การทะลุผ่านเยื่อลิพิดไบเลเยอร์และการนำส่งแอนติบอดีของอนุภาคระดับนาโนเมตรของคาร์บอนที่ถูกออกซิไดส์

นายณัฐพล ตันติเมฆิน

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LIPID BILAYER MEMBRANE PENETRATION AND ANTIBODY DELIVERY OF OXIDIZED CARBON NANOPARTICLES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
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ณัฐพล ตันติเมฆิน : การทะลุผ่านเยื่อลิพิดไบเลเยอร์และการนำส่งแอนติบอดีของอนุภาคระดับนาโนเมตรของคาร์บอนที่ถูกออกซิไดส์ (LIPID BILAYER MEMBRANE PENETRATION AND ANTIBODY DELIVERY OF OXIDIZED CARBON NANOPARTICLES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.ศุภศร วนิชเวชารุ่งเรือง, 35 หน้า.

อนุภาคระดับนาโนเมตรของคาร์บอนที่ถูกออกซิไดส์ (oxidized carbon nanoparticles, OCNs) สังเคราะห์ได้จากการออกซิไดส์คาร์บอนแบล็ค (carbon black) ด้วยวิธีของ Hummers และ Offeman ที่ปรับปรุงขึ้น ในงานวิจัยนี้ใช้ OCNs นำส่งโมเลกุลขนาดใหญ่เข้าสู่เซลล์ แอนติบอดีของมนุษย์ชนิดโมโนโคลนอล (human monoclonal antibodies, HuMAbs) เป็นโมเลกุลที่น่าสนใจที่จะนำส่งเข้าสู่เซลล์โดยใช้ OCNs เป็นตัวนำส่ง เนื่องจาก HuMAbs เป็นแอนติบอดีที่สังเคราะห์ขึ้นด้วยเทคโนโลยีไฮบริโดมา (hybridoma technology) ซึ่งสามารถยับยั้งเชื้อไวรัสเดงกี (dengue virus) ซึ่งเป็นสาเหตุของโรคไข้เลือดออกได้ทั้ง 4 ชนิด แต่อย่างไรก็ตาม HuMAbs ไม่สามารถเข้าสู่เซลล์เองได้ ดังนั้นจึงจำเป็นต้องใช้ OCNs ในการนำส่ง HuMAbs เข้าสู่เซลล์เพื่อยับยั้งไวรัสเดงกีภายในเซลล์ OCNs สามารถนำ HuMAbs เข้าสู่เซลล์วีโร (Vero cell) และ HuMAbs ที่ถูกส่งเข้าไปยังคงแสดงความสามารถในการยับยั้งไวรัสเดงกี ยิ่งไปกว่านั้นการใช้ OCNs ยังช่วยเพิ่มความสามารถในการยับยั้งของ HuMAbs เพราะว่า OCNs ทำให้ HuMAbs สามารถยับยั้งไวรัสเดงกีได้ทั้งภายในและภายนอกเซลล์ นอกจากนี้จากการสังเกตกันเองในกลุ่มวิจัยพบว่า OCNs มีการตอบสนองต่อความร้อน เราได้ตั้งสมมติฐานว่าการฉายรังสีอินฟราเรดแบบใกล้ (NIR) อาจจะสามารถเร่งอัตราการทะลุผ่านของ OCNs เข้าสู่เยื่อลิพิดไบเลเยอร์ (lipid bilayer membrane) เราได้เตรียมลิโปโซมขนาดเซลล์ (cell-sized liposome) ขึ้นมาแทนเยื่อลิพิดไบเลเยอร์ด้วยวิธีการดึงน้ำออก (dehydration) แต่การใช้ OCNs ร่วมกับการฉายรังสี NIR ไม่สามารถเร่งอัตราการทะลุผ่านของ OCNs เข้าสู่ลิโปโซมขนาดเซลล์ได้

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NATTHAPOL TANTIMEKIN: LIPID BILAYER MEMBRANE PENETRATION AND ANTIBODY DELIVERY OF OXIDIZED CARBON NANOPARTICLES. ADVISOR: PROF. DR. SUPASON WANICHWECHARUNGRUANG, 35 pp.

Oxidized carbon nanoparticles (OCNs) were synthesized by oxidizing carbon black via modified Hummers and Offeman method. In this research, OCNs were used for delivering macromolecules into cells. The human monoclonal antibodies (HuMAbs) are an interesting macromolecules that were delivered into cells by the using of OCNs as a carrier. Because HuMAbs are synthesized antibodies from hybridoma technology that can neutralize all four serotypes of dengue viruses (DENV), as the cause of dengue fever. Nevertheless, HuMAbs cannot automatically enter cells so OCNs were essentially used to deliver HuMAbs into cells for intracellular DENV neutralization. OCNs can bring HuMAbs into Vero cells and the delivered HuMAbs still showed DENV neutralization activity. Moreover, the using of OCNs increase the neutralization ability of HuMAbs because it make HuMAbs can neutralize both intracellular and extracellular DENV. In addition, observation in our research group demonstrate that OCNs respond to heat. We hypothesized that NIR irradiation may accelerate the OCNs penetration rate into lipid bilayer membrane. We prepared the cell-sized liposome as lipid bilayer membrane by dehydration method. But, the using of OCNs with NIR irradiation cannot accelerate the OCNs penetration rate into the cell-sized liposome.

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Student's Signature ....
Advisor's Signature ....

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CHAPTER I
INTRODUCTION

Many therapeutic techniques were developed to cure diseases in the world. However, the efficiency of each techniques still depend on many variables. One technique is cellular uptake of drug ingredients. Drug carriers were essentially used to deliver drug ingredients into cells. Molecules of drug ingredients such as DNA, gene cannot enter into cells automatically [1, 2]. These macromolecules need carrier to deliver them into the cell. Thus, delivery systems were developed to bring macromolecules enter cells e.g. cell-penetrating peptides, phospholipids [3, 4]. Nano carbon materials such as carbon nanotubes [5, 6], graphene oxide [7-9], carbon dots [10, 11] and nanodiamond [12, 13] can be used as a drug carrier and can be applied with bioactive ingredients or drugs. But, our research group discovered another type of carbon particle during oxidation of graphite. The newly found carbon particle is called cluster of carbon nanospheres (CCNs) [14]. CCNs can deliver macromolecules into cells. Moreover, CCNs has endosomal escape ability that is good for drug delivery applications. But the preparation of CCNs from graphite is so hard. Our research group found a way to prepare CCNs-like particle using carbon black instead of graphite. The oxidation of carbon black become oxidized carbon nanoparticles (OCNs) for antibody delivery was highly interested. Because antibody cannot automatically enter into cells. Although the human monoclonal antibodies (HuMAbs)

have been developed to neutralize all four serotypes of dengue viruses (DENV) [15]. But, HuMAbs can only neutralize DENV outside the cell. I expect that HuMAbs will be able neutralize DENV inside the cell cause the DENV neutralization efficiency increased if OCNs can transport HuMAbs into the cell. In addition, I hypothesized that the OCNs penetration rate into the lipid bilayer membrane were accelerated if irradiate with near-infrared (NIR). Because our research group found that the OCNs velocity in a suspension will be increased when increase the temperature of suspension. In order to use in organisms, I cannot increase whole temperature of cells because cells were died. This hypothesis was tested by NIR irradiation instead of heating. If NIR irradiation can accelerate the OCNs penetration rate into the lipid bilayer membrane, I can adapt to use OCNs for macromolecule delivery to target area in organisms by NIR irradiation onto target area.

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CHAPTER II
LITERATURE REVIEWS

2.1 Types of Carrier for Drug Delivery

2.1.1 Liposomes

Liposome is a spherical vesicle most often composed of phospholipids bilayer membrane. Phospholipids consist of hydrophobic and hydrophilic moieties. This self-assemble in water. Liposomes were used for administration of pharmaceutical drugs and nutrients.

In 2016, Zhao et al. found that liposome with sucrose ester could transport siRNA against luciferase for silencing gene in lung tumors of mice. This improved transfection and reduced cytotoxicity [16].

Recently, Rasoulianboroujeni et al. developed an association of cationic liposomes and DNA-liposome as gene carriers in transfection assays. This developed liposomal formulation which the cellular activity (cellular protein) was higher in the prepared lipoplex than Lipofectamine® 2000, cause a better function than Lipofectamine® 2000 as a commercialized product [2]. In the same year, Ewe et al. prepared liposome-polyethylenimine complexes which are the combination of lipid-based carriers with polymeric nanoparticles for therapeutic siRNA delivery *in vivo* [17].

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2.1.2 Cell-Penetrating Peptides

Cell-penetrating peptides (CPPs) are short cationic peptides with inherent ability to cross the plasma membrane barrier which can transport various molecular equipments into cells via endocytosis. This reason cause CPPs were widely used as drug delivery. But, the cargo needs to combine with CPPs.

Recently, Mesken et al. modified plasmid-loaded human serum albumin (HSA) –nanoparticles with CPPs for cellular uptake and enhanced gene delivery. Transfection efficiency of modified nanoparticles increased significantly comparing to the free DNA or polyplexes [18]. In the same year, Kang et al. discovered the peptide pVEC from vascular endothelial cadherin that had more effective in transporting exogenous proteins into algal cells than other peptides. This peptide was used as CPPs for protein delivery into microalgae [3].

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2.1.3 Nano Carbon Materials

Nano carbon materials were widely used as drug carrier such as carbon nanotubes, graphene oxide, etc.

In 2016, Ohta et al. discovered using single-walled carbon nanotubes (SWCNTs) associated with designed polycationic and amphiphilic peptides with PEGylation was uptaken by A549 human lung adenocarcinoma epithelial cells. This carrier was used as drug and gene delivery applications [6]. In the same year, Razzazan et al. prepared gemcitabine conjugated to SWCNT and PEGylated SWCNT.

These were used as anti-tumor *in vivo* [19]. Seemork et al. studied the penetration of oxidized carbon nanosphere (OCS), oxidized carbon nanotube (OCT), oxidized graphene sheet (OGSh) through lipid bilayer membrane. OCS can bring curcumin into cells. Moreover, OCS and delivered curcumin are not trapped by lysosome [20].

2.2 Antibody Delivery into Cells

Recently, Lim et al. modified an all-protein self-assembled nanocarrier (Hex nanocarrier) capable of delivering functional antibodies to the cytosol. The Hex nanocarrier was prepared by combining an α-helical peptide which self-assembles into a hexameric coiled-coil bundle and an fragment crystallizable (Fc)-binding Protein A fragment. The Hex nanocarrier tightly bind antibodies (anti-β-tubulin or anti-nuclear pore complex antibody) to deliver functional antibodies to the cytosol without cytotoxicity [21]. In the same year, Akishiba et al. developed endosomolytic peptides derived from the cationic and membrane-lytic spider venom peptide M-lycotoxin. This lipid-sensitive endosomolytic peptide promotes the uptake by inducing macropinocytosis and it can make antibodies (immunoglobulins G (IgGs)) to liberate from endosomes [22].

2.3 Dengue Virus

Dengue virus (DENV) is the cause of dengue fever. The dengue virus genome is a single-stranded RNA. Dengue virus consist of four closely related viruses named

DENV-1, DENV-2, DENV-3, and DENV-4. These are called serotypes because each has different interactions with the antibodies in human blood serum. All four dengue viruses are similar genomes approximately 65% but each serotype have some genetic variation [23-25].

2.4 Dengue Virus Infection and Replication

First, DENV attach to the cell. After that, cell membrane fold around the virus and form an endosome that seal around the virus particle, as endocytosis, which cell use endosome to uptake large molecules and particles from outside the cell. Next, the virus fuse with endosomal membrane to release into the cytosol. The virus particle break to release the viral genome. Then, the viral RNA is translated into a single polypeptide which is cut into 10 proteins, and the viral genome is replicated. Virus assembly occur on the surface of the endoplasmic reticulum (ER) when the structural proteins and newly synthesized RNA bud out from the ER. And, the immature viral particles are transported through the trans-Golgi network (TGN), the virus mature and convert to infectious form. Finally, the mature virus was released from the cell and can go on to infect the other cells [26].

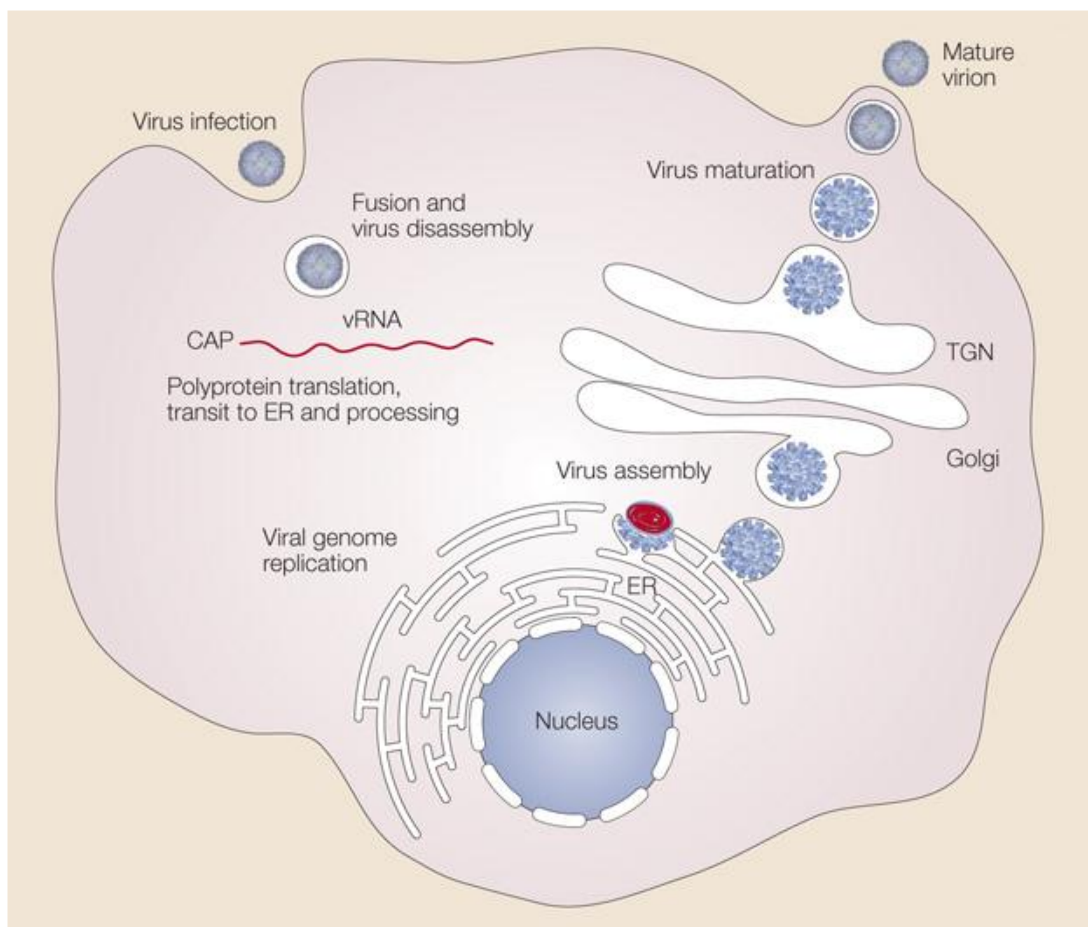
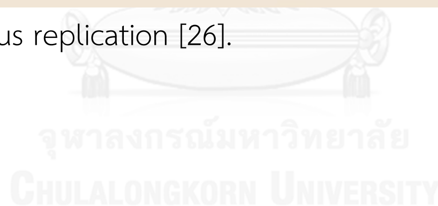


Figure 2.1 Dengue virus replication [26].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2.5 Dengue Virus Neutralization by Human Monoclonal Antibodies

In 2012, Setthapramote et al. developed human monoclonal antibodies from the fusion of lymphocytes (peripheral blood mononuclear cells, PBMCs) from patients at acute phase of the secondary infection and myeloma cells (SPYMEG) to generate hybridomas producing HuMAbs to neutralize all four serotypes of DENV [15].

2.6 Cell-Sized Liposomes

The cell-sized liposome is cell-sized vesicles with a phospholipid bilayer that was used as the real-world modeling of living cells (cell membrane). The size of the cell-sized liposome should be large enough to observe under optical microscope [27].

2.7 Research Objective

Carbon black was oxidized via modified Hummer and Offeman method to generate oxidized carbon nanoparticles (OCNs) as nano carbon materials for antibody delivery application. The oxidation will increase dispersibility in water of OCNs. Structure, morphology, and size of OCNs were also verified. OCNs were tested to deliver the human monoclonal antibodies (HuMAbs) into cells. The dengue virus neutralization activity of HuMAbs were also evaluated comparing to the using of HuMAbs without OCNs. Finally, my hypothesis that NIR irradiation can accelerate the OCNs penetration rate into the lipid bilayer membrane was investigated.

CHAPTER III

EXPERIMENTAL

3.1 Synthesis and Characterization of Oxidized Carbon Nanoparticles (OCNs)

OCNs were prepared by Hummer and Offeman method [28] with some modifications [14]. Briefly, 0.3 g of carbon black (Denka Company Limited, Denki Kagaku Kogyo Kabushiki Kaisha, Japan) was mixed with 1 g of sodium nitrate (Suksapan, Bangkok, Thailand) and 50 mL of 18 M sulfuric acid (Sigma-Aldrich, St. Louis, USA), and the mixture was sonicated at 40 kHz at room temperature for 1 h. Then, 6 g of $KMnO_4$ (Suksapan, Bangkok, Thailand) was then added with stirring for 90 min. Subsequently, 100 mL of water was added, allowing an increase of a temperature to 90 °C for 15 min. Then, water (300 mL) was added to the mixture and the mixture was stirred for another 30 min. Next, an excess $KMnO_4$ was quenched by adding 50 mL of 5% (w/v) H_2O_2 and stirring at room temperature for 5 min. After that, OCNs were separated by centrifugation at 20,000 rpm at 25 °C for 10 min. Finally, dialyzing the precipitate of OCNs in a dialysis bag (CelluSep T4, MWCO of 12,000-14,000 Da, Membrane Filtration Products, USA) against water until the pH raise to 5-6.

The morphology of OCNs were visualized by transmission electron microscopy (TEM, JEM-2100, JEOL, Tokyo, Japan, using an accelerating voltage of 120 kV) and scanning electron microscopy (SEM, JSM-6400, JEOL, Tokyo, Japan, using an accelerating voltage of 15 kV). The particle size of OCNs were measured by dynamic

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light scattering (DLS, Malvern Instruments Ltd., Worcestershire, UK). Zeta potential value of OCNs were measured by a zetasizer (nanoseries model S4700, Malvern Instruments Ltd., Worcestershire, UK). Chemical structure was evaluated by UV-visible absorption spectroscopy (UV-vis, UV2500, Shimadzu Corp., Kyoto, Japan) and fluorescent spectroscopy (Varian Cary Eclipse fluorescence spectrophotometer). Functional groups were characterized by an attenuated total reflectance fourier transform infrared spectrometer (ATR-FTIR, Nicolet 6700, Thermo Scientific, Thermo Fisher Scientific Inc., Massachusetts, USA) and X-ray photoelectron spectroscopy (XPS, Kratos AXIS Ultra DLD Instrument, Kratos, Manchester, England, using a monochromatic Al Kα X-ray source at 1486.6 eV, 150 W, 15kV and 10 mA). Bond and hybridization of carbon of OCNs were verified by Raman spectroscopy (DXR Raman microscope, Thermo Scientific, Thermo Fisher Scientific Inc., Massachusetts, USA, using a 10X objective and a 5 mW diode laser with a wavelength of 780 nm excitation source). Finally, component of OCNs were evaluated by elemental analysis (EA, combustion through the PE2400 Series II, Perkin-Elmer, Massachusetts, USA).

3.2 Fluorescence-labeling of OCNs and Human Monoclonal Antibodies (HuMAbs)

Fluorescence-labeled OCNs (TAMRA-OCNs) were prepared by mixing 40 µL of 5,6-carboxytetramethylrhodamine in dimethylformamide (1.3 mg/mL, TAMRA, Life Technologies, Carlsbad, California, USA) and 40 µL of dimethylformamide was added

at 0 °C, and 5.5 μL of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (11 mg/mL, EDCI, Acros Organics, Belgium) in water was added before incubation for 30 min. Then, 6.9 μL of *N*-Hydroxysuccinimide (5.8 mg/mL, NHS, Acros Organics, Belgium) in water was added followed by the addition of 5 mL of OCNs (0.1 mg/mL). The mixture was stirred in an ice bath for overnight. TAMRA-OCNs were dialyzed in a dialysis bag (CelluSep T4, MWCO of 12,000-14,000 Da, Membrane Filtration Products, USA) against water.

Fluorescence-labeled HuMAbs (FITC-HuMAbs) was prepared by dissolving 1 mg of fluorescein-5-isothiocyanate (FITC, CALBIOCHEM, EMD Chemicals Inc., San Diego, California, USA) in 2 mL of 0.1 M Na_2HPO_4 solution within 5-10 min. Then, 50 μL of HuMAbs (2 mg/mL, D23-4 F5E1, molecular weight (MW) ~150 kDa, provided by Center of Excellence for Antibody Research (CEAR), Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand) was mixed with 12.5 μL of 0.2 M Na_2HPO_4 solution and 60 μL of prepared FITC solution. Subsequently, the pH of the mixture was immediately brought to pH 9.5 by adding 0.1 M Na_3PO_4 solution. The mixture was stirred overnight at 4 °C. Finally, FITC-HuMAbs were dialyzed in a dialysis bag (CelluSep T4, MWCO of 12,000-14,000 Da, Membrane Filtration Products, USA) against phosphate buffered saline (PBS, HyClone, GE Healthcare Life Sciences, USA).

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3.3 Cell Culture

Vero cells (Vero E99 cell, American Type Culture Collection (ATCC), Virginia, USA) were maintained in minimum essential medium with Earle's balanced salts with L-glutamine (MEM/EBSS, HyClone, GE Healthcare Life Sciences, USA) and 10% fetal bovine serum (FBS, HyClone, GE Healthcare Life Sciences, USA) at 37 °C in a humidified 5% CO_2 atmosphere.

3.4 Neutralization Assay (NT)

Vero cells were seeded at the density of 2.5 x 10^4 cells per well in 96-well cell culture plate and allowed to attach for 16-24 h. Then, cells were washed with PBS and then infected with 100 focus-forming units (FFU) of individual DENV serotypes (provided by CEAR, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand) in 50 µL of MEM/EBSS. After incubation at 37 °C for 1 h, culture media was removed and HuMAbs with or without OCNs (15 ppm) in MEM/EBSS with 1% FBS (50 µL) were added. After incubation at 37 °C for 2 h, 100 µL of the mixture of 2X minimum essential medium (2X MEM, Gibco, Invitrogen, USA): 2% carboxymethyl cellulose (2% CMC), ratio = 1: 1, with 2.5% FBS were added. And then, cells were incubated at 37 °C for 2 (for DENV-4) or 3 days (for DENV-1, DENV-2 and DENV-3). The number of infected cells were immunostained by indirect immunofluorescence assay. The experiments were performed in triplicate. The average of triplicate were used for NT_{50} determination.

3.5 Indirect Immunofluorescence (IF) Assay

IF assay was conducted for Vero cells obtain from NT assay. Cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. After that, cells were incubated with α-dengue virus HuMAbs as primary antibody for HuMAbs blocked DENV. Finally the bound antibody was visualized by further incubation with an Alexa Fluor 488 goat anti-human IgG (H+L, molecular probes, Life Technologies, Eugene, Oregon, USA), as a cross-adsorbed secondady antibody (1:1,000).

3.6 Cytotoxicity of OCNs

Cytotoxicity of OCNs was performed to determine the cytotoxicity of OCNs to Vero cells. The assay was performed similar to NT assay, except no DENV and HuMAbs were used in the assay. PBS was used instead of 2% carboxymethyl cellulose. After three days of incubation of Vero cells with OCNs (at the neutralization assay's end), MTT dye (10 μL, 5 mg/mL) was added into cells and incubated for another 4-6 h. Next, culture medium was carefully removed. Then, 100 μL of DMSO was added and mixed gently for 5 min. The optical density at 570 nm ($OD_{570\ nm}$) of each well was measured on an absorbance plate reader (Sunrise, Tecan, Tecan Trading AG, Switzerland).

3.7 Tracking Intracellular Delivery of Antibody

Vero cells were seeded at the density of 7.5×10^4 cells per well in an 8-well chamber slide and allowed to attach for 16-24 h. Next, cells were washed with PBS and then added FITC-HuMAbs (64 µg/mL) with or without TAMRA-OCNs (15 ppm) in MEM/EBSS with 1% FBS (250 µL). After incubation at 37 °C for 30 min, 2 h and 4 h, cells were washed twice with PBS and then fixed in a 4% paraformaldehyde in PBS. Nuclei were stained with DAPI. Finally, cells were observed under confocal laser scanning microscope (CLSM, Niokon Digital Eclipse C1-Si/C1 Plus, Nikon, Tokyo, Japan, equipped with Plan Apochromat VC 100x, a 32-channel-PMT-spectral-detector). Excitation was performed by using diode lasers (405, 488 and 561 nm, Melles Griot, New Mexico, USA), and collected fluorescent signals at 450, 525 and 595 nm.

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3.8 Liposome Preparation and Lipid Bilayer Membrane Penetration of OCNs with NIR irradiation

The cell-sized liposome was prepared by dehydration method. First, 100 µL of 2 mM dioleoyl L-α phosphatidylcholine (DOPC, Avanti Polar Lipid, Alabama, USA) in chloroform was added into glass vial. Then, 60 µL of 10 mM glucose in methanol was added. The mixture was dried to make a thin film by blowing nitrogen gas. The dried film was kept in desiccator overnight, before adding with 1 mL of MilliQ water and keep at 37 °C for 3 h to hydrate the lipid film and induce the formation of the cell-sized liposome.

The lipid bilayer membrane penetration of OCNs with NIR irradiation was performed by mixing the cell-sized liposome with TAMRA-OCNs (42 ppm), and the mixture was irradiated with NIR laser with a wavelength of 980 nm (1 watt) for period of times. Finally, the results were observed under CLSM with $\lambda_{ex}/\lambda_{em}$ of 561/595.

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Synthesis and Characterization of Oxidized Carbon Nanoparticles (OCNs)

OCNs were prepared by Hummer and Offeman method with some modifications. The obtained OCNs disperse well in water when compared to carbon black which cannot disperse in water (Figure 4.1). This suspension of OCNs can be used in biological aqueous medium. Scanning electron microscopic (SEM) and transmission electron microscopic (TEM) images (Figure 4.2) revealed the morphology of OCNs as spherical shape with diameter of ~150 nm. The particle size of OCNs measured by dynamic light scattering (DLS, Figure 4.3) were 170.5 ± 2.2 nm with the polydispersity index (PDI) of 0.282 ± 0.023. This PDI value of OCNs indicated a moderately polydisperse distribution of the product. Zata potential of OCNs were -32.8 ± 0.7 mV.

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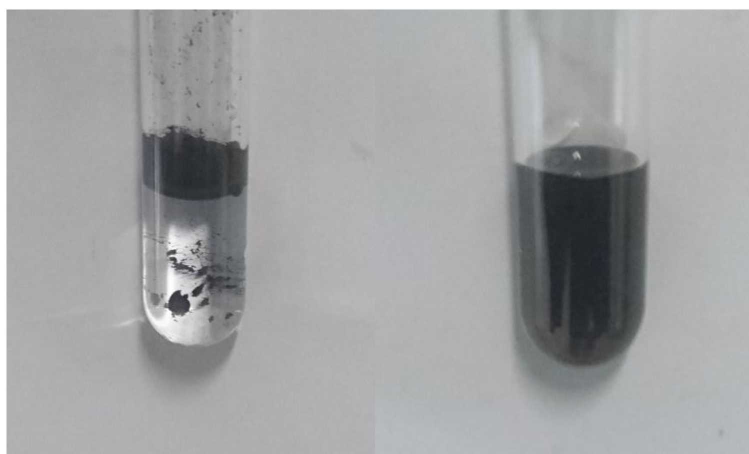

Figure 4.1 Photographs of carbon black (left) and OCNs (right) against water.

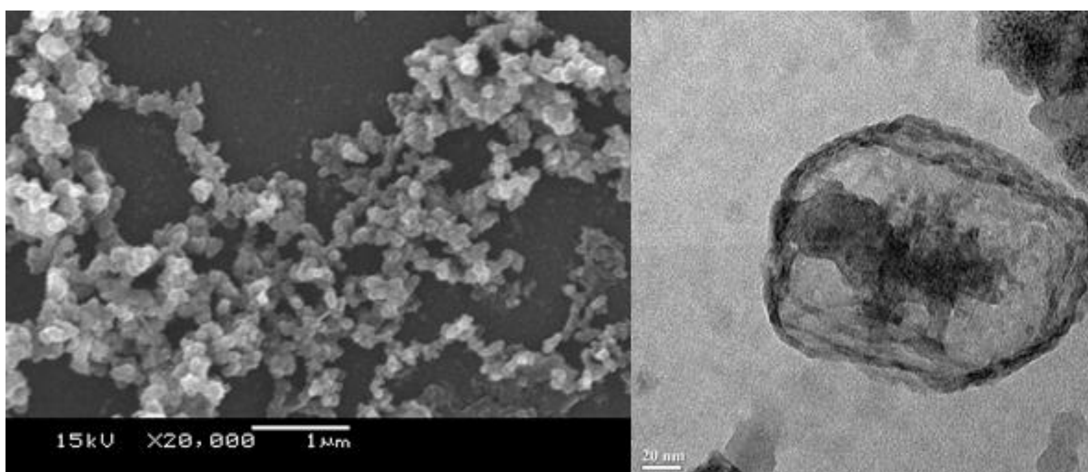

Figure 4.2 SEM (left) and TEM (right) image of OCNs.

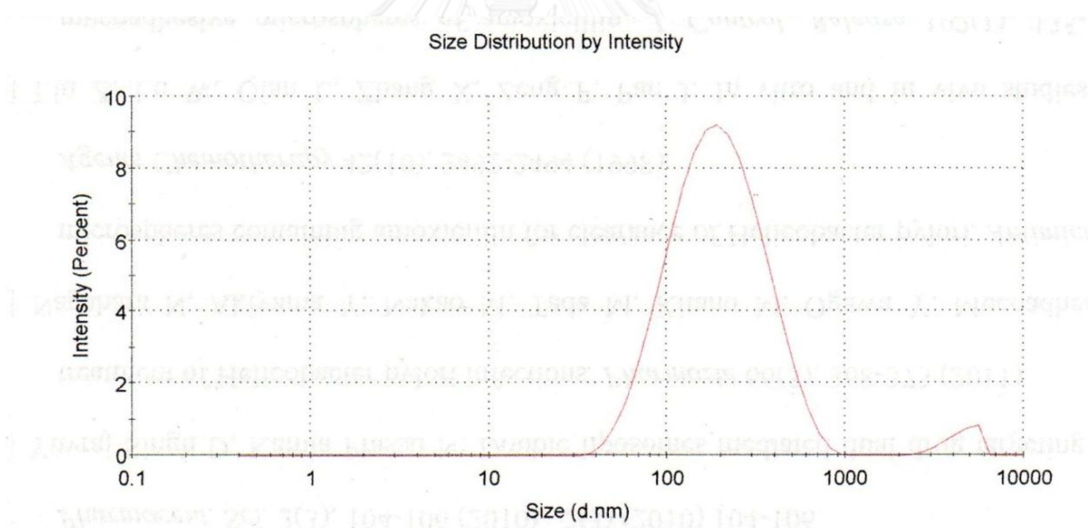

Figure 4.3 Size distribution of OCNs.

UV-Vis absorption spectra of OCNs (Figure 4.4) with a maximum absorption at 252 nm and broadened absorption up to 600 nm. The absorption profile could be

related to an extension of π-π conjugation network. Fluorescent spectra of OCNs (Figure 4.5) showed maximum emission at 504 nm when excited at 252 nm.

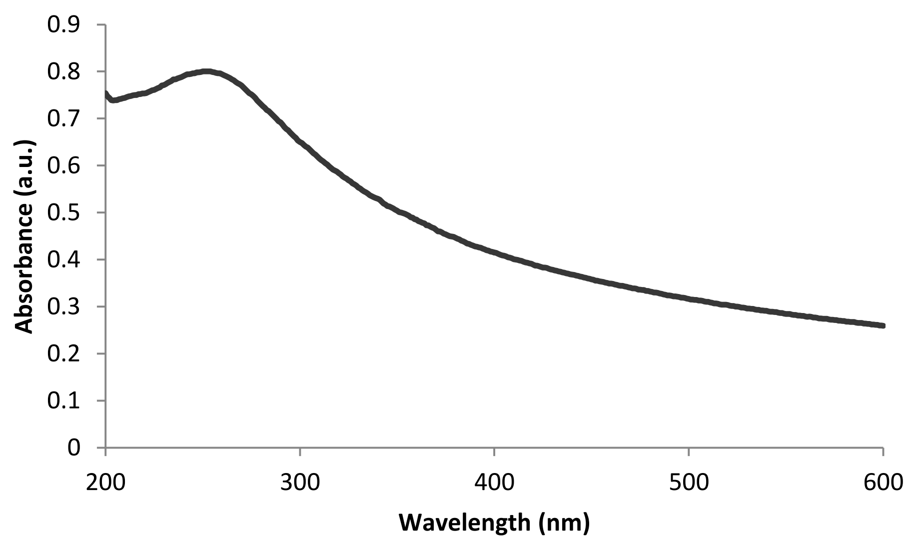

Figure 4.4 UV-Vis absorption spectra of OCNs.

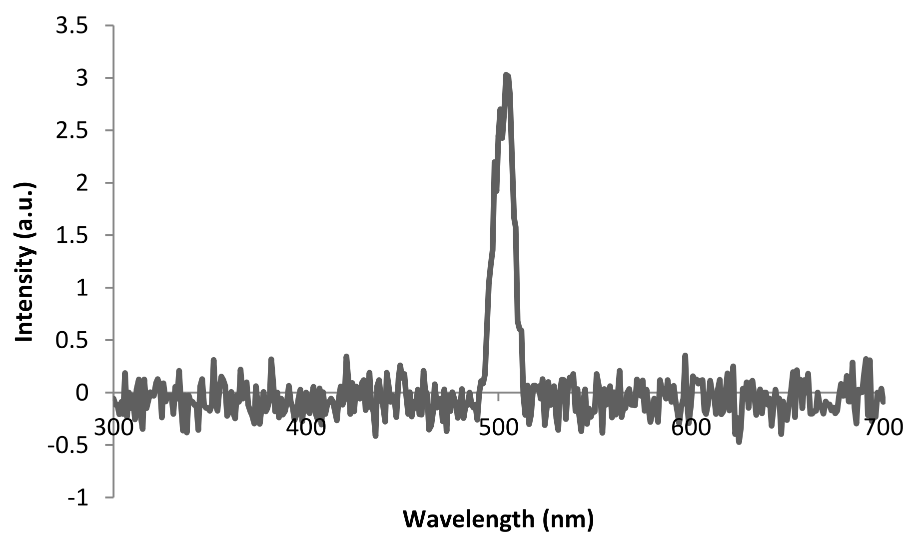

Figure 4.5 Fluorescent spectra of OCNs.

FT-IR spectra of OCNs (Figure 4.6) revealed a broad OH stretching at 3,000-3,600 cm^{-1}, C=O and C=C stretching at 1,600-1,800 cm^{-1} and C-O stretching/ C-H bending/ O-H bending at 1,000-1,450 cm^{-1} comparing to carbon black which wasn't detected OH and CO functional groups. This demonstrated that oxidation of carbon black can generate OH and COOH functional groups in OCNs.

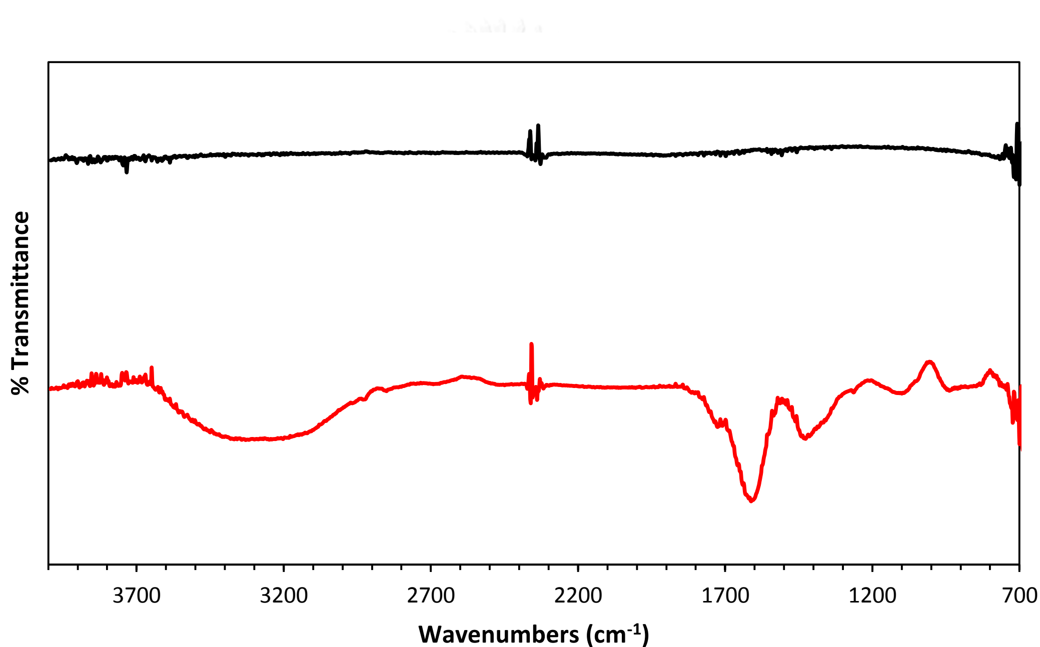

Figure 4.6 FT-IR spectra of carbon black (black) and OCNs (red).

Raman spectra of OCNs (Figure 4.7) possessed two strong broad absorption bands at 1,333 cm^{-1} (D band, disordered sp^2 bonded carbons) and 1,570 cm^{-1} (G band, sp^2 bonded carbons) with a three small peaks at 2,500-3,300 cm^{-1} (2D or G' band, disordered sp^2 planes). OCNs spectra showed a broader D and G bands and

the increased D band intensity, comparing to carbon black spectra. The D band to G band peak area ratio of carbon black increased from 1.04 to 1.54 in OCNs. This result revealed that the oxidation of carbon black to OCNs can increase the disordered carbon network planes in the structure. This result related to the previously reported oxidized carbon particles prepared from graphite [14].

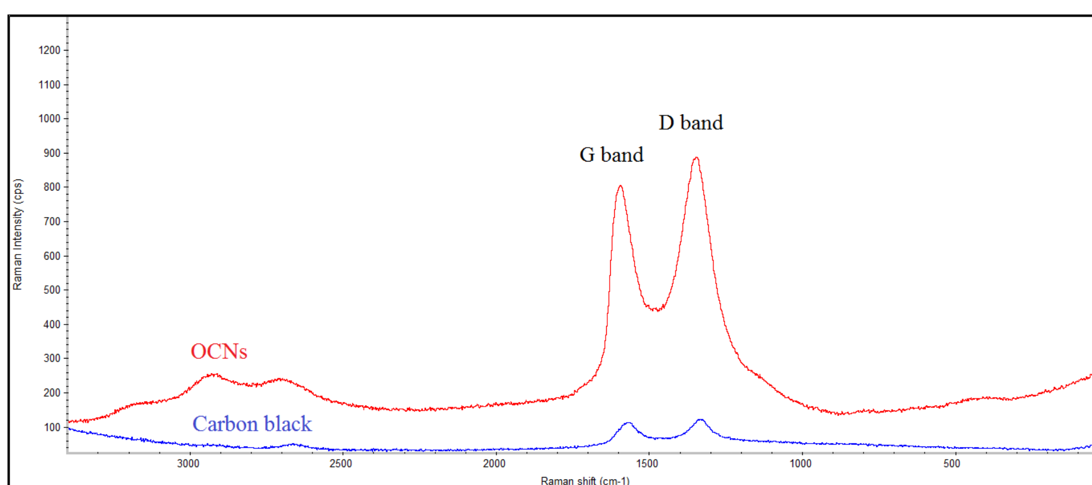

Figure 4.7 Raman spectra of carbon black and OCNs.

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X-ray photoelectron spectroscopy (XPS, Figure 4.8) revealed the increase of O1s intensity from carbon black (A) to OCNs (D) refered to the increase of oxygen by oxidation. Carbon black showed low signal intensity of C-O and C=O (A, B, C). Whereas, C1s spectra of OCNs showed high signal intensity of the C-C, C=C, C-O (from C-O-C and C-OH), C=O and COOH functional groups at the binding energy (BE) of 283.9, 285.3, 286.2, 287.5 and 289.7 eV, respectively (E). O1s spectra of OCNs showed high signal intensity of the C-O (from C-O-C and C-OH), C=O and COOH functional groups at the binding energy (BE) of 530.8, 530.2 and 529.5 eV, respectively (F). The

elemental analysis (EA) were performed to confirm the successfully oxidation of carbon black to OCNs. The C, H and O molar ratio of OCNs are 1.0: 0.02: 0.86 comparing to carbon black that EA could detected only carbon component. This result demonstrated the increase of oxygen component in OCNs by oxidation of carbon black.

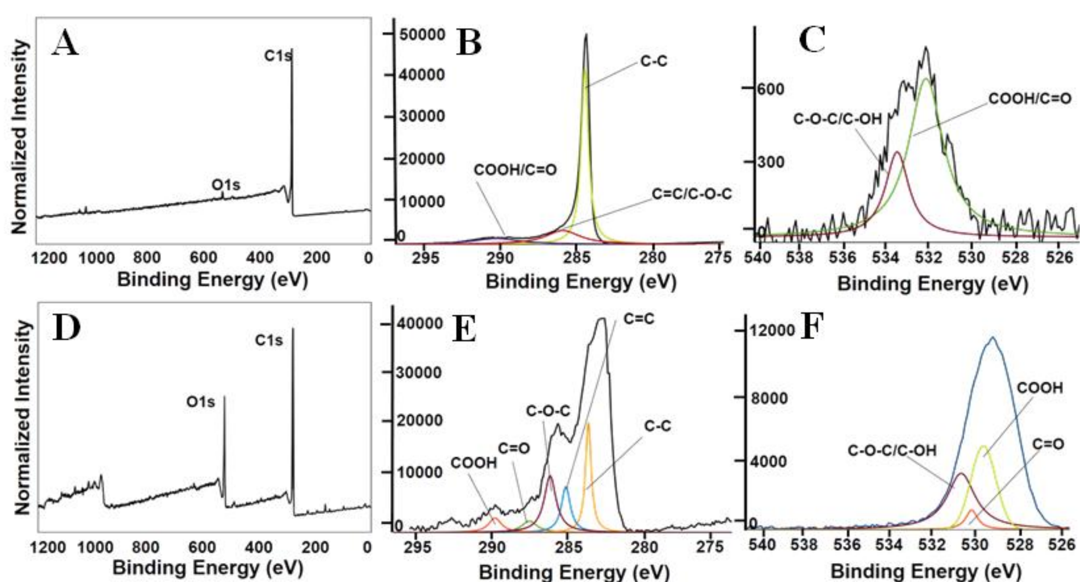

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Figure 4.8 XPS survey scan spectra, the XPS derived deconvoluted C1s and O1s spectra of carbon black (A, B, C) and OCNs (D, E, F).

4.2 Lipid Bilayer Membrane Penetration of OCNs with NIR irradiation

After the cell-sized liposome as lipid bilayer membrane was prepared by dehydration method. Then, TAMRA-OCNs (42 ppm) were added to the cell-sized liposome at room temperature, and the mixture was irradiated with NIR laser for period of times. I expected that NIR can accelerate the OCNs penetration rate into the cell-sized liposome by exciting OCNs, which can respond with NIR such as the increase velocity of OCNs in suspension. Unfortunately, CLSM images (Figure 4.9) showed that no difference of the OCNs penetration rate into the cell-sized liposome between using OCNs with NIR irradiation, and using OCNs without NIR irradiation after 1 h. Probably, the low concentration of OCNs is not enough to be respond by NIR irradiation. However, for application of OCNs in organisms, the concentration of OCNs cannot increase because more than 50 ppm of OCNs are toxic against Vero cell line. The second possibility is actually NIR irradiation onto the OCNs suspension cannot accelerate the OCNs penetration rate into the cell-sized liposome, but NIR irradiation may increase Brownian motion of OCNs in suspension just like the increase distribution.

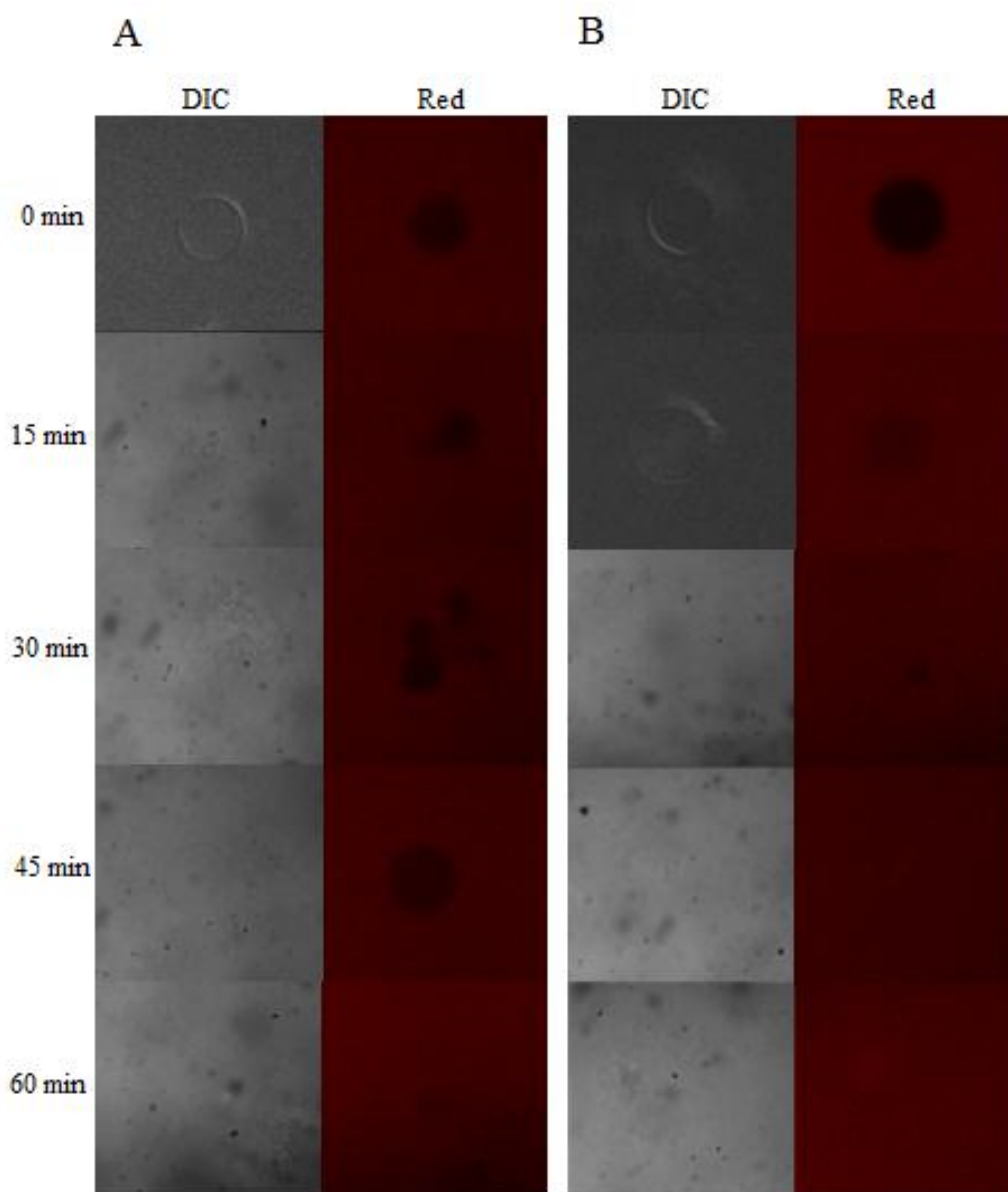

Figure 4.9 CLSM images of the cell-sized liposome incubated with TAMRA-OCNs (A), and the cell-sized liposome incubated with TAMRA-OCNs and NIR irradiation (B) at 0 (row 1), 15 min (row 2), 30 min (row 3), 45 min (row 4), and 60 min (row 5). Fluorescence signals of TAMRA-OCNs represented as red, when DIC is differential interference contrast mode.

4.3 Cytotoxicity of OCNs

Cytotoxicity of OCNs were evaluated against Vero cell line by MTT assay. Colorimetric assay was used for assessing cell metabolic activity. The highest concentration of OCNs was tested at 49.5 ppm. The results (Figure 4.10) showed that viability of Vero cell was around 80% at 20 ppm of OCNs. This demonstrates that OCNs can be used in Vero cell at up to 20 ppm with relatively non-toxicity. I chose the concentration of OCNs at 15 ppm for using OCNs with Vero cells.

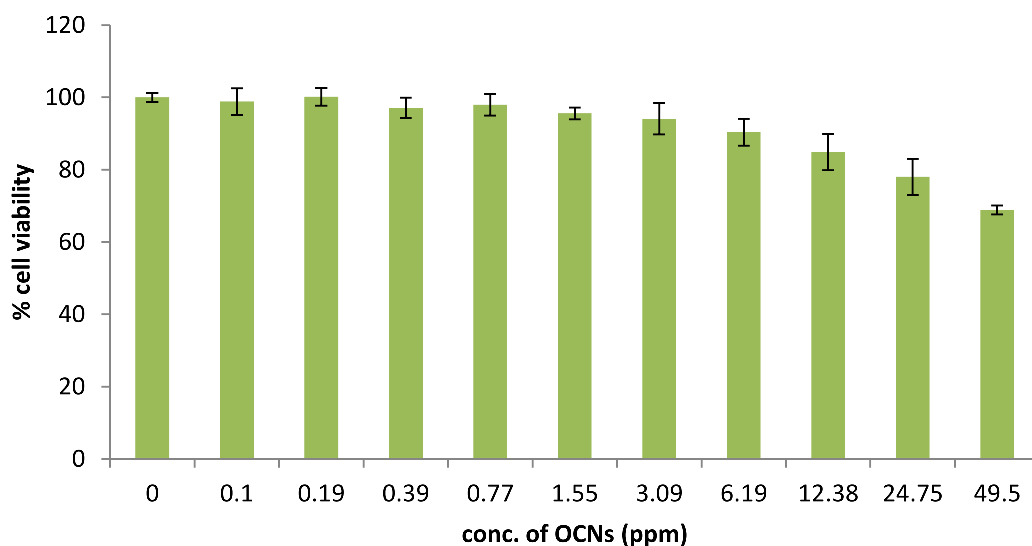

Figure 4.10 Cell viability of Vero cell line against OCNs.

4.4 Tracking Intracellular Delivery of HuMAbs

As oxidized carbon particles have been demonstrated to be able to make membrane leak [14, 20]. Thus, our particle can also do that. Therefore, our OCNs

were tested to bring the giant antibody (D23-4 F5E1, MW ~150 kDa, provided by CEAR, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand) into cells. Intracellular delivery of HuMAbs into Vero cell line was observed under confocal laser scanning microscope (CLSM). HuMAbs and OCNs were visualized by fluorescence-labeling. HuMAbs were labeled with fluorescein-5-isothiocyanate (FITC) and OCNs were labeled with 5,6-carboxytetramethylrhodamine (TAMRA). FITC-HuMAbs (64 μg/mL) with or without TAMRA-OCNs (15 ppm) were added into Vero cells and incubate at 37 °C for 30 min, 2 h and 4 h. After that, cells were washed and fixed to remove FITC-HuMAbs and TAMRA-OCNs outside the cells. Finally, nuclei were stained with DAPI. CLSM images (Figure 4.11) demonstrated that usage of FITC-HuMAbs with TAMRA-OCNs showed fluorescence signal of FITC-HuMAbs (green) and TAMRA-OCNs (red) inside cells, and fluorescence intensity of FITC-HuMAbs and TAMRA-OCNs increase when incubation time increase. Whereas, usage of FITC-HuMAbs without TAMRA-OCNs showed no green fluorescence signal of FITC-HuMAbs inside cells all the incubation time. These results with neutralization activity of HuMAbs verified that HuMAbs were brought into cells by OCNs, and intracellular HuMAbs still showed neutralization activity to dengue virus.

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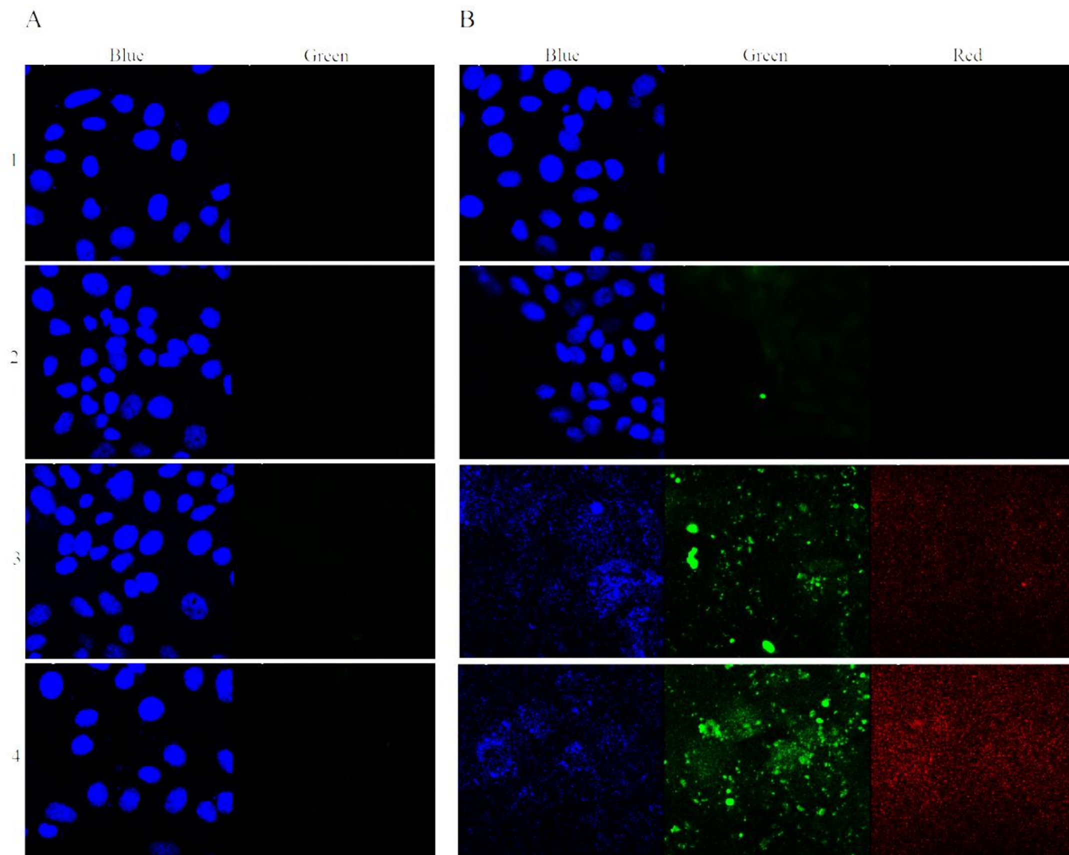

Figure 4.11 CLSM images of the incubation of FITC-HuMAbs without TAMRA-OCNs (A) and FITC-HuMAbs with TAMRA-OCNs (B) against Vero cells at 0 (row 1), 30 min (row 2), 2 h (row 3), and 4 h (row 4). Fluorescence signals of FITC-HuMAbs and TAMRA-OCNs represented as green and red, respectively. Nuclei were stained with DAPI (blue).

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4.5 Dengue Virus Neutralization

The ability of HuMAbs to neutralize DENV in Vero cell was evaluated by neutralization test by focus reduction test [29]. This assay evaluate amount of infectable DENV. After Vero cells were seeded in 96-well cell culture plate for 20 h.

Then, Vero cells were infected with 100 focus-forming units (FFU) of individual DENV serotypes. After 1 h, some of Vero cell was infected by DENV. Then, culture media was removed to eject DENV outside cells. This can decrease non-attached DENV outside cells. Next, HuMAbs with or without OCNs (15 ppm) were put into the cell culture (post infection) and incubated for 2 h. I expected that OCNs brought HuMAbs to neutralize DENV inside cells. After that, viscous culture media was added to limit virus spreading from infected cells. Cells were incubated at 37 °C for 2 (for DENV-4) or 3 days (for DENV-1, DENV-2 and DENV-3) to generate the group of DENV-infected cells. The group of infected cells were visualized by indirect immunofluorescence assay. First, cells were washed and fixed by 3.7% formaldehyde. Then, cells were permeabilized by 0.1% Triton X-100 to allow HuMAbs (primary antibody) interact to DENV-infected cells. Finally the bound antibody was reacted with an Alexa Fluor 488 goat anti-human IgG (H+L) to visualize the group of DENV-infected cells (focus, Figure 4.12). These results (Figure 4.13-4.16) showed the difference of neutralization activity of HuMAbs against each DENV serotypes. However, I observed reduction of focus of using HuMAbs with OCNs higher than using HuMAbs without OCNs about 2 times for all DENV serotypes, more focus reduction mean more neutralization activity of HuMAbs against intracellular DENV and extracellular DENV. From these results, I hypothesized that OCNs can bring HuMAbs into cells and then HuMAbs can neutralize intracellular DENV.

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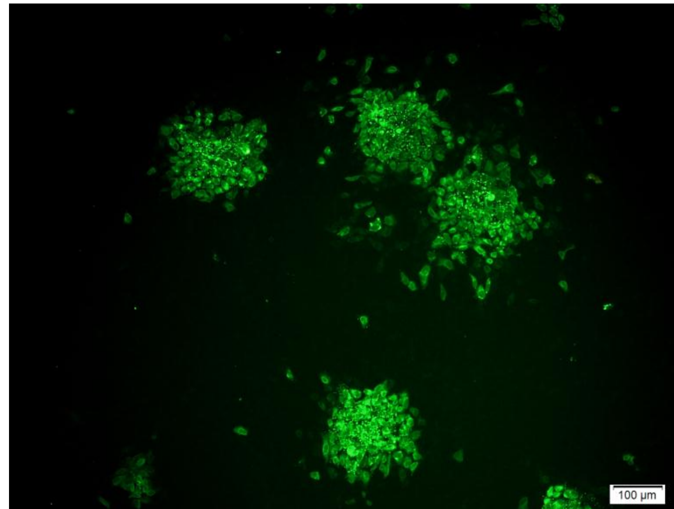


Figure 4.12 A photograph of the group of DENV-infected cells after a visualization.

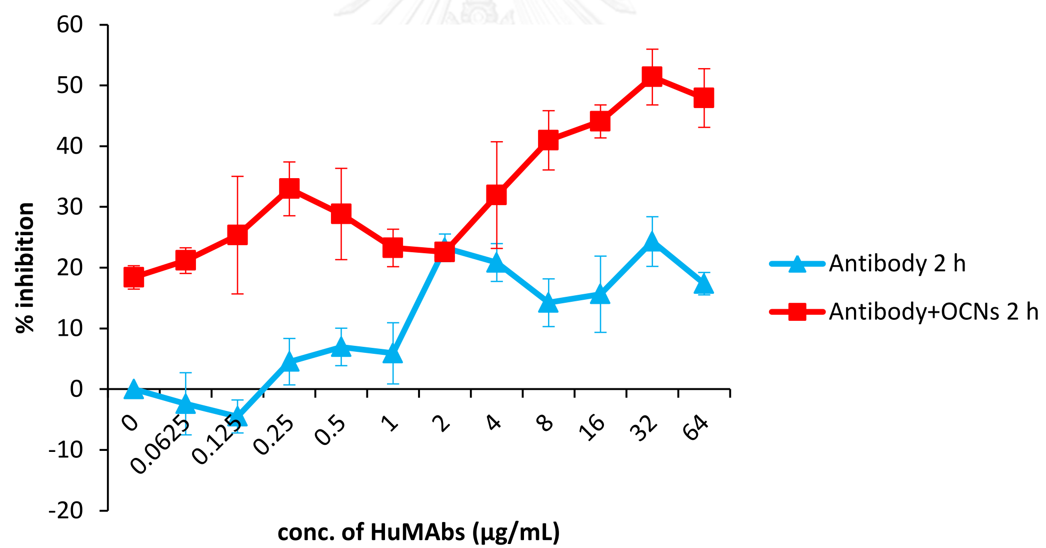


Figure 4.13 Neutralization activity of HuMAbs against DENV-1.

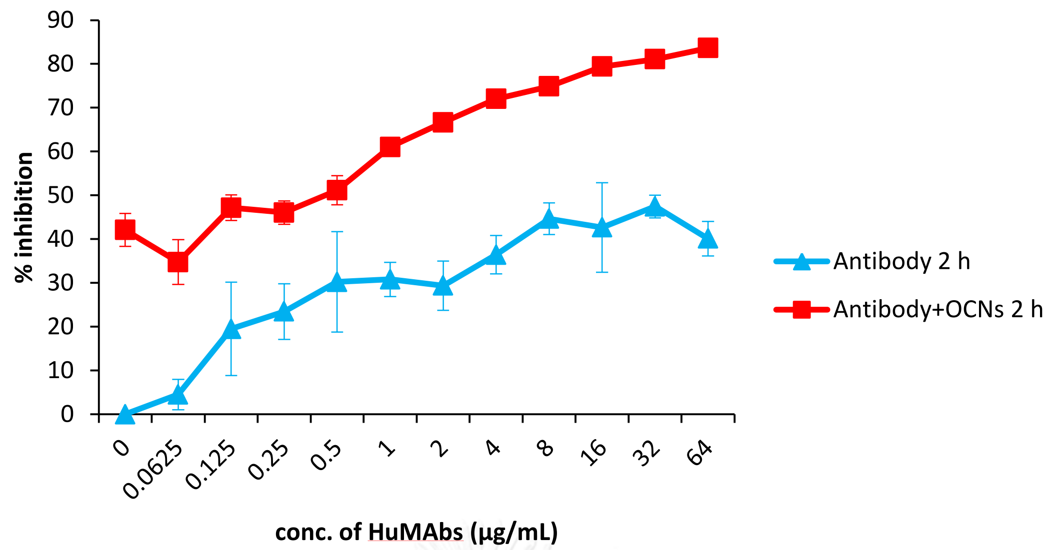


Figure 4.14 Neutralization activity of HuMAbs against DENV-2.

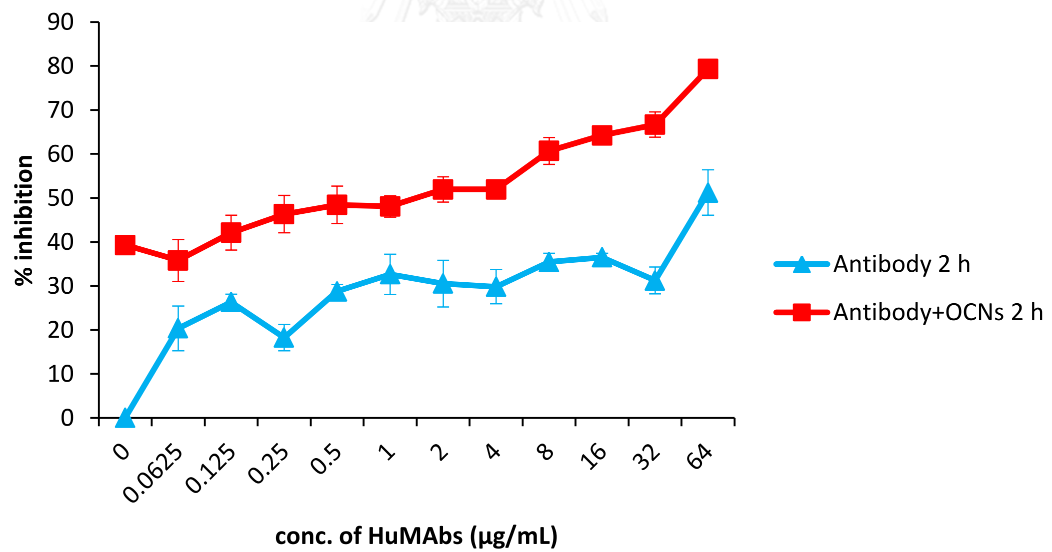


Figure 4.15 Neutralization activity of HuMAbs against DENV-3.

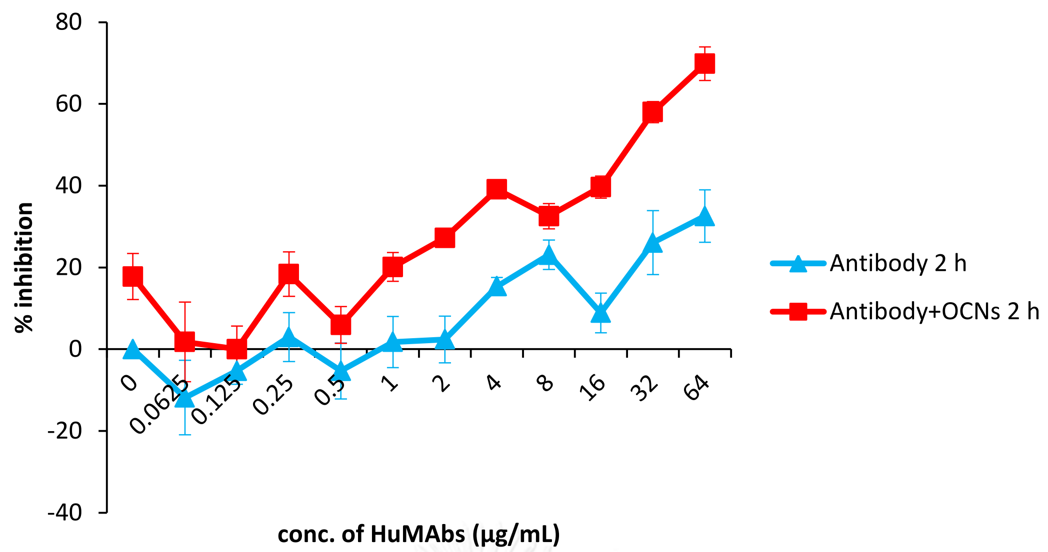
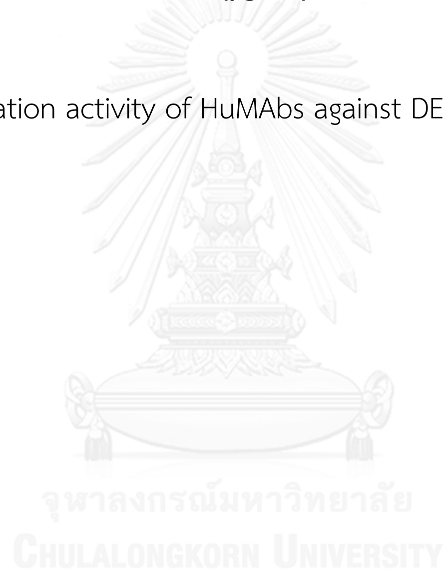


Figure 4.16 Neutralization activity of HuMAbs against DENV-4.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CHAPTER V
CONCLUSION

In this research, I can prepare oxidized carbon nanoparticles (OCNs) which can disperse in water from carbon black by modified Hummer and Offeman method. This suspension of OCNs are compatible with any aqueous medium. OCNs include the spherical shape of carbon network with the diameter of ~170 nm. OCNs exhibited a non-toxic against Vero cell line. I can use OCNs with human monoclonal antibodies (HuMAbs) to increase its neutralization activity to dengue virus at attachment step for all four dengue virus serotypes. Then, the translocation of OCNs and HuMAbs were tracked to confirm that OCNs can deliver HuMAbs into cells. The delivered HuMAbs can neutralize dengue virus inside the cell. Whereas, the using of HuMAbs without OCNs, HuMAbs cannot enter into cells. Furthermore, lipid bilayer membrane penetration of OCNs were investigated, which OCNs can penetrate the cell-sized liposome as lipid bilayer membrane. However, the using of OCNs with NIR irradiation cannot accelerate the OCNs penetration rate into the cell-sized liposome.

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