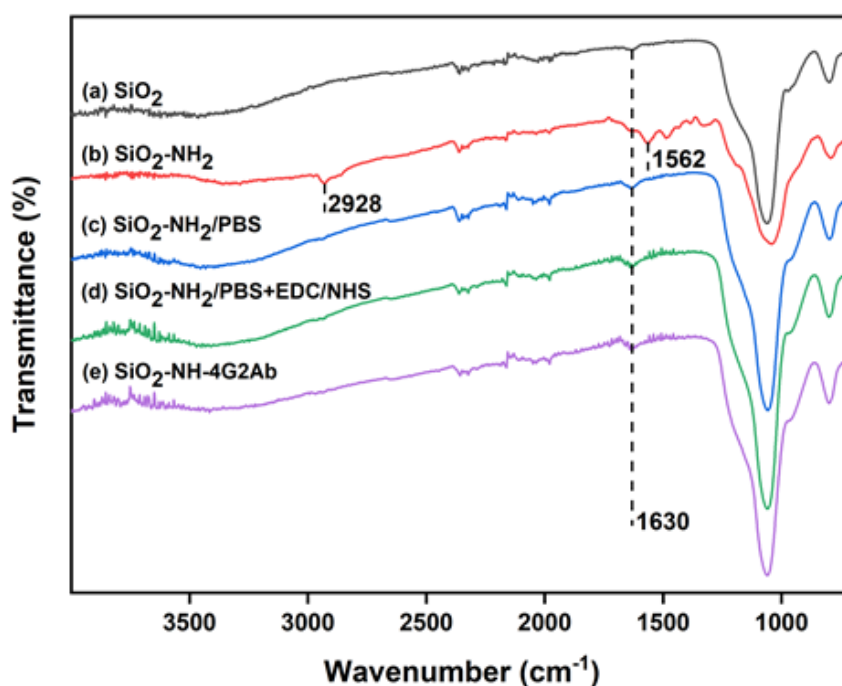
Figure 13 FT-IR spectra of SiO$_2$ (a), SiO$_2$-NH$_2$ (b), SiO$_2$-NH$_2$/PBS (c), SiO$_2$-NH$_2$/PBS+EDC/NHS (d) and SiO$_2$-NH-4G2Ab (e).

Table 3 The chemical bonds or functional groups were identified by FT-IR/ATR mode.

Wavenumber (cm^{-1})	Type of bond
1540	N-H bending
1562	N-H bending of amine group on APTES silane
1630	O-H bending of H$_2$O molecules
1653	C=O stretching of amide group
2928	C-H stretching of sp^3 carbon on APTES silane
2963	
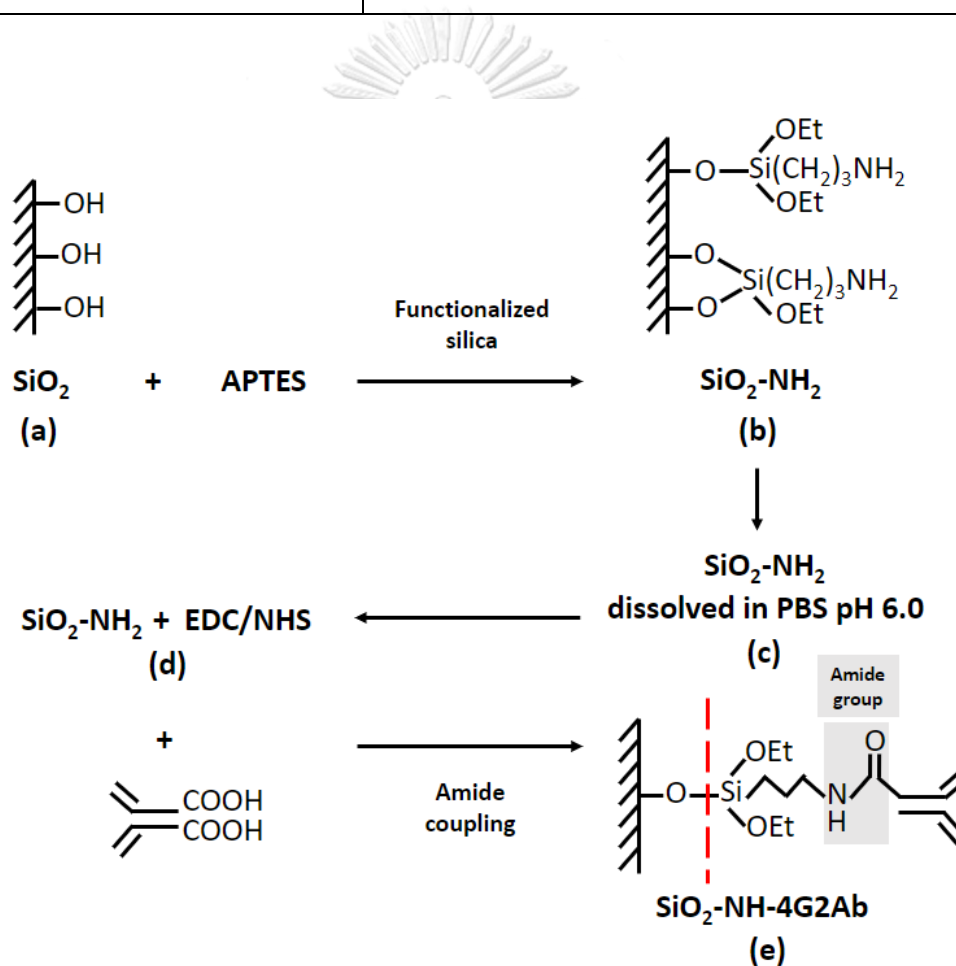
Schematic representation of the amide coupling reaction scheme for immobilization of the 4G2 antibody on functionalized silica.

(The red dotted line indicated the location where silane detached in SiO$_2$-NH-4G2Ab.)

3. Adsorption efficacy test of DENV-specific antibody integrated to alginate beads

3.1. Dengue virus adsorption efficiency test in 6-well plate

3.1.1. The DENV2-spiked 1% MEM incubated with alginate beads without monoclonal antibody

We initially examined the effect of the alginate beads to the initial DENV2 inoculum 1.7×10^{11} PFU/ml. The 2% alginate beads without monoclonal antibody was added into each well of the 6-well plate and incubated for 3 hours with continuously gentle rocking. The virus medium without a bead (blank) was used as a negative control. Supernatants collected after the run showed the viral titer reduction at 35 and 34 percents by RT-qPCR (Figure 14a) and plaque titration (Figure 14b), respectively. The blank control did not show viral titer reduction after 3 hours of incubation. This result indicated that the empty alginate beads was capable of nonspecific viral adsorption at 34-35 percent. Data represented the means and standard deviation of the technical quadruplicated results.
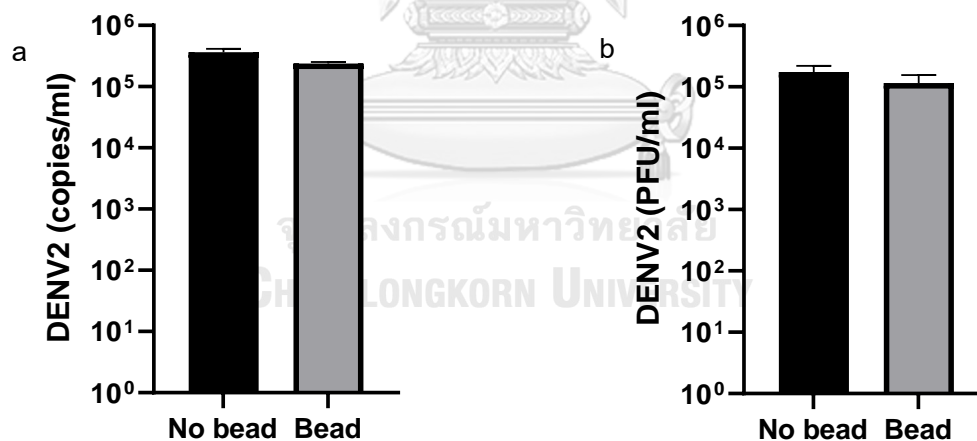
Figure 14 DENV2 after 3 hours incubated between blank (no bead) and empty alginate bead that quantified by RT-qPCR (a) and plaque titration assay (b).

3.1.2. The differential titers of DENV2-spiked 1% MEM incubated with alginate beads without monoclonal antibody

Next, the differential titers of DENV2-spiked 1% MEM medium were prepared using 10-fold serial dilution from the initial inoculum at 1.7×10^{11} to 17 PFU/ml. The serially diluted virus was incubated with and without empty alginate beads (300 bead/well). Results showed that three titration levels (1.7×10^{9} to 1.7×10^{11} PFU/ml) exhibited the cytopathic effect quantifiable by the plaque titration assay (Figure 15). In addition, results showed that the empty alginate beads was capable of adsorbing the virus nonspecifically at 50, 11, and 33 percents at 1.7×10^{9}, 1.7×10^{10}, and 1.7×10^{11} PFU/ml input, respectively. Data represented the means and standard deviation of the technical quadruplicated results. Noted that the highest titer (1.7×10^{11} PFU/ml) was at the most convenient for visualization and quantification; therefore, the highest titer was selected for further investigation.
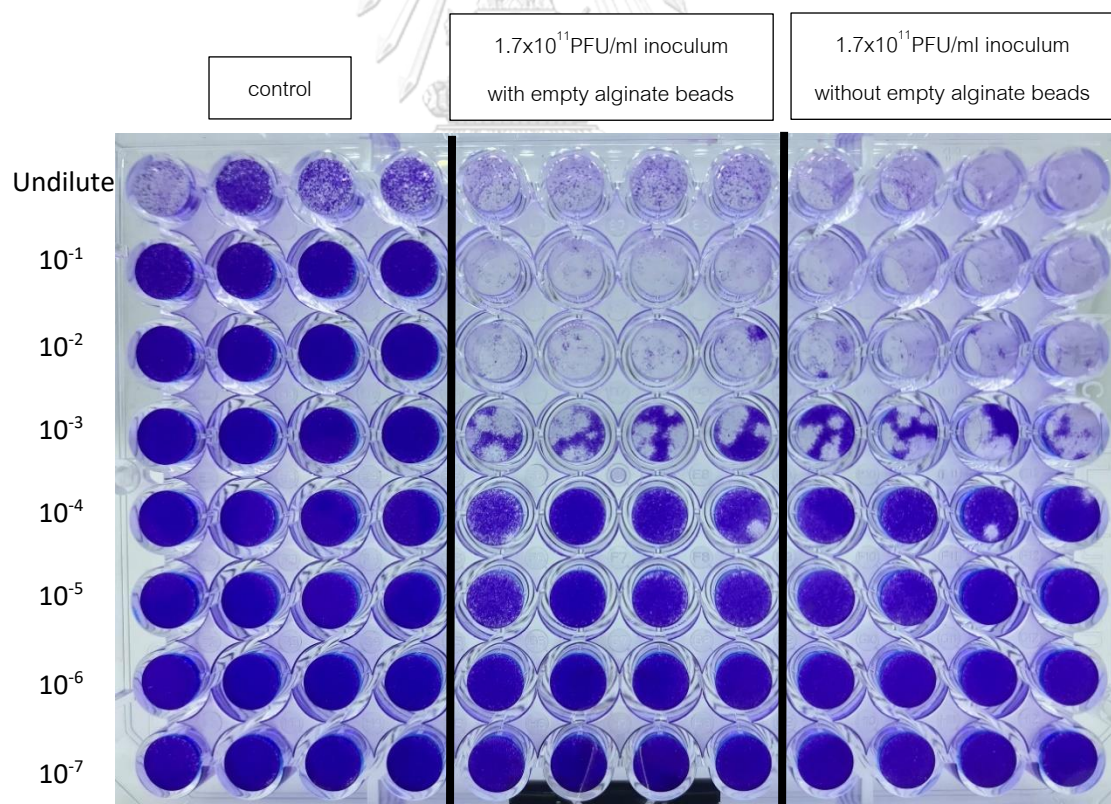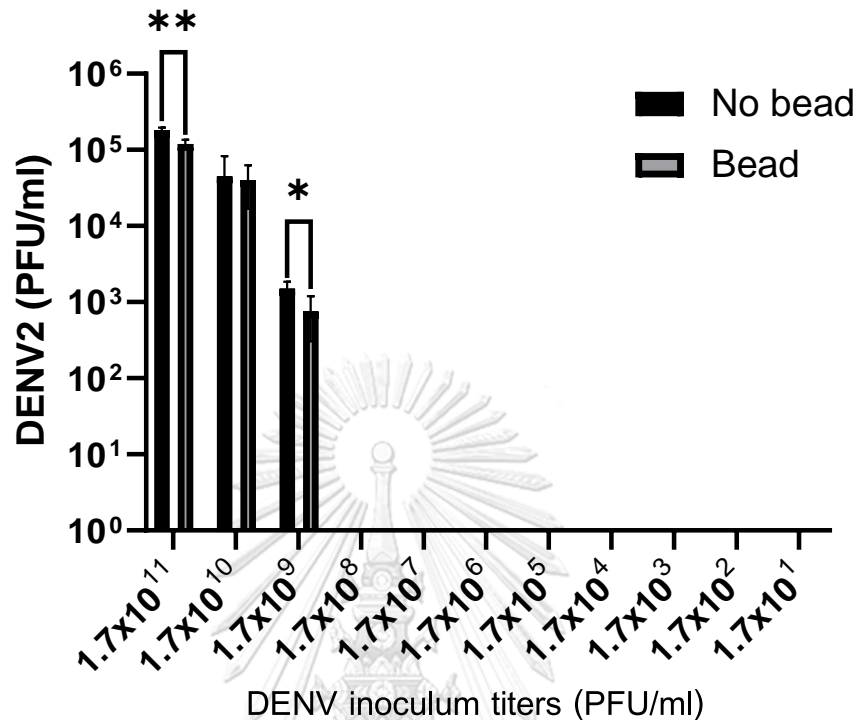
Figure 15 DENV2 after 3 hours incubated with the differential titers DENV inoculum optimization between blank (no bead) and empty alginate bead that quantified by plaque titration assay (shown in upper figure).

(*,** represented significant value at p-value < 0.1, 0.01, respectively.)

3.1.3. The DENV2-spiked 1% MEM incubated with alginate beads with monoclonal antibody

The 2% alginated beads (300 beads/well) with 1 mg/ml 4G2 monoclonal antibody were incubated with DENV2-spiked 1% MEM medium in each well of the 6-well plate for 3 h under continuous rocking. The 2% alginate beads (300 beads/well) without the monoclonal antibody and the virus medium without bead (blank) were used as negative controls. Samples were taken after the 3 h incubation for quantification by RT-qPCR and plaque titration (Figure 16). Results showed that the 4G2 alginate beads could significantly adsorb the virus at the percentage of 74, and 100 by RT-qPCR and

plaque titration. The empty alginate beads was capable of adsorbing the virus at the percentage of 50, and 24 by RT-qPCR and plaque titration, similar to the previous experiment. Data represented the means and standard deviation of the technical quadruplicated results. Noted that the limit of detection of plaque titration assay was at 100 PFU/ml.
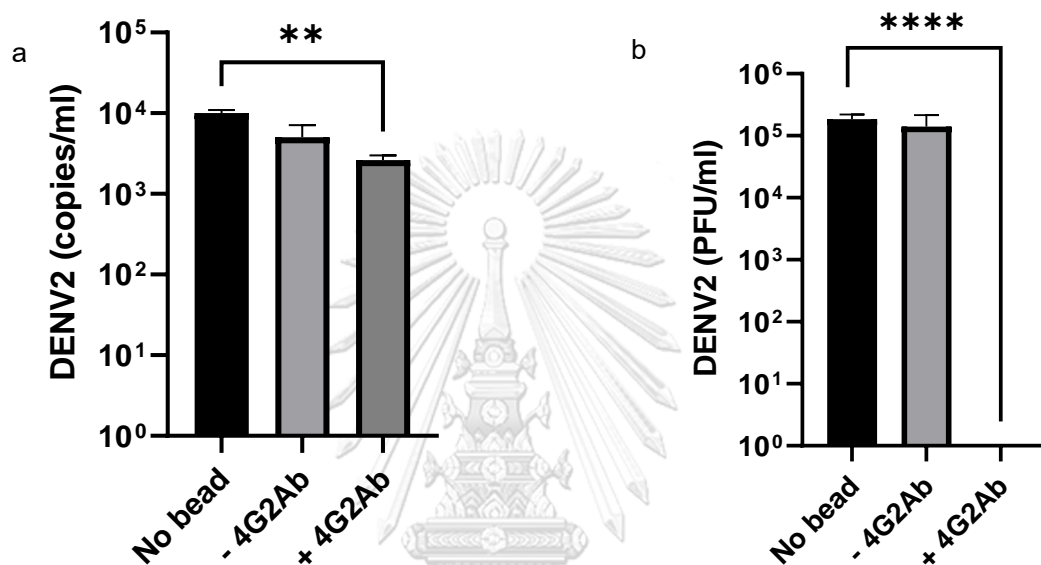
Figure 16 DENV2 after 3 hours incubated with blank (no bead), empty alginate bead, and 4G2 alginate bead that quantified by RT-qPCR (a) and plaque titration assay (b). (**,**** represented significant value at p-value < 0.01, 0.0001, respectively.)

3.1.4. The two concentrations of 4G2 on alginate beads with DENV2-spiked MEM medium.

Next, the two different concentrations of 4G2 (0.5, and 1 mg/ml) were prepared in the 2% alginated bead and tested against the DENV2-spiked MEM medium in 6-well plate format. The conditions without a bead and with the empty alginate beads were used as negative controls. The samples collected after run were quantified by RT-qPCR and plaque titration (Figure 17). Results showed that the 0.5, and 1 mg/ml 4G2 alginate beads were capable of adsorbing the virus at the percentage of 66, and 77 by RT-qPCR. As of the plaue titration, both 0.5 and 1 mg/ml 4G2 alginate beads were capable

of adsorbing the virus exceeded the detection limit (100 PFU/ml). The empty alginate beads were capable of adsorbing the virus at the percentage of 40, and 21 by RT-qPCR and plaque titration, similar to the previous experiment. Data represented the means and standard deviation of the technical quadruplicated results. Noted that the limit of detection of plaque titration assay was at 100 PFU/ml.
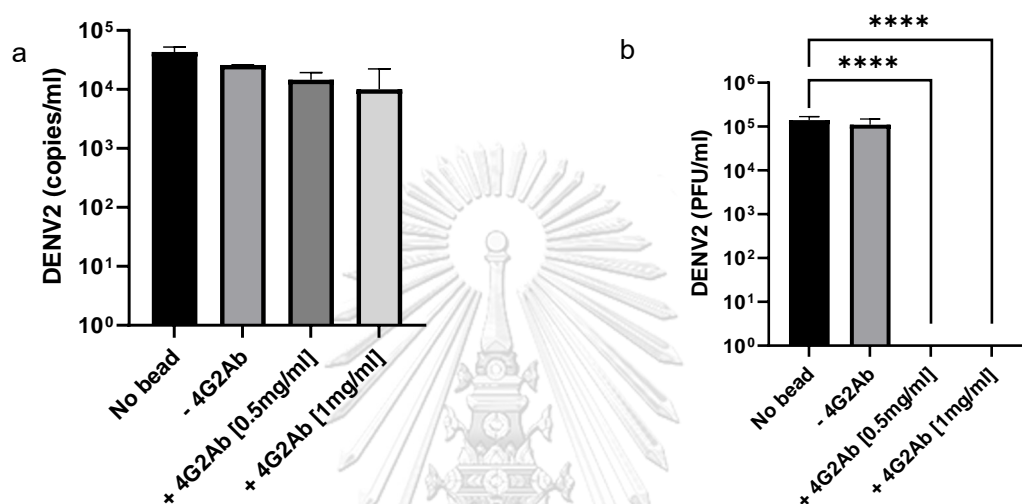
Figure 17 DENV2 after 3 hours incubated with blank (no bead), empty alginate bead, and 4G2 alginate bead (concentration at 0.5 mg/ml, 1 mg/ml) that quantified by RT-qPCR (a) and plaque titration assay (b).

(**** represented significant value at p-value < 0.0001.)

3.1.5. The fetal bovine serum-based incubation of alginate beads with 4G2 monoclonal antibody and DENV2 virus.

In this experiment, fetal bovine serum was used as a based medium because its biological properties were more similar to human plasma when compared with the MEM medium previously used. The DENV2 virus (1.7×10^{11} PFU/ml) was spiked into the fetal bovine serum prior to the experiment in a 6-well plate. The conditions without a bead, and with the empty alginate beads were used as negative controls. The samples collected after run were quantified by RT-qPCR and plaque titration (Figure 18). Results showed that the 1 mg/ml 4G2 alginate beads were capable of adsorbing the virus at the

percentage of 88 by plaque titration, but not by RT-qPCR as the adsorbing percentage was at 31. The empty alginate beads was capable of adsorbing the virus at the percentage of 38, and 28 by RT-qPCR and plaque titration, similar to the previous experiment. Data represented the means and standard deviation of the technical quadruplicated results.
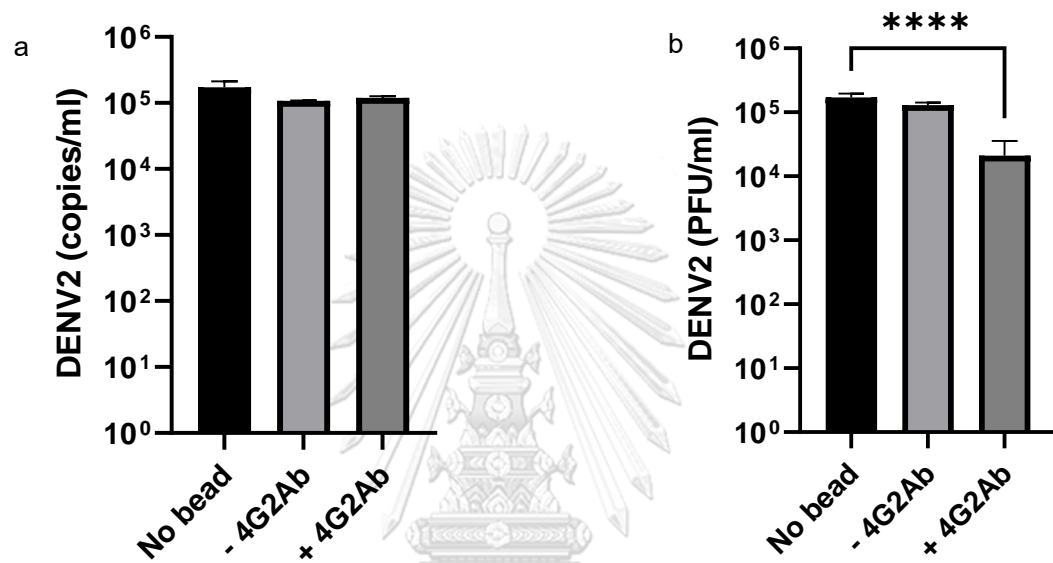
Figure 18 DENV2 after 3 hours incubated with blank (no bead), empty alginate bead, and 4G2 alginate bead (concentration at 1 mg/ml) that quantified by RT-qPCR (a) and plaque titration assay (b).

(**** represented significant value at p-value < 0.0001.)

3.2. The adsorption efficiency in a simulated circuit

The simulated circuit is equipped with the syringe containing the bead, peristaltic pump, stop valve for sampling the circulating liquid, etc. as shown in the Figure 10. The DENV2 virus (6×10^7 PFU/ml) was spiked into the 50 ml medium or fetal bovine serum before loading into the circuit. The 2% alginate beads with or without 4G2 (1000 beads/run) were prepacked in the 5 ml syringe before running on a simulated circuit with a continuous flow rate of 1 ml/min controlled by the peristaltic pump. The system was run for 3 h at ambient temperature and samples were taken for viral quantification by RT-qPCR and plaque titration. The 2% alginate beads without 4G2 was used as negative controls (Figure 19).

The results with MEM medium showed that 4G2 alginate bead was capable of adsorbing the virus significantly at the percentage of 78, and 100 by RT-qPCR and plaque titration. The empty alginate beads was capable of adsorbing the virus at the percentage of 53, and 61 by RT-qPCR and plaque titration, significantly superior to the previous 6-well plate experiments. Data represented the means and standard deviation of the technical quadruplicated results. Noted that the limit of detection of plaque titration assay was at 100 PFU/ml.

The results with FBS-based medium showed that that 4G2 alginate bead was capable of adsorbing the virus significantly at the percentage of 63, and 94 by RT-qPCR and plaque titration. The empty alginate beads was capable of adsorbing the virus at the percentage of 50, and 53 by RT-qPCR and plaque titration, similar to the previous MEM-based circuit experiment. Moreover, the simulated circuit without bead was adsorbing the virus at the percentage of 44, and 43 by RT-qPCR and plaque titration suggesting that the circuit equipment was capable of adsorbing the virus nonspecifically. The RT-qPCR and plaque titration was done in four technical replicates. This experiment was performed in two biological replicates and the data were shown as means and standard deviation of the two biological replicates.

Figure 19 Percentage of DENV2 after run on simulated circuit for 3 hours that quantified by RT-qPCR (a) and plaque titration assay (b).

(*,**** represented significant value at p-value < 0.1, 0.0001, respectively.)

According to the concerns of 4G2 escape, the beads and MEM medium were collected before and after run for western blot analysis (Figure 20). The result showed that the 4G2 antibody indeed escaped from the bead as the band reduced by 59 percent. The band was presented in the MEM-based mdium only after the run confirmed the 4G2 excape hypothesis.
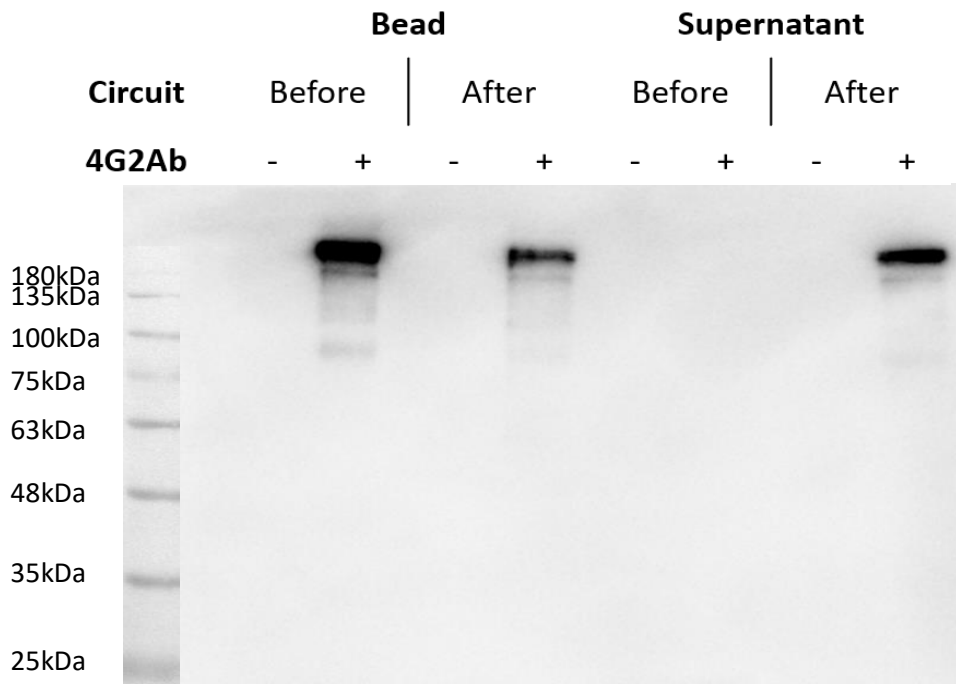
Figure 20 The monoclonal antibody was detected by Western blot.

4. Adsorption specificity of 4G2 DENV-specific antibody to other pathogens inducing systemic infection

4.1. *Escherichia coli*

The alginate beads with and without monoclonal antibody were incubated with *E. coli* DH5-Alpha (1.12×10^7 CFU/ml) spiked into L.B. broth and incubated in 6-well plate. The results with L.B. medium showed that 4G2 alginate bead was incapable of adsorbing the bacterium at the percentage of -15 by colony forming unit assay. The empty alginate beads were ineffective at the percentage of -36. Data represented the means and standard deviation of the technical duplicated results (Figure 21).
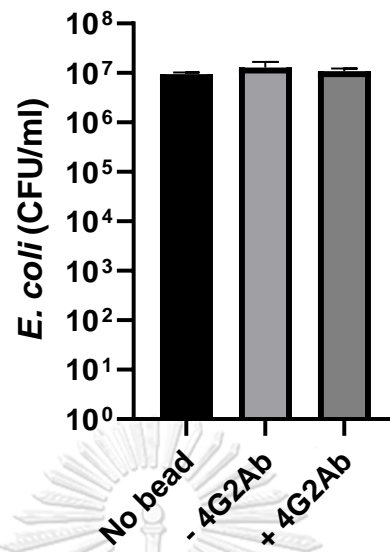
Figure 21 *E. coli* after 3 hours incubated with blank (no bead), empty alginate bead, and 4G2 alginate bead that quantified by colony forming unit assay.

4.2. Enterovirus A71

In this experiment, fetal bovine serum was used as a based medium for a simulated circuit. The EV-A71 virus (1.14×10^7 copies/ml) was spiked into the 50 ml fetal bovine serum prior to a 3 h continuous run in the simulated circuit. The conditions without bead, and with the empty alginate beads were used as negative controls. The samples collected before and after run were quantified by RT-qPCR and plaque titration (Figure 22). Results showed that the 1 mg/ml 4G2 alginate beads were capable of adsorbing the virus at the percentage of 62, and 40 by RT-qPCR and plaque titration. The empty alginate beads showed similarly adsorption pattern at the percentage of 68, and 42 by RT-qPCR and plaque titration. Without bead, the simulated circuit was capable of nonspecific viral adsorption at the percentage of 50, and 46 by RT-qPCR and plaque titration. Data represented the means and standard deviation of the

technical quadruplicated results. The results indicated the nonspecific adsorption of the simulated circuit to the enterovirus.

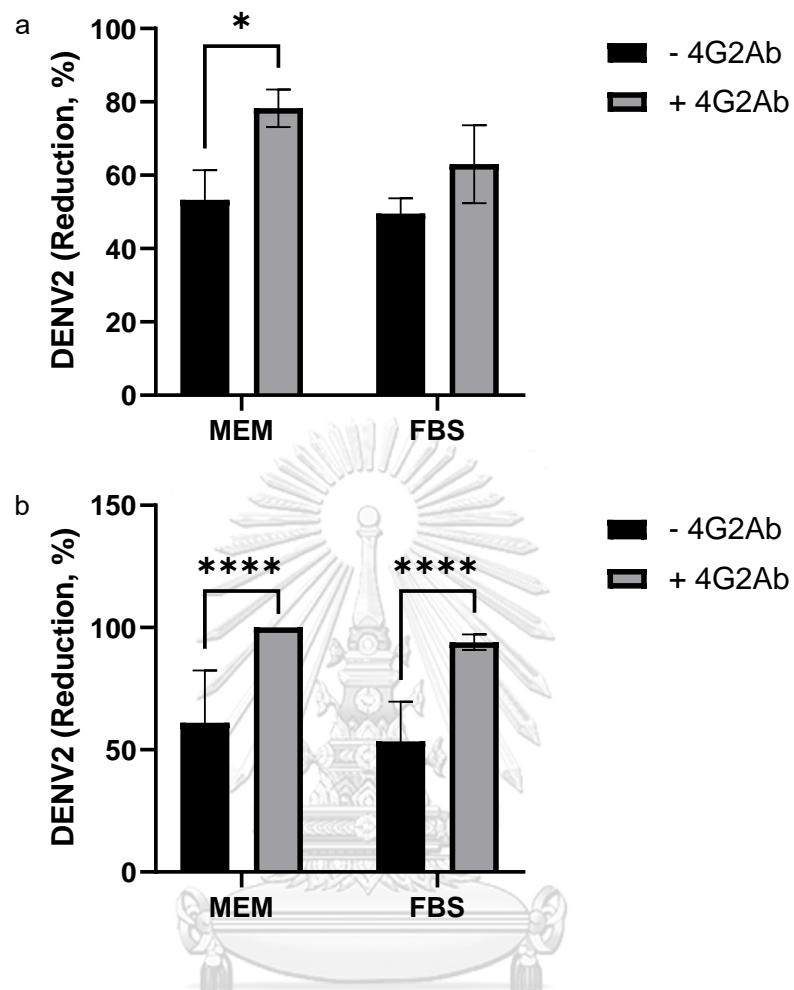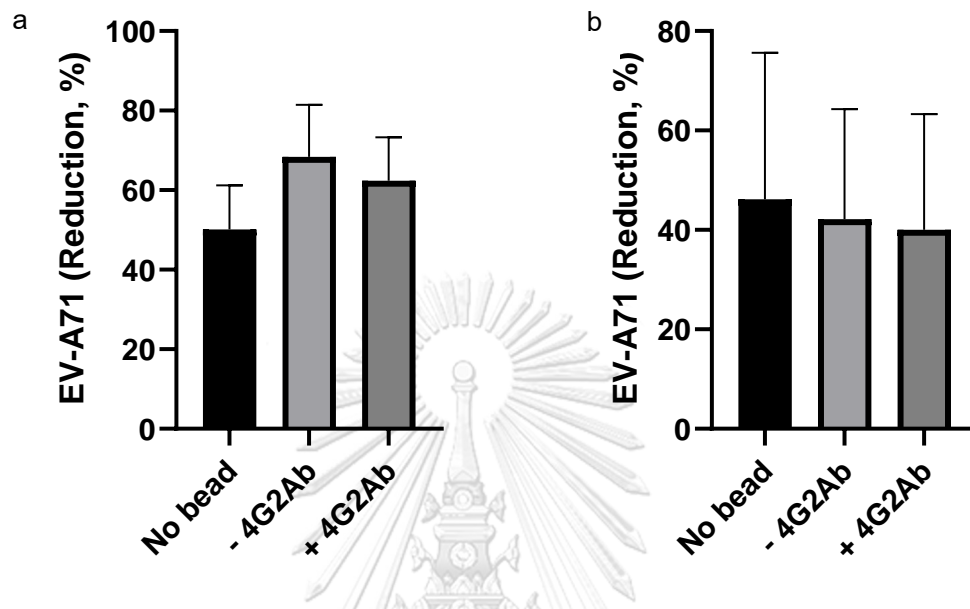**Figure 22** Percentage of EV-A71 after run on simulated circuit for 3 hours that quantified by RT-qPCR (a) and plaque titration assay (b).

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CHAPTER 5

DISCUSSION

Dengue fever is a significant public health problem affecting almost a hundred countries, especially in tropical region. However, no specific antivirals is available and supportive treatment and fluid therapy still played a crucial role. Although there is a Dengvaxia® vaccine, some restrictions are applied as the vaccine can only be given to persons with a confirmed history of a prior dengue virus infection. The therapeutic plasma filtration using specific antibodies could be an alternative treatment, especially in severe cases. The potential benefit of immunoadsorption over a drug administration is that the adverse drug reaction and drug toxicity could be prevented. A simulated circuit showed that the plasma filtration with a specific antibody can absorb dengue virus, thereby reduce viral load. This could be useful in patients with high severity risk and clinically impending shock. A previous report suggested that the patients with severe dengue were linked to higher virus levels (43). In fact, a clinical study reported that of infection in severe patients had ten times higher levels of viremia in the early stage compared to those of the nonsevere patients (44). Furthermore, in the defervescence stage, the level of plasma dengue viral RNA was undetectable in most nonsevere patients. In contrast, the viral load remains high in severe patients (45). Therefore, we hypothesized that reducing the DENV viremia could reduce the risk of severe dengue progression.

In general, therapeutic plasmapheresis is the process of removing plasma and replacing it with appropriate replacement fluid. It is a standard treatment of diseases caused by abnormal components in the plasma such as autoantibodies, circulating immune complexes, soluble inflammatory factors, lipids like LDL, paraproteins (46), and the systemic Ebola infection (10). However, removing all plasma compartment and replacing with albumin or donated plasma could introduce another problem: losing the essential proteins, nutrients, and drugs or increasing risk of infection. Therefore, the primary attempt was to design a simulated circuit of a plasma filtration system adapting from the Cytosorb model, a size-exclusion, nonselective adsorption device.

The covalent coupling of the DENV-specific (4G2) monoclonal antibody with silica-based matrix was primarily attempted via covalent crosslinking using carbodiimide crosslinking and identified by infrared spectroscopy. Results showed that the albumin was successfully coupled with the functionalized silica from the 1653, 1540, and 2963 cm^{-1} peaks indicating C.O. stretching, N.H. bending, and C.H. stretching. However, significant peaks were not detected in the immobilization of 4G2 antibodies on the functionalized silica, indicating the silane group detachment. The silane detachment could be resulting from an imbalanced buffer condition (47). It was also likely that the immunoglobulin was two times larger than BSA albumin, thus contributing to weak bond formation (48). Therefore, the covalent linkage formation between the immunoglobulin and the silica was disrupted by high entropy created by the ionized buffer. Therefore, the immobilization of immunoglobulin to the functionalized silica still requires further optimization.

An entrapment system using 2% alginate bead as a matrix for the specific antibody was used as an alternative matrix instead of the functionalized silica. The specific adsorption was successfully demonstrated in both culture medium and fetal bovine serum continuously run in with more than 90% adsorption the simulated circuit. The FBS circuit was repeated with two biological replicates and the results were consistent. Moreover, the binding was exclusive to the DENV as the effect was not observed in an *E. coli* bacterium, or an enterovirus A71. The results indicated that the specific binding was established thus reducing viral load in the circulation. The system could be improved by replacing the 4G2 with more specific antibodies. Furthermore, the blank circuit demonstrated the viral adsorption at 40-50% possibly due to nonspecific attachment to the silicone tube. This factor was consistent throughout the experimental setting but was expected to inversely correlate with the total running volume. This confounding factor should be reevaluated in further *in vivo* experimental setup. Parameters affected by the nonspecific binding were essential nutrients, clotting factors, antibodies, and other macromolecules (49). However, the nonspecific binding could be

beneficial as it helps adsorb excessive cytokines in the circulation during cytokine storm.

In addition, the 4G2 entrapment system was not stable as the free antibody was detected in the circulating plasma after run, correlated with the decreasing bead-entrapped antibody. Therefore, the escaped mouse antibody could directly bind to mast cell and activate degranulation by crossing mechanism, subsequently lead to vascular leakage *in vivo* (50).

Curently, a commercial viral particle adsorption device called Hemopurifier® was used as one of the treatments in an Ebola-infected patient (10, 51). This device was a *Galanthus nivalis* lectin affinity column which is capable of adsorbing heavily glycosylated virions. Enveloped viruses are affinity-captured by lectins. The purified plasma returned to the patient. After three cycles, the patients recovered and eventually discharged from the hospital. However, only limited *in vitro* data was available in dengue cases and the filtration columns used in the trials were all equipped with nonspecific binding matrix. (52). Therefore, our device is the first in class with a specific adsorbent selectively for the viral particle. The antibody was a mouse origin, anti-flavivirus envelope protein antibody bind to a conserved epitope on the E protein of the flavivirus family. This filtration column can also be used in other flaviviruses such as West Nile virus, Japanese Encephalitis Virus, and Zika Virus. Similarly, the system can be used in different disease setting by replacing 4G2 with another specific antibody or even antibodies cocktail suitable for those particular disease.

Additionally, the system could be improved by increasing the column volume, decreasing alginate bead size so as to expand the surface area for higher binding potency. Finally, the antibody escape could be removed by addition of membrane filtration or centrifugal device to the circuit before returning the plasma to the patient.

However, the therapeutic plasma exchange is not suitable for all patients as it is an invasive procedure requiring intensive care from medical specialists. Plasmapheresis with one total blood volume exchange removes the pathogenic agent at 63% (53); therefore several runs were usually expected in the clinical setting. The utilization of our

immunoadsorption column could imitate the plasmapheresis clinical setting. Major concerns are cost-effectiveness as several columns are expected for effective treatment in a patient besides the safety issue regarding the escaped antibody and nonspecific binding silicone tubing system. Moreover, another limitation is that the system cannot remove the intracellular pathogens as most cellular components were returned to the patients.

In conclusion, we reported for the first time that our specific adsorptive device successfully removed more than 90% of the dengue virus particle in an *in vitro* simulated plasma filtration circuit.

APPENDIX A

REAGENTS, MATERIALS AND INSTRUMENTS

A. Media and Reagents

0.5 Tris-HCl Buffer (Biorad, USA)

1.5 Tris-HCl Buffer (Biorad, USA)

2,4,6-Tris(dimethylaminomethyl)phenol (DMP-30) (Electron microscopy sciences, USA)

2-Mercaptoethanol (BioRad, China)

2xLaemmli Sample Buffer (BioRad, China)

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Bio Basic, Canada) (HEPES, Free acid)

40% Acrylamide/Bis (BioRad, USA)

Acetic acid (Emsure, Germany)

Ammonium Peroxodisulfide (APS) (Panreac Applichem, Germany)

Bovine serum albumin (Sigma, USA)

Coomasie Blue R-250 (Thermo Scientific, USA)

Crystal Violet (Merck, Germany)

Dimethyl sulfoxide (DMSO) (Panreac Applichem, Germany)

Ethanol (Emsure, Germany)

Ethyl alcohol 70% (Defence Pharmaceutical Factory, Thailand)

Fetal bovine serum (FBS) (Gibco, USA)

Formedehyde 37% m/v (CARLO ERBA, France)

Glycine (BioRad, USA)

Isopropanol (2-Propanol) (Emsure, Germany)

Medium MEM (Gibco, USA)

Methanol (MeOH) (Univar, Australia)

Penicillin G (Bio Basic, Canada)

Potassium chloride (KCl) (Merck, Germany)

Potassium phosphate monobasic (KH2PO4) (Bio Basic, Canada)

Sodium Chloride (Bio Basic, Canada)

Sodium dodecyl sulfate (SDS) (Bio Basic, Canada)

Sodium hydroxide (NaOH) (Sigma, USA)

Sodium phosphate (Bio Basic, Canada)

Streptomycin (Sigma, USA)

TEMED (N,N,N′,N′-Tetramethylethylenediamine) (Thermo scientific, USA)

Tris-HCl (Vivantis, USA)

Trypan blue (Invitrogen, USA)

Trypsin (Bio Basic, USA)

B. Materials

1.5, 15 and 50 mL tube (JetBioFil, China)

6 well-plate (Thermo, China)

96 well-plate (Thermo, China)

Cell culture flask (T25 and 75) (Thermo, China)

Hemocytometer (Spencer, USA)

Syringe-driven filters (JetBioFil, China)

C. Instruments

Autoclave (Hirayama, Japan)

Biohazard safety cabinet (Flufrance, France)

CO_2 incubator (Thermo, USA)

Heat box (Bioer technology, china)

Incubator (Memmert, Germany)

Inverted microscopy (Leica, Germany)

Microcentrifuge (SelectBioProducts, UK)

Microplate reader (Perkin Eimer, USA)

Mixer-vortex (Scientific industrial, USA)

pH meter (Accmet Basic, Singapore)

Refrigerated Centrifuge (Thermo, USA)

Spindown (Hercuvan, Malaysia)

Step One Plus Real-Time PCR System (Applied Biosystems. USA)

Water bath (Julabo, Germany)

APPENDIX B

REAGENTS PREPARATION

REAGENTS AND MEDIA FOR CELL CULTURE

1. 2x Hybri-Care Medium

Hybri-Care Medium	12 g
Sterilized Double-distilled water (DDW)	1000 ml
Sterilized by filtration (0.2 μm) and stored at 4ºC	

2. 20% Hybri-Care Medium (Growth media for HB112 cell)

2x Hybri-Care Medium	50 ml
Fetal Bovine Serum (FBS)	20 ml
Pen/Strep antibiotics (10^5 unit/mL)	1 mL
10% $NaHCO_3$	0.3 mL
Sterilized DDW	29 mL
Stored at 4ºC	

3. 2x MEM

MEM with L-glutamine	19.2 g
Sterilized DDW	1000 ml
Sterilized by filtration (0.2 μm) and stored at 4ºC	

4. 10% MEM (Growth media for C6/36 cell)

2x MEM with L-glutamine	50 ml
Fetal Bovine Serum (FBS)	10 ml
10 mM HEPES	0.7 ml
Pen/Strep antibiotics (105 unit/mL)	1 mL
10% $NaHCO_3$	0.5 ml
Sterilized DDW	37.5 ml
Stored at 4ºC	

5. 1% MEM (Maintenance media for C6/36 cell)

2x MEM with L-glutamine	50 ml
Fetal Bovine Serum (FBS)	1 ml
10 mM HEPES	0.7 ml

Pen/Strep antibiotics (105 unit/mL)	1 mL
10% NaHCO3	0.5 ml
Sterilized DDW	45.5 ml
Stored at 4ºC	

6. Pen/Strep antibiotic (10^5 units/mL)

Penicillin G	0.6 g
Streptomycin	1.5 g
Sterilized DDW	200 mL
Sterilized by filtration (0.2 µm) and stored at -20ºC	

7. 10 mM HEPES

HEPES	11.915 g
Sterilized DDW	50 mL
Sterilized by autoclave and stored at 4ºC	

8. 10% $NaHCO_3$

$NaHCO_3$	5 g
Sterilized DDW	50 mL
Sterilized by autoclave and stored at 4ºC	

9. 10x PBS, pH 7.4

NaCl	40 g
KCl	1 g
Na_2HPO_4	5.75 g
KH_2PO_4	1 g
DDW 500 mL	
Sterilized by autoclave and stored at room temperature	

10. 1x PBS

10x PBS	20 mL
Sterilized DDW	180 mL
Stored at room temperature	

11. 5% Trypsin

Trypsin	5 g

Sterilized DDW	100 mL

Sterilized by filtration (0.2 μm) and stored at -20ºC

12. 0.25% Trypsin

5% Trypsin	2 ml
1% EDTA	0.8 ml
1x PBS	37.2 ml

Stored at -20ºC

13. 0.05% Trypsin

0.25% Trypsin	4 ml
1% EDTA	0.320 ml
1x PBS	15.68 ml

Stored at 4ºC

REAGENTS FOR SDS-PAGE

1. 10% Running gel (10% Acrylamide concentration)

DDW	3.8 mL
40% Acrylamide	2 mL
1.5M Tris pH 8.8	2 mL
10% SDS	80 μL
10% APS	80 μL
TEMED	8 μL

2. 4% Stacking gel (5% Acrylamide concentration)

DDW	3.1 mL
40% Acrylamide	0.5 mL
0.5M Tris pH 6.8	1.25 mL
10% SDS	50 μL
10% APS	50 μL
TEMED	5 μL

3. 10% Ammonium persulphate (APS)

APS	0.03 g

| DDW | 300 µL |

4. Coomassie blue R-250 staining solution

Coomassie blue R-250 stock	15 mL
Methanol	250 mL
Distilled water (35)	200 mL
Acetic acid	50 mL

5. 10x Running buffer for SDS-PAGE (10x Tris/Glycine/SDS Buffer)

Tris base	30 g
Glycine	144 g
SDS	10 g
Distilled water (35)	1000 g

pH should be 8.3-8.4

6. 1x Running buffer (1x Tris/Glycine/SDS Buffer)

| 10x Running buffer | 100 mL |
| Distilled water (35) | 900 mL |

7. SDS loading buffer (Sample buffer)

| 2x laemmli sample buffer | 190 µL |
| 2 mercapto ethanol | 10 µL |

8. Destaining solution for Coomassie blue

Methanol	200 mL
Acetic acid	100 mL
Distilled water (35)	700 mL

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