



CHAPTER III EXPERIMENTAL

3.1 Materials

3.1.1 Clay Mineral

Commercial sodium activated bentonite Mac-Gel[®] (GRADE SAC), Na-BTN, with cationic exchange capacity (CEC) of 44.5 meq/100g clay, was supplied by Thai Nippon Co., Ltd. Thailand.

3.1.2 Surfactant

Cetyl trimethyl ammonium bromide (CTAB) was purchased from Italmar Co., Ltd.

Dodecylamine (DDA) was purchased from Aldrich.

3.1.3 Chemical Substances

Tetraethoxysilane (TEOS) was purchased from Aldrich.

3-Aminopropyltriethoxysilane (APTES) was purchased from Sigma Aldrich.

3.1.4 Polymer

Polypropylene under trade name Moplen HP525N (MFI 11 dg/min) was purchased from HMC Polymers Co., Ltd.

Low density polyethylene under trade name (PETLIN LD C150Y) (MFI 5 dg/min) was purchased from PETLIN (MALAYSIA) Sdn Bhd.

3.1.5 Compatibilizer

Sodium-neutralized ethylene-co-methacrylic acid (MFI = 4 dg/min) under trade name Surlyn[®] PC350 was purchased from DuPont Co., Ltd.

3.1.6 Indicator

Methyl red ($C_{15}H_{15}N_3O_2$, MW 269.3 g/mol) was purchased from Panreac Quimica, Spain.

Bromothymol Blue ($C_{27}H_{28}Br_2O_5S$, MW 624.4 g/mol) was purchased from Ajax Finechem, Australia.

3.1.7 Reagent

Hydrochloric acid 37% was purchased from Calro Erba.

Boric acid was purchased from (H_3BO_3) was purchased from Calro Erba.

Potassium carbonate (K_2CO_3) was purchased from Calro Erba.

Trichloroacetic acid (TCA) was purchased from Calro Erba.

3.1.8 Solvent

Methanol was purchased from RCI Labscan Limited.

3.1.9 Sample

Fresh Barramundi was obtained from “SamYarn” market and filleted with aseptic techniques such as the use of disposable gloves and flame sterilized scalpel were used to avoid sample contamination.

3.2 **Equipment**

3.2.1 X-ray Diffractometer (XRD)

The X-ray diffractometer using Bruker AXS model Diffractometer D8 was used to identify the interlayer spacing of Na-bentonite and modified clay. The experiment was performed in the 2θ range of 2-10 degrees with scan speed 2 degree/min and scan step 0.01 degree.

3.2.2 Fourier Transform Infrared Spectrometer (FT-IR)

The functional group of Na-bentonite and modified clay were identified by Nicolet Nexus 670 FTIR spectrometer. FTIR was carried out in the transmission mode with 64 scans between $4000-400\text{ cm}^{-1}$ at a resolution of 4 cm^{-1} .

3.2.3 Scanning Electron Microscope (SEM)

The surface morphology of Na-bentonite and modified clay were observed by using S-4800 field emission scanning electron microscope. Then, the dispersion of the PCH in polymer matrix was determined. The specimens were coated with platinum before observation.

3.2.4 Surface Area Analyser (SAA)

The pore size, surface area and pore volume of porous samples were measured by nitrogen adsorption-desorption isotherms on surface area analyzer using Quantachrome Autosorb-1. The samples were degassed at 250°C for 17 h in a vacuum furnace before analysis.

3.2.5 CHNS Analyzer

The content of nitrogen in functionalized PCH was determined by CHNS analyzer (Leco, Two spec) and APTES content in functionalized PCH was calculated.

3.2.6 UV-Vis Spectrometer

The indicator dye (methyl red) was dissolved in water to prepare standard solution. The absorbance at 524 nm of the methyl red standard solutions and the functionalized PCH after adsorbed methyl red solution was observed by UV-Vis using Shimadzu Model UV-1800.

The indicator dye (methyl red or bromothymol blue) was dissolved in water to prepare standard solution. Optical absorption spectrum of standard solution and the solution of leaching of dye from nanocomposite film was observed by UV-Vis using Shimadzu Model UV-1800.

3.2.7 Twin Screw Extruder

The nanocomposites were prepared by using co-rotating twin-screw extruder (Labtech) with $L = 80$ and $D = 20$ mm. The operation temperature was performed at 170, 175, 180, 185, 190, 195, 200, 205, 210 and 215°C from hopper to die respectively and the screw speed was 50 rpm for prepared PP/Clay nanocomposites. The operation temperature was performed at 130°C from hopper to die respectively and the screw speed was 25 rpm for prepared LDPE/Clay nanocomposites.

3.2.8 Compression Molding Machine

The nanocomposite films were prepared by compression molding machine using Wabash V50H. The mold containing the pellet was preheated at 210°C for 5 minutes and then compression at 5 tons of force for 5 minutes for prepared PP/Clay nanocomposite films. The mold containing the pellet was preheated at 130°C for 5 minutes and then compression at 5 tons of force for 5 minutes for prepared LDPE/Clay nanocomposite films. Then, the molds were cooled to 50°C.

3.2.9 Gas Permeability Testing

Oxygen Permeation Tester, Illinois model 8000, was used to determine the oxygen gas transmission rate through PP films, PP/clay nanocomposite films,

LDPE films and LDPE/clay nanocomposite films. Gas permeation experiment was investigated following the procedure described in ASTM D 3985-05 at 23°C. The films were prepared by compression molding and were cut into circular shape with 15 cm in diameter.

3.2.10 Calorimetric Spectrophotometer

The nanocomposite films were measured the color change by the HunterLab Model Colorflex with 45°/0° optical geometry and EasyMatch[®] QC software. The result was expressed as Hunter color system (L, a, b) values and total color difference (TCD) or ΔE . The nanocomposite films were cut into the circular shape with diameter of 4 cm. The TCD value was calculated by following equation:

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

Where, ΔL = The brightness difference between sample and target

Δa = The redness difference between sample and target

Δb = The yellowness difference between sample and target

The target color is (93.13, -0.96, 1.69) corresponding to (L, a, b) for white standard color in Hunter system.

3.2.11 Thermogravimetric Analyzer (TGA)

Thermogravimetric analysis (TGA) was used to study the thermal stability of PP/clay nanocomposite films and LDPE/clay nanocomposite films compared to PP films and LDPE films. The degradation temperature of samples was determined by Perkin Elmer Pyris Diamond TG/DTA instrument. The pellets were loaded on platinum pan and heated from 30 to 900°C at heating rate 10°C/min and flow under N₂ 200 ml/min.

3.2.12 Differential Scanning Calorimeter (DSC)

The crystallization and melting behavior of PP/clay nanocomposite films and LDPE/clay nanocomposite films compared to PP films and LDPE films were measured by Differential Scanning Calorimeter (DSC) using Met.tler DSC822 STARe System. First, the pellet samples was heated from 30°C to 200°C at heating rate of 10°C/min in order to eliminate the influence of thermal history and then

cooled down from 200°C to 30°C to observe melt crystallization behavior. After that, reheated to 200°C to observe melting behavior.

3.2.13 Lloyd Universal Testing Machine

Tensile test of nanocomposite films was measured under ASTM D 683. The specimen was cut into rectangular shape with 10x100 mm and cross head speed of 50 mm/min.

3.3 Methodology

3.3.1 Fish Freshness Indicator

3.3.1.1 *Preparation of Organomodified Bentonite*

A 0.1 M solution of the surfactant (Cetyl trimethyl ammonium bromide, CTAB) was prepared. 3 g of clay (Sodium bentonite, Na-BTN) was added to 50 ml of this solution and the mixture was stirred for 24 h at 50°C. After the exchange cation reaction, the clay was filtrated from the solution and washed with the mixture of distilled water and methanol (1:1) until pH of 9 was reached. The modified clay was dried at 50°C for 24 h and ground into powder using centrifugal ball mill with 400 rpm for 30 min before screened through a mesh #325.

The interlayer spacing of Na-bentonite and modified clay were studied by small angle X-ray diffractometer. The intercalation of cationic surfactant between the layers of bentonite was studied using FTIR spectra.

3.3.1.2 *Preparation of Functionalized Porous Clay Heterostructure*

The synthesized organoclay was added to a neutral amine such as dodecylamine and the mixture of tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES) in the follow molar ratio: organoclay/dodecylamine/TEOS: APTES = 1/20/150. In first step, organoclay was mixed with dodecylamine and stirred at 50°C for 30 min. After that the mixture of TEOS and APTES in mole fraction of 0.97: 0.03, 0.95: 0.05 and 0.90: 0.10 was added to the mixture allowed reacting for 4 h at room temperature under continuous stirring by co-condensation method. After reaction, the modified clay was filtrated from the solution and washed with methanol. The modified clay was dried at room temperature for 24 h. For extraction process, 1 g of as-synthesized PCH was added to 45 ml of methanol and 5

ml of concentrated HCl and refluxed for 2 h. Then, the modified clay was filtered off and washed with methanol and water and air-dried at room temperature overnight before screened through a mesh #325. The modified clay by APTES was named APPCH.

The organic group incorporated into porous structure of APPCH was studied by FTIR spectra. The content of nitrogen in functionalized APPCH was determined by CHNS analyzer and APTES content in functionalized PCH was calculated. The surface morphology of porous clay compared to the pristine clay was observed by SEM images. The pore diameter, pore volume and surface area of porous clay compared to the pristine clay were characterized by surface area analyzer.

For dye adsorption experiment, 0.1 g of APPCH was mixed with 50 ml aqueous solution containing 2×10^{-4} M MR. After one day, the residual MR concentration was determined compared with the initial concentration of MR by UV-Vis using Shimadzu Model UV-1800 at 524 nm. The amount of methyl red (MR) adsorbed onto the APPCH compared to PCH was calculated following equation (Wu *et al.*, 2006)

$$q = \frac{V(C_0 - C)}{mS}$$

Where, q	=	The amount of MR adsorbed onto porous sample (mol/m ²)
C ₀	=	The initial concentration of MR solution (mol/l)
C	=	The residual concentration of MR solution (mol/l)
V	=	The solution volume (l)
m	=	Mass of porous sample (g)
S	=	BET surface area of porous sample (m ² /g)

3.3.1.3 Preparation of Chromophores Modified Functionalized PCH

The functionalized PCH from 3.3.1.2 (APPCH) was consequently modified with pH dye (methyl red) at various weight ratio of APPCH: dye (10:1, 20:1 and 30:1). First, methyl red is dissolved in 0.05M HCl solution. Then, methyl red solution was added into the functionalized PCH under continuous stirring to obtain chromophores modified functionalized PCH. After that the

modified clay was filtered off and dried in vacuum oven before screened through a mesh #325. The methyl red modified APPCH was named APPCH-MR.

The morphology of APPCH-MR was observed by SEM images. The results of pore diameter, pore volume and surface area after incorporated methyl red onto APPCH were measured by surface area analyzer.

3.3.1.4 Preparation of Nanocomposite Film

The nanocomposite was prepared by using twin-screw extruder (Labtech) with L = 80 and D = 20 mm. The operation temperature was performed at 170, 175, 180, 185, 190, 195, 200, 205, 210 and 215°C from hopper to die respectively and the screw speed was 50 rpm.

First, polypropylene (PP) was blended with 6%wt surlyn® in order to obtain PP/surlyn® pellet. Then, PP/surlyn® pellet was mixed with 2%wt of chromophores modified functionalized PCH to obtain the nanocomposite. Each composition was dried in vacuum oven for moisture removal and premixed in tumble mixer before extruded through the twin screw extruder. Then, the extruded nanocomposite was quenched immediately in water and pelletized. The obtained pellet was dried in vacuum oven.

Nanocomposite films of PP/chromophores modified functionalized PCH was prepared by compression molding machine at 5 tons of force for 5 minutes. The processing temperature was 210°C.

The clay contents and thermal behavior of nanocomposite was investigated by TGA. Crystallization and melting behavior of PP compared to nanocomposite were observed by DSC.

The mechanical properties of PP compared to nanocomposite were observed by Lloyd Universal Testing Machine.

Oxygen gas transmission rate through PP films and PP/clay nanocomposite films was measured by Oxygen Permeation Tester.

3.3.1.5 Indicator Films Response to Standard Ammonia

Study of sensor response to standard ammonia was adopted based on the method used by Xiuchen (2000).

The main classes of volatile compounds which characterize the spoilage odor of fish such as amine were tested to estimate the sensitivity of the color

response. Ammonia was selected from amine group. 25 ml of standard ammonia solution in various concentrations (10, 25, 50, 100, 250 and 500 ppm) were pipette into the well which was sealed to prevent the leakage of amine. The indicator film was faced down inside the well that contained standard ammonia. The experiment was kept at room temperature until the color response of indicator film saturated to change color. The color response of nanocomposite films at various weight ratio of APPCH and methyl red (10:1, 20:1 and 30:1) was measured by the calorimetric spectrophotometer. The result was expressed as Hunter color system (L, a, b) values and total color difference (TCD) or ΔE .

3.3.1.6 Determination of Total Volatile Basic Nitrogen (TVB-N)

The method of determination of TVB-N from fresh fish in order to determine the fish spoilage will be adopted from the method used by Fish Inspection and Quality Control Division (FIQD), The Department of Fisheries (DOF), Thailand (Seephung A., 2008).

3.3.1.6.1 Sample Preparation

Grind the sample by homogenizer and 3 g of homogenized sample was placed into a centrifuge tube. Then, 12 ml of 4% trichloroacetic acid (TCA) solution was added to the centrifuge tube and the tube was sealed and shaken to make sure that it was properly mixed. After that, the sample was left at room temperature for 30 min with stirring from time to time. The sample mixture was filtrated using Whatman paper no.1. When freshly prepared samples were not used within a day for further analysis, the filtrated solution must be kept at -18°C in vials.

3.3.1.6.2 Measuring of TVB-N

Sealing agent (Vaseline) was first applied to the top edge of convey's unit. The inner ring solution (1% boric acid mixed with 1 ml of indicator) was pipette and placed into the inner ring of conway's unit (volatile compounds from sample extract would diffuse into boric acid salt and these salts would be reduced to HCl-salts by strong HCl during titration). Then, 1 ml of filtered sample extract was pipette into the outer ring of conway's unit. 1 ml of saturated K_2CO_3 solution was pipette into the outer ring of conway's unit and placed on the opposite side of the sample solution (to made sample extract into alkalines condition

similar to that of volatile compound). The convey's unit was immediately shaken in order to mixed sample solution and K_2CO_3 solution without contaminating the inner ring of convey. Stand the samples were kept at room temperature for 3 h. After the color of boric solution changed from pink to green (Figure 3.1), following the generation of volatile base, this sample was titrated with 0.01 N HCl containing in a micro-burette until the color changed back to pink (Figure 3.2).

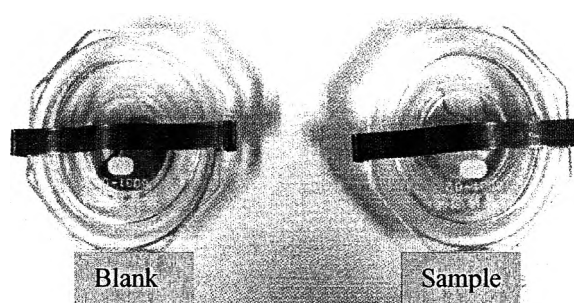


Figure 3.1 The color of boric solution changed from pink to green.

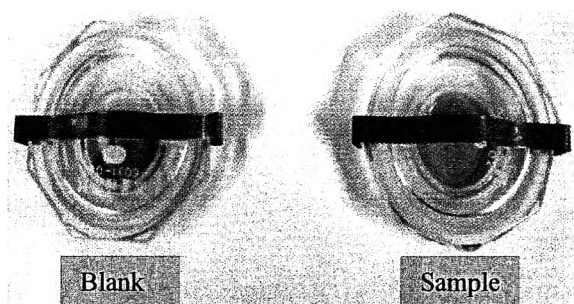


Figure 3.2 The color changed back to pink after titrated with 0.01 N HCl.

Note: Blank test was carried out using 1 ml of 4%TCA instead of sample extraction

3.3.1.6.3 Calculation

$$\text{TVB-N (mg/100g)} = \frac{(V_S - V_B) \times (N_{\text{HCl}} \times A_N) \times [W_S \times (M/100) + V_E] \times 100}{W_S}$$

Where, V_S = Titration volume of 0.01 N HCl for sample extract (ml)

V_B = Titration volume of 0.01 N HCl for blank (ml)

N_{HCl} = Normality of HCl (= 0.01 N \times factor of HCl)

A_N	=	Atomic weight of nitrogen (14.00)
W_S	=	Weight of tissue sample (g)
M	=	Percentage moisture of tissue sample (Assume 80%)
V_E	=	Volume of 4% TCA used in extraction

3.3.1.7 Indicator Films Response to Fresh Fish Tissue

Fish tissue samples of approximately 20 g were placed in separated individual wells. Each well was sealed to prevent the leakage of amine. Indirect contact between indicator films and fish sample was obtained. The response of indicator film with fish sample was observed at room temperature every 3 hours for 12 hours. The color response of nanocomposite films at various weight ratio of APPCH and methyl red (10:1, 20:1 and 30:1) was measured by the calorimetric spectrophotometer. The result was expressed as Hunter color system (L, a, b) values and total color difference (TCD) or ΔE .

3.3.1.8 Leaching Studies

Indicator dye (methyl red) was dissolved in water to prepare standard solutions of 1, 2, 5 and 10 ppm. Then the absorbance of standard solution was investigated by UV-Vis Spectroscopy (Shimadzu Model UV-1800). The indicator nanocomposite films were cut into the rectangular shape with 4x4 cm and soaked with 10 ml of water in a small container for approximately 48 h. Then, the wavelength spectrum in range of 400 to 700 nm was carried out to detect the presence of methyl red.

3.3.2 Preparation of Climacteric Fruit Freshness Indicator

3.3.2.1 Preparation of Porous Clay Heterostructure (PCH)

The obtained organoclay prepared with the surfactant (Cetyl trimethyl ammonium chloride, CTAC) was added to a neutral amine such as dodecylamine and tetraethoxysilane (TEOS) in the follow molar ratio: organoclay/dodecylamine/TEOS = 1/20/150. In first step, organoclay was mixed with dodecylamine and stirred at 50°C for 30 min. After that tetraethoxysilane (TEOS) was added to the mixture allowed reacting for 4 h at room temperature under continuous stirring. After reaction, the modified clay was filtrated from the solution and washed with methanol. The modified clay was dried at room temperature for 24 h. For extraction process, 1 g of as-synthesized PCH was added to 45 ml of methanol

and 5 ml of concentrated HCl and refluxed for 2 h. Then, the modified clay was filtered off and washed with methanol and water. After that, it was air-dried at room temperature overnight before screened through a mesh #325.

The interlayer spacing of modified clay was studied by small angle X-ray diffractometer. The intercalation of cationic surfactant between the layers of bentonite was studied using FTIR spectra.

The surface morphology of porous clay compared to the pristine clay was observed by SEM images. The pore diameter, pore volume and surface area of porous clay compared to the pristine clay were characterized by surface area analyzer.

3.3.2.2 Preparation of Chromophores Modified PCH

The PCH was modified with pH dye (bromothymol blue) at weight ratio of PCH: dye of 10:1, 20:1 and 30:1. First, bromothymol blue was dissolved in solution of 0.05 M NaOH. Then, bromothymol blue solution was added into the PCH under continuous stirring to obtain chromophores modified PCH. After that the modified clay was filtered off and dried in vacuum oven before screened through a mesh #325. The bromothymol blue modified PCH was named PCH-BTB.

The morphology of APPCH-BTB was observed by SEM images. The results of pore diameter, pore volume and surface area after incorporated bromothymol blue onto PCH were measured by surface area analyzer.

3.3.2.3 Preparation of Nanocomposite Film

The nanocomposite was prepared by using twin-screw extruder (Labtech) with $L = 80$ and $D = 20$ mm. The operation temperature was performed at 130°C from hopper to die respectively and the screw speed was 25 rpm.

First, low density polyethylene (LDPE) was blended with 6%wt surlyn[®] in order to obtain PP/surlyn[®] pellet. Then, PP/surlyn[®] pellet was mixed with 2 %wt of chromophores modified PCH to obtain the nanocomposite. Each composition was dried in vacuum oven for moisture removal and premixed in tumble mixer before extruded through the twin screw extruder. Then, the extruded nanocomposite was quenched immediately in water and pelletized. The obtained pellet was dried in vacuum oven.

Nanocomposite films of LDPE/chromophores modified PCH was prepared by compression molding machine at 5 tons of force for 5 minutes. The processing temperature was 130°C.

3.3.2.4 Indicator Films Response to Standard Carbon dioxide

The main products from respiration of climacteric fruits such as carbon dioxide and water were used to test the sensitivity of color response of indicator films. The indicator film was placed in glass chamber with contained standard carbon dioxide and water. Standard carbon dioxide in various concentrations (0, 30, 60, 90, 120 and 150 ppm) and the excess water was prepared into 600 ml glass chamber. Then, the glass chamber was closed by the rubber stopper with the rubber septum in the middle of the stopper. Color changes of indicator film at weight ratio of PCH: dye of 10:1, 20:1 and 30:1 after reacted with carbon dioxide and water was measured by the calorimetric spectrophotometer. The result was expressed as Hunter color system (L, a, b) values and total color difference (TCD) or ΔE .

3.3.2.5 Leaching Studies

Indicator dye (bromothymol blue) was dissolved in water to prepare standard solutions of 1, 2, 5 and 10 ppm. Then the absorbance of standard solution was investigated by UV-Vis Spectroscopy (Shimadzu Model UV-1800). The indicator nanocomposite films were cut into the rectangular shape with 4x4 cm and soaked with 10 ml of water in a small container for approximately 48 h. Then, the wavelength spectrum in range of 400 to 700 nm was carried out to detect the presence of bromothymol blue.