PIEZOELECTRIC-BASED IMMUNOSENSOR FOR OXIDIZED LOW DENSITY LIPOPROTEIN DETERMINATION

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ABSTRACT

Oxidized low density lipoprotein (OxLDL) is a crucial contributor in the progression of atherosclerosis. The elevation of OxLDL in circulating is associated with pathogenesis of atherosclerosis. Therefore, OxLDL is a biomarker for preventing and monitoring atherosclerosis progression. A piezoelectric immunosensor was developed for OxLDL detection. The OxLDL immunosensor was fabricated by immobilizing anti-OxLDL on silver electrode surface of 10 MHz AT-cut quartz crystal via self-assembled monolayer (SAM) of mercaptopropionic acid (MPA). The resonant frequency shift of this sensing was monitored by using RQCM under liquid system when the mass accumulated on the electrode surface. The optimal condition of immobilizing was 100 µg/ml anti-OxLDL for 30 minutes. The immobilized antibody in OxLDL immunosensor performed binding with OxLDL. Dose response curve for OxLDL measurement showed a linear response from 0.5 to 3.10 µmole/l. The limit of detection of OxLDL was 0.5 µmole/l. An analytical accuracy of this sensing was 97.02%. The intra-assay and inter-assay precision (CVs) were 9.37 and 23.04%, respectively. This system was successfully developed to determine the amount of OxLDL. Therefore, this immunosensor would be proposed for measuring OxLDL concentration in clinical diagnosis.

KEY WORDS: ATHEROSCLEROSIS / BIOSENSOR / IMMUNOSENSOR / OXIDIZED LOW DENSITY LIPOPROTEIN (OXLDL)

90 pages

อิมมูโนเซนเซอร์สำหรับการวัดปริมาณไลโปโปรตีนชนิดความหนาแน่นต่ำที่ถูกออกซิไดส์
PIEZOELECTRIC-BASED IMMUNOSENSOR FOR OXIDIZED LOW DENSITY LIPOPROTEIN DETERMINATION

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บทคัดย่อ

ปัจจุบันโรคหลอดเลือดแดง แข็งตัวเป็นสาเหตุของการตายอันดับหนึ่งของประชากรโลก การเสื่อมของหลอดเลือดแดงทำให้ขาดเลือดไปเลี้ยงหัวใจและสมองซึ่งทำให้เสียชีวิต ได้อย่างเฉียบพลัน OxLDL เป็นปัจจัยหนึ่งในการเริ่มต้นที่ทำให้เกิดภาวะหลอดเลือดแข็งตัว หากมีปริมาณเพิ่มขึ้นสามารถนำมาใช้เป็นตัวชี้วัดความเสี่ยงในการเกิดโรคของหลอดเลือดแดงได้การศึกษานี้เป็นการพัฒนาระบบอิมมูโนเซนเซอร์เพื่อใช้ในการตรวจวัดปริมาณ OxLDL การพัฒนาอิมมูโนเซนเซอร์นี้ทำโดยการตรึงแอนติบอดีต่อ OxLDL บนผิวอิเล็กโทรดของผลึกควอตซ์ชนิด AT-cut ที่มีความถี่พื้นฐาน 10 MHz โดยอาศัยหลักการ self-assembled monolayer ของ mercaptopropionic acid ซึ่งความถี่ที่เปลี่ยนไปเกิดจากมวลของสารที่สะสมอยู่บนผิวของผลึกควอตซ์ภายใต้ระบบของไหล นั้นสามารถติดตามได้จากเครื่องวัดความถี่ RQCM จากการศึกษาพบว่าปริมาณและเวลาที่เหมาะสมในการตรึงแอนติบอดีต่อ OxLDL คือ 100 µg/ml ที่ 30 นาที โดยมีความสามารถในการวัดปริมาณ OxLDL อยู่ในช่วงความเข้มข้น 0.5–3.10 µmole/l โดยข้อจำกัดความเข้มข้นของ OxLDL ที่น้อยที่สุดในระบบที่สามารถวัดได้คือ 0.5 µmole/l ความถูกต้องในการวิเคราะห์คือ 97.02% ส่วนความแม่นยำในการวัดชนิด intra-assay และ inter-assay เท่ากับ 9.37 และ 23.04% ตามลำดับ ระบบอิมมูโนเซนเซอร์ต้นแบบนี้สามารถใช้ในการตรวจวัดปริมาณ OxLDL ได้

90 หน้า

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LIST OF ABBREVIATIONS

Anti-OxLDL	Antibody for oxidized low density lipoprotein
BSA	Bovine serum albumin
^{o}C	Degree Celsius
C.V.	Coefficient of variation
DW	Distilled water
ELISA	Enzyme-linked immunosorbent assay
F_0	Fundamental frequency
ΔF	Resonant frequency shift
g	Gram
Hz	Hertz
L.	Liter
M	Molar
mm	Millimeter
mg	Milligram
min	Minute
nm	Nanometer
PBS	Phosphate buffer saline
QCM	Quartz crystal microbalance
RQCM	Research quartz crystal microbalance system, MAXTEK INC.
RT	Room temperature
S.D.	Standard deviation
µg	Microgram

CHAPTER I
INTRODUCTION

Atherosclerosis is an inflammation disease of the arterial wall involving formation of plaque in the intima layer or subendothelial space. It facilitates cardiovascular disease (CVD) leading causes of death in worldwide (1). There are many risk factors associated with atherosclerosis such as low density lipoprotein (LDL) level, high density lipoprotein (HDL) level, blood pressure, obesity, diabetes mellitus, and smoking. The high circulating LDL in plasma can be trapped into intima layer of the arterial wall and these LDL molecules can become to oxidation by many mechanisms such as free radicals, metal ions, lipoxygenase, and myeloperoxidase, called, oxidized LDL (OxLDL) (2, 3). Then, the OxLDL is taken up by macrophages via scavenger receptors, leading to accumulate of foam cells in atherosclerosis plaque and inflammation in the arterial wall (4). Therefore, OxLDL is the important contributor and major risk factor of atherosclerosis. The elevated levels of OxLDL in circulation can be indicated the pathogenesis of atherosclerosis. Thus, OxLDL would be one of biomarker for predicting and monitoring atherosclerosis events (5, 6).

Several investigation methods have been used to measure plasma OxLDL level such as thiobarbituric acid reactive substances (TBARS) assay, conjugated diene assay, electrophoretic mobility method, nuclear magnetic resonance spectroscopy (NMR), high performance liquid chromatography (HPLC), mass spectrometry (MS), and enzyme-linked immunosorbent assay (ELISA) (7-10). TBARS assay and conjugated diene are general used for measurement the plasma OxLDL (11, 12). TBARS assay can be measured the reaction of thiobarbituric acid and malondialdehyde (TBA-MDA) complex to yield the secondary product of lipid peroxidation. However, MDA can be generated from the interferences therefore it is not the final reaction product of lipid peroxidation (13). For conjugated diene assay, the principle of this method is used to measure rearrangement of double bonds in polyunsaturated fatty acid (12). Both methods are simple and rapid, but they are non-specific and low limit detection for LDL oxidation (7, 14). The electrophoretic

mobility method is used to detect OxLDL based on the charge migration of molecules in the agarose gel. This method is high sensitivity for in vitro, but it is low sensitivity in the clinical sample (7). Moreover, the amount of plasma OxLDL can be determined by nuclear magnetic resonance spectroscopy (NMR), high performance liquid chromatography (HPLC), and mass spectrometry (MS). These methods are inconvenience for clinical laboratory regarding to the complexity of technique and the running cost of analysis. Recently, enzyme-linked immunosorbent assay (ELISA) was developed to measure plasma OxLDL using monoclonal antibodies (mAbs) such as E06, FOH1a/DLH3, and 4E6 antibodies that recognized with specific structure of OxLDL (15, 16). However, this method provides high sensitivity and specificity for the analytical performance, but it has high cost per unit and long assay time. Therefore, the alternative detection methods of plasma OxLDL should be developed to solve the weakness of those methods.

Piezoelectric immunosensor is one type of biosensing device that has been developed by integrating the immunoassay coupled with the sensing device. The piezoelectric-based immunosensor is based on antigen-antibody interaction which causes mass deposition on the surface of sensing element. This measurable mass change has direct correlation to the frequency change sensing device. It is highly sensitive to mass change without the requirement of labeling system. Regarding to this simple detection concept, the assay volume of both reagent and sample is generally less than other technique. This system has been applied in environment, chemical analysis, and clinical detection including lipoprotein particle measurement such as LDL and HDL (17-19). Moreover, this measuring system can operate both in gaseous and liquid phases (20, 21).

The aim of this study is to develop the OxLDL immunosensor for detecting plasma OxLDL in the circulation. This OxLDL immunosensor was developed based on piezoelectric quartz crystal for measuring plasma OxLDL by immobilizing anti-OxLDL onto modified quartz crystal surface via self-assembled monolayer (SAM) method. The anti-OxLDL played the key role of binding interaction between OxLDL particle and anti-OxLDL. This binding interaction created the accumulation of mass on the surface of the sensing device which was monitored the resonant frequency shift of quartz crystal by the oscillator frequency counter. The

system optimization of anti-OxLDL immobilization and other involved parameters in this study were also investigated such as accuracy, precision, specificity, limit of detection and others. In this study, the immunosensor based on piezoelectric technology was developed to be a new method for OxLDL measurement and it would be useful for monitoring the atherosclerosis events in clinical work in the future.

CHAPTER II
OBJECTIVES

The main objectives of this study consist of:

1. To create the OxLDL immunosensor for quantification of OxLDL by using piezoelectric-based quartz crystal immunosensor in liquid phase.
2. To optimize the OxLDL immunosensor system for quantification of OxLDL, including flow rate, antibody immobilization, and binding ability of antibody.
3. To evaluate the reliability of OxLDL immunosensor assay in term of detection range, accuracy, precision, sensitivity, and specificity.

The expected outcome of this study:

The expected result of this OxLDL immunosensor is efficacy to monitor OxLDL. Moreover, the main propose of this study is to obtain a new analytical method for OxLDL detection that would be utilized in the clinical diagnostic.

CHAPTER III
LITERATURE REVIEWS

3.1 Atherosclerosis

Atherosclerosis is a disease of the arterial wall which is characterized by lipid accumulation, chronic inflammation, cell death, and thrombosis. This disease is an important problem of cardiovascular disease (CVD) that claims lives in the developing counties. The progression of atherosclerosis develops from the high circulating LDL in plasma. The LDL molecules transport across the endothelial cell and trap into intima layer of the arterial wall. These LDL are modified to oxidized LDL (OxLDL) by cell-mediated oxidation such as free radicals, lipoxygenase, and myeloperoxidase. Then, OxLDL stimulates immune system to release the circulating monocyte leading to monocyte trap into intima layer and transform themselves to macrophages. After that, the OxLDL uptake by macrophages via scavenger receptor leading to accumulation of foam cell called atherosclerosis plaque. Finally, the formation of thrombus and the inflammation in the arterial wall are developed after the plaque ruptured resulting in CVD (1, 22, 23). Therefore, the pathogenesis of atherosclerosis associated with the oxidative modification of LDL. It can be produced the formation of plaque in intima layer leads to reduce blood supply and developing inflammatory in the arterial wall. Moreover, OxLDL is a biomarker for monitoring atherosclerosis risk factor. The progression of atherosclerosis in the arterial wall is shown in Figure 3.1.

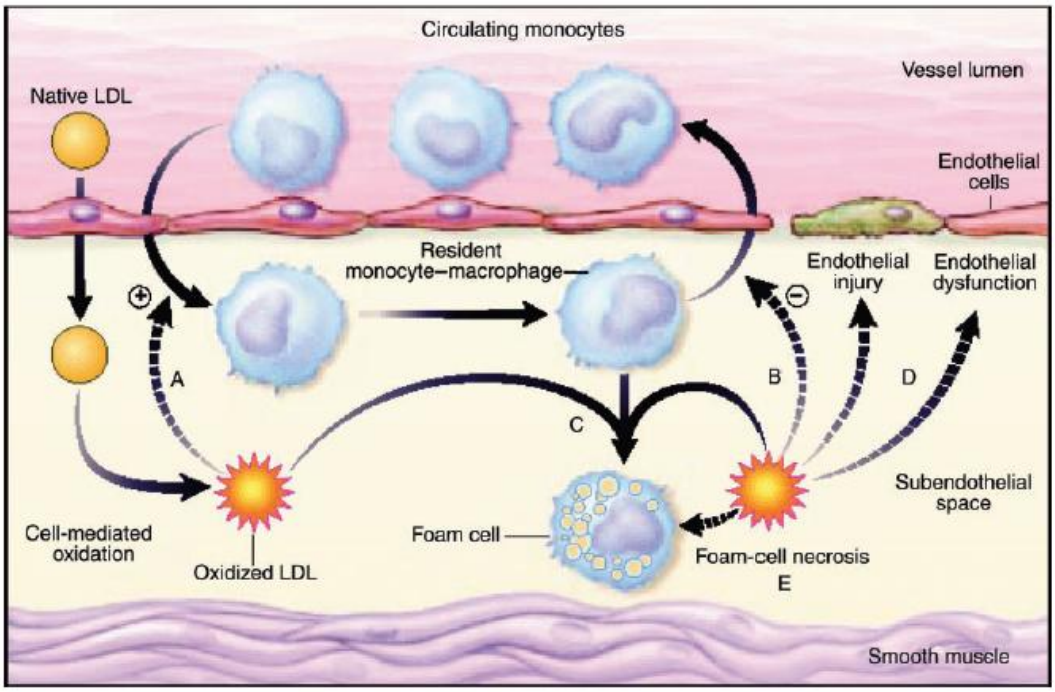

Figure 3.1 The progression of atherosclerosis in subendothelial space of the arterial wall (24). Native LDL trapped in the subendothelial space and oxidized to oxidized LDL (OxLDL) by cell-mediated oxidation. OxLDL stimulated immune system to release monocyte that trapped into subendothelial space and become macrophage (A). Macrophage ingested OxLDL resulting in formation of foam cell (C). OxLDL can cause endothelial injury and dysfunction leading atherosclerosis (D).

3.1.1 Risk factors of cardiovascular disease

There are many risk factors that used to predict the incident of cardiovascular disease such as

3.1.1.1 Age

The risk of developing cardiovascular disease is average for a 30 to 34 year old male when compared with other individual aged. Therefore, age is a one factor for incident cardiovascular disease (25).

3.1.1.2 Gender

Many studies reveal that males exhibit excess risk for cardiovascular disease compared with age-matched women. Estrogens act as a protective effect of cardiovascular disease in woman after menopause leading to the decreasing of cardiovascular disease risk factor (26).

3.1.1.3 Obesity

Stoker R et al. described that the excess body weight with an abnormal high preponderance of body fat is a condition that increases the incident risk of cardiovascular disease (27).

3.1.1.4 Cigarette smoking

The increasing cigarette smoking is linked to a cause of heart disease, myocardial infarction, atherosclerosis, and coronary heart disease (28).

3.1.1.5 Hypertension

The hypertensive patients are related to the risk of cardiovascular disease. The elevation of blood pressure leads to the increased incident of atherosclerosis (29).

3.1.1.6 Diabetes mellitus

The diabetes patients, the risk of coronary artery disease and atherosclerosis are three to five fold greater than in non-diabetics despite controlling for other risk factors (30).

3.1.1.7 Serum cholesterol

The increasing LDL cholesterol is associated with atherosclerosis due to a functional of LDL receptor impaired and defected in clearance of LDL (1).

3.2 Lipoprotein

Lipoprotein is macromolecular complex that contains both proteins and lipids. The protein and lipid are hold together by non-covalent force. The functional of lipoprotein is transport lipid into the blood circulation. Lipoproteins are water-soluble spherical particles including the hydrophobic core and the outer surface monolayer. The hydrophobic core consists of triglyceride (TG) and cholesteryl ester (CE) and the

outer surface monolayer includes phospholipid (PL), unesterified cholesterol (UC), and specialized protein called apolipoprotein (apo) as shown in Figure 3.2. Lipoproteins are classified on the basis of density, size, and electrophoretic mobility into 5 major classes including chylomicron (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). The different of each lipoprotein classes is depended on the amount of the component and the different apolipoprotein type on their outer surface (31, 32). Therefore, the characteristics of the major classes of lipoprotein are represented in terms of density, molecular weight, diameter, migration during electrophoresis, and functional of each lipoprotein as shown in Table 3.1. In addition, the chemical compositions of each lipoprotein are shown in Table 3.2.

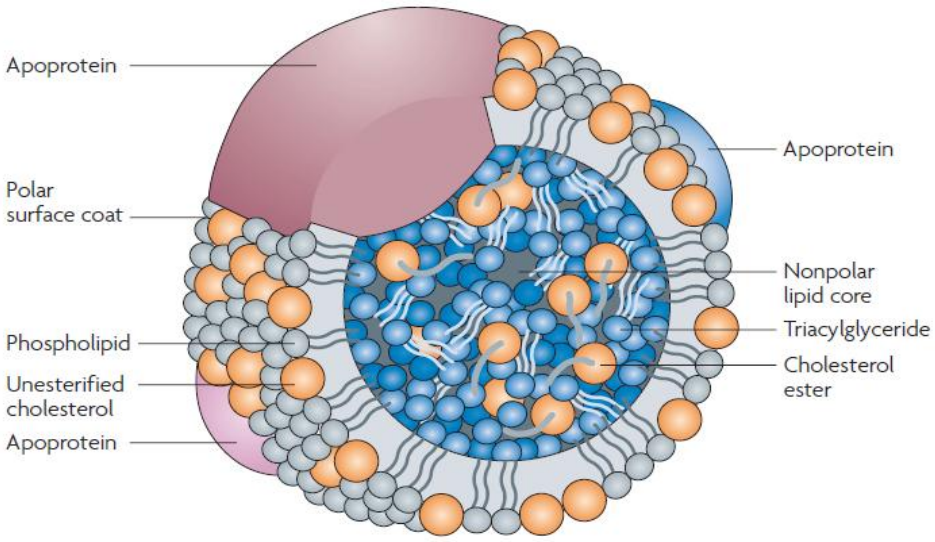

Figure 3.2 Structure of lipoprotein particle (31). This particle contains hydrophobic core: triglyceride and cholesteryl ester and outer surface monolayer: phospholipid, unesterified cholesterol, and apolipoprotein. Reprinted from Journal of Nature Reviews Drug Discovery, Vol. 7, Wasan KM, Brocks DR, Lee SD, Sachs-Barrable K, Thornton SJ, Impact of lipoproteins on the biological activity and disposition of hydrophobic drugs: implications for drug discovery, P.84-99, Copyright (2008), with permission from Nature Reviews Drug Discovery (Permission Number: 3120590060327).

Table 3.1 Characteristics and function of major lipoprotein (31-33)

Characteristics	Chylomicron	VLDL	IDL	LDL	HDL
MW×10^{-6}	504	19.6	nr	2.3	0.36
Density (g/mL)	0.93	0.930 –1.006	1.006 –1.019	1.019–1.063	1.063–1.210
Diameter (nm)	75-1200	30-80	25-35	18-28	5-12
Electrophoretic Mobility	Origin	Pre-β	Slow Pre-β	β	α
Apolipoprotein	B48, A2, C2	B100, C2,C2	B100, C3, E	B100	A1, A2
Function	Transport of exogenous triglyceride and cholesterol	Transport of endogenous triglyceride	-	Cholesterol transport to all tissues	Reverse cholesterol transport

Table 3.2 Chemical component molecules of each lipoprotein (32)

Lipoprotein particle	Amino acid	PL	FC	CE	TG	Equivalent radius (Å)
Chylomicron	102,000	45,160	25,840	27,700	507,000	600
VLDL	15,656	4,445	3,539	3,600	11,500	200
LDL	4,830	653	475	1,310	298	95.9
HDL	961	51	1.2	8	8.3	37.5

3.2.1 Lipoprotein classification

Lipoproteins are classified in 5 major classes based on their relative densities as shown in Figure 3.3.

3.2.1.1 Chylomicron (CM)

Chylomicron is the largest diameter and the lowest density of lipoproteins. This particle is mostly composed of 80-88% (w/w) triglycerides and represented protein content less than 3% (w/w). However, mostly protein in this particle is apolipoprotein B48 (apo B48). Chylomicron is generated from absorb dietary lipid that esterifies to form fatty acid in the intestinal mucosa cell. Fatty acids are incorporated into triglyceride and packed with phospholipid choresterol ester, choresterol, and apo B48 to form chylomicron. This particle is secreted into the

intestinal lymph and delivered directly to the blood circulation. Lipoprotein lipase (LPL) hydrolyzed triglyceride in chylomicron and the particle remnant is processed by the liver via specific receptor. Additionally, chylomicron contains apolipoprotein A1 (apo A1) which is almost immediately transfers to the HDL particle when chylomicron reaches the circulation (31).

3.2.1.2 Very low density lipoprotein (VLDL)

Very low density lipoprotein is mostly composed of triglycerides 45-50% (w/w) and also has free and unesterified cholesterol. VLDL contains a single intact apo B protein. This particle is produced and released by the liver. The removal of triglyceride content in VLDL is activated by LPL. This molecule is a transporter of cholesterol and triglyceride from the liver to organs and tissues in the body (31).

3.2.1.3 Intermediate density lipoprotein (IDL)

Intermediate density lipoprotein is the product from VLDL catabolism by the activity of LPL. IDL is smaller than VLDL particle. IDL combines with cholesterol, triglyceride, and apolipoprotein B (apo B) that circulates through the body and transports cholesterol throughout the body. IDL particle is rapidly removed from blood circulation by the liver and receptor pathway. Moreover, this particle can be further degraded to LDL particle (34).

3.2.1.4 Low density lipoprotein (LDL)

Low density lipoprotein is the major transporter cholesterol from the liver to tissues and account for 70-80% of the circulating cholesterol in human (35). LDL particle is a stable product from VLDL catabolism that composed of triglyceride, cholesterol ester, unesterified cholesterol, and phospholipid. This particle contains a single molecule of apolipoprotein B100 (apo B100) which is the major protein component of LDL. In addition, LDL particle is the major risk factor of cardiovascular disease that can be produced the formation of plaque in the arterial wall (31).

3.2.1.5 High density lipoprotein (HDL)

High density lipoprotein is the smallest diameter of the lipoproteins and the highest protein component. It contains a large number of different apolipoproteins including apo A1, apo A2, apo C1, apo C2, apo D, and apo E. This

particle is produced in the liver and the intestine and acted like a scavenger of cholesterol. The role of HDL is the unesterified cholesterol transporter from the peripheral tissue to the liver. Additionally, HDL is an antioxidant to protect against LDL oxidation and reduce the risk factor of atherosclerosis (31, 38).

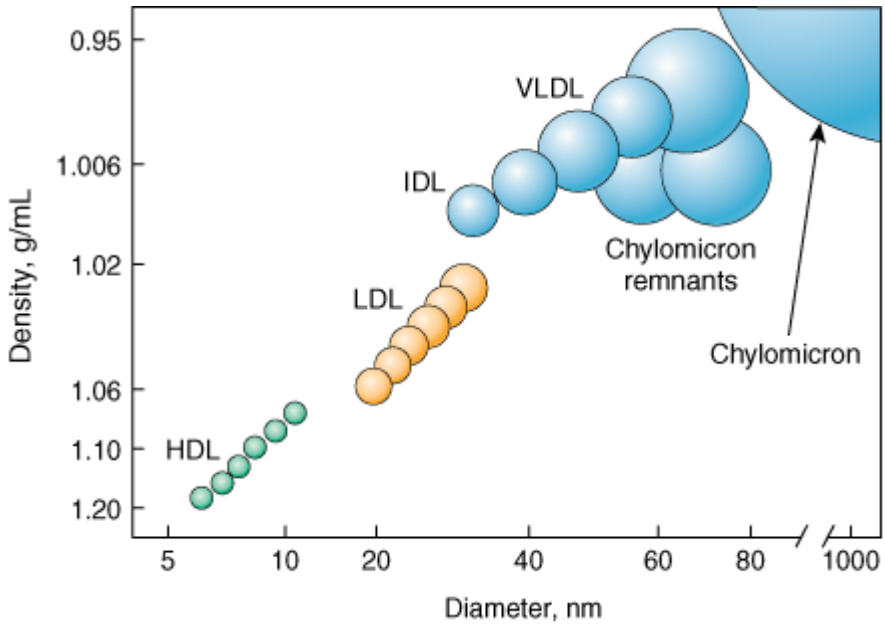

Figure 3.3 The density and size-distribution of the major lipoprotein classes (33). Lipoproteins are classified into 5 major classes by density that composed of chylomicron, VLDL, IDL, LDL, and HDL.

3.2.2 Apolipoprotein

Apolipoprotein is a protein that is attached to the surface of lipid or cholesterol to form lipoprotein. It is regularly synthesis in the intestine by fat content of the diet. The function of apolipoprotein is transport cholesterol and triglyceride in the blood circulation. They act as ligand for specific interaction receptor and act as coenzyme which is activator or inhibitor of specific enzyme. Additionally, an apolipoprotein is directly responsed for lipoprotein to its correct destination in the body. There are many types of apolipoprotein that are found on the different classes of lipoprotein. For example, apolipoprotein A1 (apo A1) which is found on virtually all HDL and apolipoprotein B (apo B) is the major protein of chylomicron, VLDL, IDL, and LDL as shown in Table 3.3.

Table 3.3 Major apolipoprotein (31, 33)

Apolipoprotein	Primary Source	Lipoprotein Association	Function
Apo A1	Intestine, Liver	HDL, chylomicron	Structural protein for HDL, Activates LCAT
Apo A2	Liver	HDL, chylomicron	Structural protein for HDL
Apo A4	Intestine	HDL, chylomicron	Activates LCAT
Apo A5	Liver	VLDL, chylomicron	Promotes LPL-mediated triglyceride lipolysis
Apo B48	Intestine	Chylomicron	Structural protein for chylomicron
Apo B100	Liver	VLDL, IDL, LDL, Lp(a)	Structural protein for VLDL, LDL, IDL, and Lp(a), Ligand for binding to LDL receptor
Apo C1	Liver	Chylomicron, VLDL, HDL	Activates LCAT
Apo C2	Liver	Chylomicron, VLDL, HDL	Cofactor for LPL
Apo C3	Liver	Chylomicron, VLDL, HDL	Inhibits lipoprotein binding to receptor
Apo D	Spleen, Brain, Testes, Adrenal	HDL	Many postulation
Apo E	Liver	Chylomicron remnant, IDL, HDL	Ligand for binding to LDL receptor
Apo H	Liver	Chylomicron, VLDL, LDL, HDL	B_2 glycoprotein I

3.2.3 The role of low density lipoprotein

Low density lipoprotein (LDL) is the major cholesterol transporter in the human circulation. It can promote atherosclerosis due to forming of fatty deposits in the arterial wall. LDL originates from VLDL that synthesizes and releases by the liver. VLDL is converted to LDL by the activity of lipoprotein lipase (LPL), an enzyme that hydrolyzes triglyceride in VLDL and releases free fatty acid. The removal of triglyceride from VLDL by LPL leaving a greater proportion of cholesterol, increasing the density of particle, and changing it to LDL as shown in Figure 3.4. LDL particle is uptaken by cells via LDL receptor and nonspecific endocytosis. In addition, LDL binds to LDL receptor which is presented on the surface of the most cells. The highest concentration of LDL receptor is found in the liver and the three quarter of LDL is removed from the blood circulation by the liver (14).

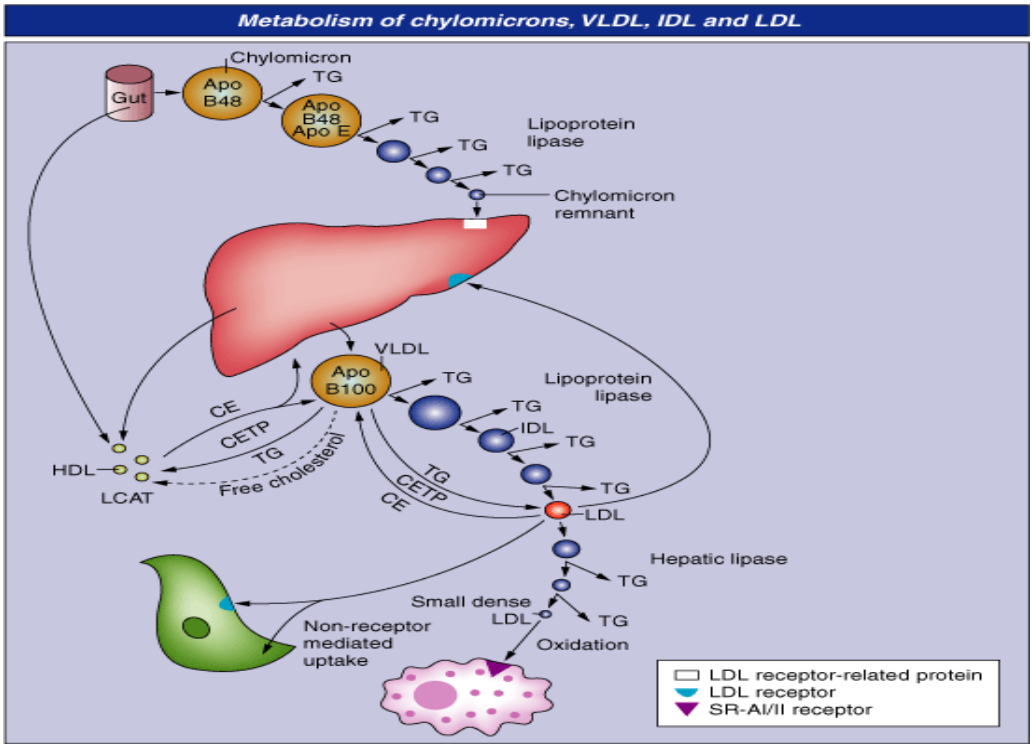

Figure 3.4 The metabolism pathway of LDL (36). LDL originates from VLDL catabolism that hydrolyzed triglyceride by lipoprotein lipase (LPL).

LDL molecules are large spherical particle with an average diameter of 22 nm and have the density limit of 1.019-1.063 g/ml. The structure of LDL particles consist of a core that including 170 triglyceride and 1,600 cholesteryl ester molecules. This core is surrounded by a surface monolayer composed of about 700 phospholipid molecules and 600 molecules of unesterified cholesterol (37). In addition, the LDL particle contains a large protein that is embedded in the outer surface, called apolipoprotein B100 (apo B100). This protein embraces on the whole surface of the LDL particle. It does not sit like a cap on the LDL particle, but it looks like an octopus (14) as shown in Figure 3.5. The apo B100 is a large protein consisting of 4,536 amino acids. The main phospholipid components of LDL are composed of 450 phosphatidylcholine (PC) and 185 sphingomyelin (SM) molecules. In addition, LDL particle contains 80 lysophosphatidylcholine (lyso-PC) and 10 phosphatidyl-ethanolamine (PE) molecules (14, 37). LDL particle also carries polyunsaturated fatty acids (PUFAs), mainly linoleic acid with less amount of arachidonic acid and docosahexaenoic acid (38). Moreover, PUFAs are protected against free radical and oxidation by antioxidants, mostly α-tocopherol (6 molecules/LDL particle) with minor amounts of γ-tocopherol, carotenoid, oxycarotenoid, and ubiquinol-10 (14).

In healthy people, the plasma LDL concentration is about 3 mg/ml circulates in the bloodstream for about 2 days before they are cleared (14). Accordingly, high plasma LDL in circulation is possible to the formation of plaque in the intima layer of the arterial wall refers to atherosclerosis. The formation of plaque is produced from the modification of LDL components called oxidized LDL (OxLDL) which is one of major risk factor in atherosclerosis.

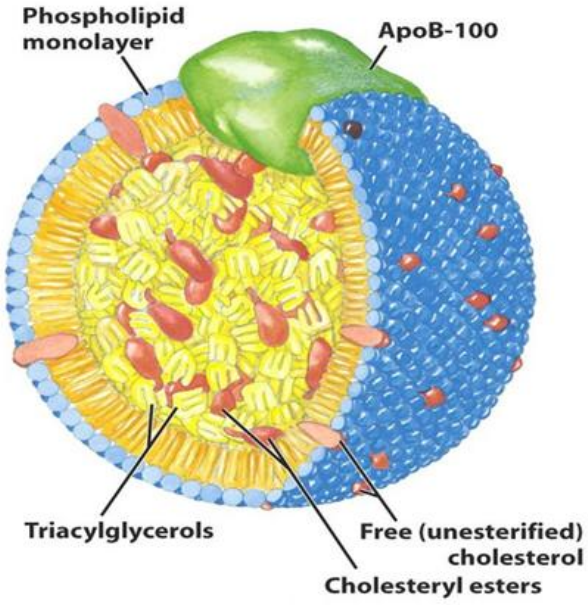

Figure 3.5 Structure of LDL composed of triglyceride, cholesteryl ester, phospholipid, unesterified cholesterol, and apo B100 (37).

3.3 Oxidized Low Density Lipoprotein

Oxidized low density lipoprotein (OxLDL) or oxidatively modified LDL is a major risk factor in atherosclerosis. It is generated by variety modification of LDL in lipid and protein component. Therefore, OxLDL is an important atherogenesis contributor in the arterial wall. The oxidation of LDL occurs when free radicals or enzymatic oxidation such as myeloperoxidase and lipoxygenase which induced lipid peroxidation and protein changed in LDL become to OxLDL (2). In generally, oxidation of LDL occurs in intima layer or subendothelial space of the arterial wall rather than in circulation according to in the circulation has a strongly enriched in antioxidants (39). However, fully oxidized LDL has a very short half-life in plasma because it is rapidly remove from the circulation by reticuloendothelial system (40). The small amounts of OxLDL (minimally modified LDL) represent in plasma. In addition, OxLDL autoantibodies and soluble immune complexes are indirect markers of OxLDL which are presented in the circulation (15). The major chemical and

physicochemical of OxLDL undergo oxidative changes was shown in Table 3.4. In addition, the biological properties of OxLDL were shown in Table 3.5.

Oxidative modification of LDL in the arterial wall, it is uptaken via scavenger receptors through monocyte-derived macrophages leading to the accumulation of foam cell in atherosclerotic lesions (41). The accumulation of foam cells which induced fatty steak formation, called formation of plaque. After that, the plaque ruptures to thrombosis due to cause of cardiovascular disease. Therefore, OxLDL represents in the early state of atherogenesis.

Table 3.4 The major chemical and physicochemical of OxLDL undergo oxidative changes (14)

Chemical and Physicochemical Properties
• Complete loss of antioxidants • More or less complete loss of PUFAs • Loss of phosphatidyl choline and cholesteryl ester • Increased content of lysophosphatidyl choline and oxysterols • Increased content of hydroxy- and hydroperoxy-PUFAs • Increased content of conjugated dienes • Increased content of MDA, hexanal, HNE, and other aldehydes • Strong fluorescence at 430 nm with excitation at 360 nm • Partial loss of free amino groups in apo B • Fragmentation of apo B to smaller peptides • MDA and HNE epitopes on apo B recognized by specific antibodies • Increased electrophoretic mobility and increased density (1.06-1.08) • Increased tendency to aggregate heterogeneity in size • Conformational rearrangement of apo B structure and phospholipid monolayer

Table 3.5 The biological properties of OxLDL (14)

Biological Properties
• Increases uptake and degradation by macrophages • Cytotoxic to most cells • Chemotactic for monocytes and smooth muscle cells • Inhibits NO activation of guanylatecyclase • Inhibits in isolated smooth muscle strips relaxation induced by acetyl choline, nitric oxide, and nitroglycerin • Increases in cultured endothelial cells tissue factor activity (TF) and suppresses protein C (which increases thromboresistance) activity • Suppresses the production of PDGF-mRNA and PDGF secretion by monocyte-macrophages • Increases in macrophages glutathione content about two fold; HNE has a similar effect • Systemic administration into hamsters causes immediate leukocyte adhesion to capillary endothelium • Treatment of cultured endothelial cells with MM-LDL stimulates production of a number of biological active factors, such as monocyte chemotactic factor, MCP-1; monocyte binding molecules (so-called endothelial-leukocyte-adhesion-molecules, ELAMs); growth factors for monocytes, M-CSF, and granulocytes, G-CSF, and tissue factors (TF) essential for coagulation. • MM-LDL injected into mice increases in serum and tissue levels of MCP-1 and CSF • MM-LDL inhibits mitogenesis in SMC and stimulates (low concentration) or inhibits PGI2 synthesis in SMC

3.3.1 Mechanisms of LDL oxidation

LDL can be oxidized by non-enzymatically such as free radicals, metal ions and other catalysts (2). LDL can be also oxidized by oxidizing enzymes such as lipoxygenase and myeloperoxidase. However, all of these mechanisms can be converted LDL to OxLDL which is involved in pathogenesis of atherosclerosis.

3.3.1.1 Metal ions

LDL can be modified by metal ions (e.g., Cu^{2+} and Fe^{2+}) that occurred in 3 phases including an initial lag phase (consumption of endogenous antioxidant), a propagation phase (rapid oxidation of unsaturated fatty acid to lipid hydroperoxides) and a decomposition phase (hydroperoxides are converted to reactive aldehyde). The primary product of lipid peroxidation consists of a variety of different aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE). All of aldehyde products, they undergoes carbon-carbon bond cleavage in the presence of transition metals forming short chain unesterified aldehyde. In addition, when LDL is incubated with iron, the formation of hydroperoxides and aldehydes are enhanced as increased cysteine concentration lead to the generation of oxygen-or sulfur-centered free radicals (2, 38, 42).

3.3.1.2 Reactive species

Reactive species are atoms or molecules that are generated by enzymatic and chemical interactions. There are three main classes of reactive species including reactive nitrogen species (RNS), reactive chloride species (RCS), and reactive oxygen species (ROS). However, each class remains free radical that has one or more unpaired electron. The highly reactive can cause lipoprotein damage to modified lipoprotein, it is involved in atherosclerosis. LDL is oxidized by reactive nitrogen species. In addition, nitric oxide (NO) is a stable radical that releases by various vascular cells and it can inhibit lipid peroxidation. NO fails to oxidize LDL but it is rapidly inactivated by superoxide anion to form peroxynitrite which is promoted in LDL oxidation (43). Moreover, oxidation of LDL by reactive oxygen species can be removed a H^+ from a double bond in PUFA and the molecule is rearranged by ROS resulting in the formation of conjugated double bonds called conjugated dienes (CD) (14, 38, 44).

3.3.1.3 Lipoxygenase

Lipoxygenase (LOX) is a non-heme iron containing dioxygenase which is one of intracellular oxidation enzymes. It directly oxygenates polyunsaturated fatty acids to lipid hydroperoxide. In vitro, 15-lipoxygenase directly oxidizes LDL particle and LDL exposed to fibroblasts transfection with the 15-lipoxygenase gene that exhibited high level of lipid peroxides. Therefore, the amounts of lipoxygenase in vascular cells are involved in atherogenesis, while the amounts of lipoxygenase in global cells are exerted anti-inflammatory functions (2, 45).

3.3.1.4 Myeloperoxidase

Myeloperoxidase (MPO) is generated from neutrophils and monocytes when phagocytes activated during inflammation. MPO is involved in inflammation and oxidative stress that is linked to pathogenesis of atherosclerosis. This enzyme can be released various reactive species including hypochlorous acid (HOCl), chloramines, tyrosyl radicals, and nitrogen dioxide (NO_2). In addition, HOCl reacts with the ε-amino group of apo B lysine residues, resulting in the formation of N-chloramines. Atherosclerotic lesion has been found reactive species products that are induced by MPO. It interacts with both LDL and HDL to induce oxidation. Although, MPO is more closely associated with inflammation and presented the evidence of MPO role to induce strong oxidants that is found in atherosclerotic plaque as refer in term biomarker of oxidative stress (38, 44, 46).

3.3.1.5 Glycated LDL

Glycation of protein is a complex series of Maillard reaction. It has early phase reactions that are subjected to lysine and NH_2-terminal amino acid residues. The late phase reactions form the advance glycation end product. The non-enzymatic glycation of LDL occurs in lysine residues of apo B which is the major amino acid undergoes glycation, making 2-17% of LDL-lysine glycated. In addition, glucose can enhance LDL peroxidation by oxidative pathway and it raises the possibility of lipoprotein oxidation in chronic hyperglycemia. However, glycation and oxidation take place in vivo concurrently because free radicals can be generated by glycation itself from glucose and Amadori products leading the enhanced susceptibility of glycated LDL to further oxidation (2, 47, 48).

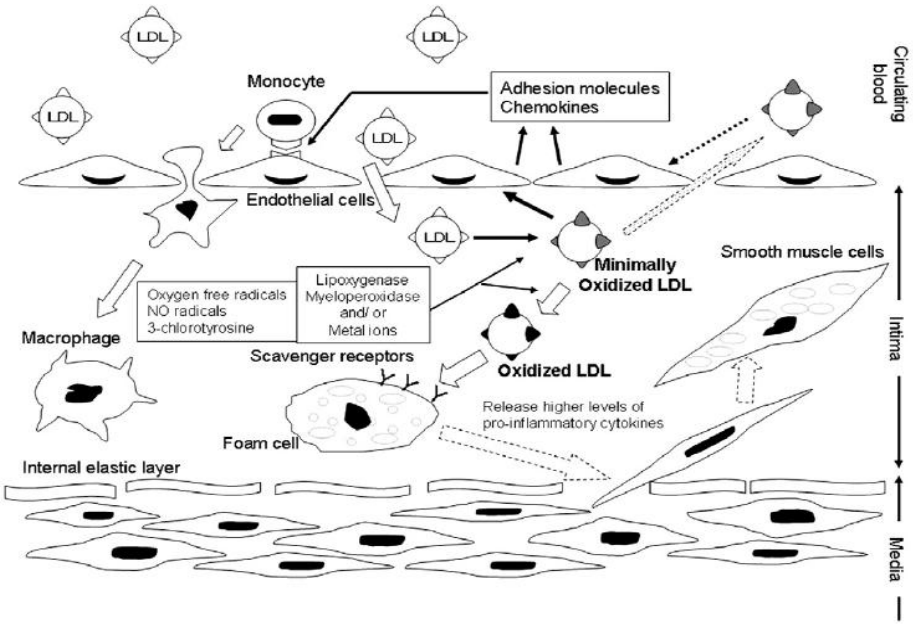

Figure 3.6 Mechanisms of LDL oxidation (2). LDL modified to OxLDL by many mechanisms such as metal ions, lipoxygenase, myeloperoxidase, and free radical. All of these mechanisms occur in the arterial wall. Reprinted from Journal of Clinica Chimica Acta, Vol. 411, Yoshida H, Kisugi R, Mechanisms of LDL oxidation, P.1875-1882, Copyright (2010), with permission from Elsevier (Permission Number: 3120670369665).

3.3.2 Biological products of LDL oxidation

The oxidative modification of LDL undergoes biological activity that generates a variety of new products such as aldehyde products, phospholipid products, and free fatty acid products.

3.3.2.1 Aldehyde products

LDL oxidation can be produced many short-chain aldehyde products that cleavage from unsaturated fatty acid. The major form of aldehyde consists of hydroxynonenal (HNE) and malondialdehyde (MDA). HNE is formed from the degradation of n-6 fatty acid, while MDA is formed from the peroxidation of all unsaturated fatty acid. Aldehydes have relatively contribution to cell death. In

addition, MDA can be measured by thiobarbituric acid reactive substances (TBARS) assay which is the most widely used for measuring lipid peroxidation (13, 40).

3.3.2.2 Phospholipid products

The major phospholipid products from LDL oxidation are lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA). LPC is one of the first components of OxLDL that presents at low concentration in normal LDL. The majority of LPC in OxLDL is formed from the action of Ca^{2+}-independent lipoprotein-associated Phospholipase A2 (PLA2) that efficiently hydrolyzes the oxidized acyl groups from the *sn*-2 position of phospholipids. LPC is a chemotactic agent for monocytes and help recruit circulating monocytes into the arterial wall. However, LPA is generated from LPC by the action of lysophospholipase D and it is a mitogen that acts through specific G-protein coupled receptors (40). In addition, it has variety phospholipid products such as *sn*-2 short chain 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) products, *sn*-2 epoxy products and PAF-like products (40, 49).

3.3.2.3 Free fatty acid products

The isoprostane derivatives are discovered that a series of structurally unique prostaglandin F_2 (PGF_2)-like compounds (F_2-isoprostanes) are produced in human by a noncyclooxygenase mechanism involving free radical-catalyzed peroxidation of arachidonic acid. The formation of F_2-isoprostanes is induced in plasma and LDL is exposed to oxidative stress in vitro. Moreover, the release of PGF_2 is increased in LDL oxidation by macrophages, endothelial cells, or copper (7, 40).

3.3.2.4 Oxysterols and cholesteryl ester products

The oxidative modification of LDL can be generated the numerous oxysterols and cholesterol ester products. The major oxysterols are 7 keto-cholesterol, 7α-OH, and 7β-OH cholesterol and cholesterol epoxides. The oxysterols are accumulated in atherosclerosis lesions and can exert several biological activities in the arterial cells including apoptosis, cytotoxicity, and regulation of gene expression (40). The majority of the di-and polyunsaturated fatty acids in LDL are presented as cholesterol ester. In addition, the hydroperoxides and hydroxides of cholesteryl ester are the major lipid oxidation products that are found in atherosclerotic lesions (50, 51).

3.3.2.5 Products of apo B modification

Apo B is modified by various functional groups of amino acids such as lysine, cysteine, histidine, tryptophan, and tyrosine (40). The modification of lysine residues of apo B by MDA has been shown the loss of recognition by LDL receptor and the appearance of epitopes for recognition by scavenger receptor (40, 52).

3.3.3 Measurement methods of LDL oxidation

OxLDL is an important biomarker for atherosclerosis progression. It can be monitored the atherosclerosis events. Therefore, there are many detection methods to investigate plasma OxLDL such as TBARS assay, conjugated diene, HPLC, and ELISA (7, 10).

3.3.3.1 Thiobarbituric acid reactive substances (TBARS) assay

TBARS assay is the most commonly method that is used to measure LDL oxidation. This method investigated malondialdehyde (MDA) molecule which is an indicator and a secondary product of lipid peroxidation. The chromogen is formed by one molecule of MDA to interact with two molecules of thiobarbituric acid (TBA). This method involves heating under acidic conditions and reading the absorbance of MDA-TBA complex that is formed the formation of pink-colored products at 532 nm with spectrophotometer. TBARS assay can be interfered by other compounds in biological so it is not specific for lipid peroxidation. In addition, several other compounds including sugar, amino acid, and bilirubin are also reactive toward TBA (11, 13, 14).

3.3.3.2 Conjugated diene (CD)

This method is the fundamental measurement method of lipid peroxidation similar to TBARS assay. The peroxidation of polyunsaturated fatty acid is accompanied by the formation of conjugated dienesthat absorbs ultraviolet light at 234 nm. The reaction occurs in three phases including, lag phase, propagation phase, and decomposition phase. This method is the mostly used to monitor oxidation of LDL in vitro. It is rapid and simple to perform with instruments and generally available in clinical laboratories but it is low limit of detection (7, 53).

3.3.3.3 Electrophoretic mobility

Electrophoretic mobility is a separation technique which is based on the movement of charged particles in an electric field. The electrophoretic mobility of OxLDL is determined by agarose gel electrophoresis. The increasing negatively charges is induced by the oxidation of LDL more than the native LDL leading to electrophoretic mobility increased (44, 54). This method can be interfered by aldehydes modification (7).

3.3.3.4 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance spectroscopy is a one of method for measuring lipoprotein level. It can be measured the quantification of lipoprotein profiles based on the amplitudes detection of spectral signal emitted by lipoprotein subclasses of different sizes. Each subclass signal leave from the aggregate number of terminal methyl groups on the lipid contained within the particle. For the cholesterol ester and triglyceride in the core each contribute three methyl groups and in the surface, phospholipid and unesterified cholesterol each contribute two methyl groups. It can provide the information of atherogenic lipoprotein profile. This method is used the expensively equipment cost and not suitable in routine work (8, 55).

3.3.3.5 High performance liquid chromatography (HPLC)

HPLC is an alternative method for classifies and quantifies lipoprotein on the basis of difference particle sizes. The component analysis of lipoprotein profiles after HPLC can provide useful almost information of the above mentioned atherogenic lipoprotein subclasses. It can be applied to measure OxLDL product such as aldehydes and conjugated dienes. This method can be detected in lipoprotein fraction but it has inconvenience in clinical laboratory (56, 57).

3.3.3.6 Mass spectrometry (MS)

Mass spectrometry is one of the most powerful devices to identify posttranslational modification of protein. It has been demonstrated the determination of stable oxidative product in protein. This method can be identified the oxidation pattern of apo B100 in LDL and other lipoproteins. In addition, this method is usedto measure other product of OxLDL such as aldehydes. The difficulty in the analysis of modification sites of apo B100 in OxLDL by this technique is complexity of the heterogeneous modifications of the apolipoproteins. Moreover, the sample

preparation method for apolipoproteins oxidation in OxLDL should be improved to perform MS-based molecular analysis (9, 13).

3.3.3.7 Fluorescence

The fluorescence is a spectrochemical analytical method for direct quantitation of oxidized lipoprotein by fluorescence spectrometry with excitation in UV or visible range and emission detected at the different wavelength. Fluorescently labeled lipoproteins have provided information on both physical and cellular binding properties of lipoprotein. Fluorescence detection method of lipoproteins has two major dyes including protein marker dyes that fluorochromes are conjugated apolipoproteins through functional groups and lipophilic dyes for lipoprotein analysis. This method is rapid, used small sample volume, and no need any expensive antibodies. However, it is not suitable for routine work since it need the professional worker (7, 58).

3.3.3.8 Enzyme linked immunosorbent assay (ELISA)

The ELISA assay has been used for the qualitative and semi-quantitative detection of antibodies to OxLDL in human serum. The development of the specific OxLDL assay is based on monoclonal antibodies recognizing to specific structures including E06, FOH1a/DLH3, and 4E6 (15). The E06 assay represents the measurement of oxidized phospholipids (OxPL)/apo B100 particle (59). The FOH1a/DLH3 assay demonstrates the oxidized phosphatidylcholine epitopes on LDL (60). The 4E6 assay measures an unknown OxLDL epitope, it appears to bind both copper-OxLDL and malondialdehyde (MDA)-LDL epitopes (61). These ELISA assay can be used for measuring the circulation OxLDL in direct clinical sample. This method can indicate the level of plasma OxLDL and it has many commercial kits to detect plasma OxLDL which is available to use. However, it may be not completed in sensitivity and specificity due to the various oxidation-specific epitopes, time-consuming, and high analytical cost (15, 16).

3.4 Biosensor

The biosensor is an analytical device that combines a biochemical substance with a transducer to convert the biochemical response into electrical signal as shown in Figure 3.7. This device has been used in many fields such as in environment, chemical analysis, food analysis and clinical detection (17, 62). It has many advantages for example, high sensitivity and specificity to target molecule, used small volume of sample and reagent, and portable. The biosensor system consists of two major components: a biological recognition element which is directly response to the selectivity of sensor after the specific binding between target molecule and biological recognition occurs. A transducer can convert the biological signal to the electrical signal (63, 64). Therefore, the important characteristic of biosensor is the selectivity of biological recognition specific for target analyte.

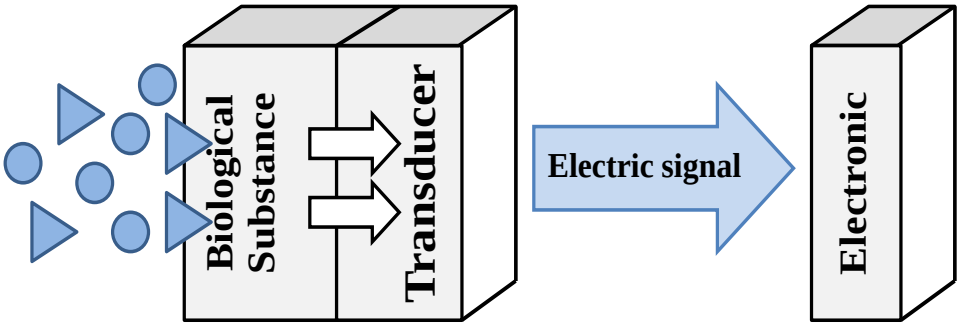

Figure 3.7 A typical biosensor configuration; biological recognition and tranducer modified from Peter et al. (64).

In addition, the biological components are the chemical components of living organism that is used a biochemical mechanism for recognition. There are many types of the biological recognition element including enzymes, antibodies, whole cells, receptors, affinity ligand, peptide, and nucleic acids (65, 66). The biological component can be selective for the analyte target.

Moreover, a transducer is a one important component of biosensor device which is used to convert the interaction changes between biological recognition and target molecule into the electrical signal. Transducer can be classified to four main

groups following electrochemical (potentiometry, amperometry or conductimetry), mass (piezoelectric crytal or acoustic wave), heat (colorimetry), and optical change (luminescence, fluorescence, reflective index, surface plasmon resonance, and waveguide) (66).

3.4.1 Piezoelectric quartz crystal – based immunoassay

Piezoelectric immunosensor is a one of advance devices in transducer technology. This transducer converts immunoreaction events into different physical signals and can sense antigen/antibody concentration by direct or indirect changes in transducer output. The direct format determines the measuring physical changes that are induced by the formation of the immune complex and the indirect is a sensitively detectable label that combines with the antibody or antigen of target (67). The piezoelectric crystal device is based on antigen-antibody interactions that can be directly identified by measuring the frequency changes corresponding to the mass changes onto the modified crystal surface. This assay is simple and easy to use (68).

The most commonly piezoelectric device are classified into two classes including surface acoustic wave (SAW) device and quartz crystal microbalance (QCM) device. In a SAW device, it can oscillate at normally frequency above 100 MHz. The electrodes are on the same side of the crystal. Mass loading on the acoustic path between two sets of electrode will alter the phase wave velocity and cause a shift in the frequency. This device is mass sensitive more than piezoelectric because mass sensitivity is directly related to the operating frequency. However, SAW device presents several problems when it is applied to a biological sensing system because biological solutions can severely reduce surface acoustic wave (69). QCM is alternative term that describes the mass sensitivity of the crystal. This system determines the target molecules via the frequency change of quartz crystal after mass deposition on the surface. It is highly mass sensitive device which is not required or labeled reagent for target analyte. The QCM device has been applied for bioanalytical assay such as bacteria and virus, protein, nucleic acids, and small molecules (70).

The QCM device consists of a quartz crystal wafer sandwiched between two metal electrodes. The electrodes are provided to connect the device to external oscillator circuit that measured its resonant frequency. The quartz crystal is a

crystalline form of silicon dioxide (SiO_2) which is abundant in the nature. For the majority of piezoelectric work, piezoelectric quartz sheets are made from AT-cut (at +35°15′ angle from the z-axis of quartz wafer) that are stable property with a zero temperature coefficient between 10-50°C and operated at 5-30 MHz (71, 72). The most commonly used crystals are 5-15 MH with a quartz disks at 10-16 mm diameter (63). The quartz crystal surfaces are covered with metal electrode such as gold and silver and prepared by thermal evaporation onto the quartz surface. Major function of the electrode transform electron via applied voltage to the crystal. In addition, the function of electrode is used as immobilization area for affinity interaction between biological recognition and specific target. Figure 3.8 was shown the structure of QCM.

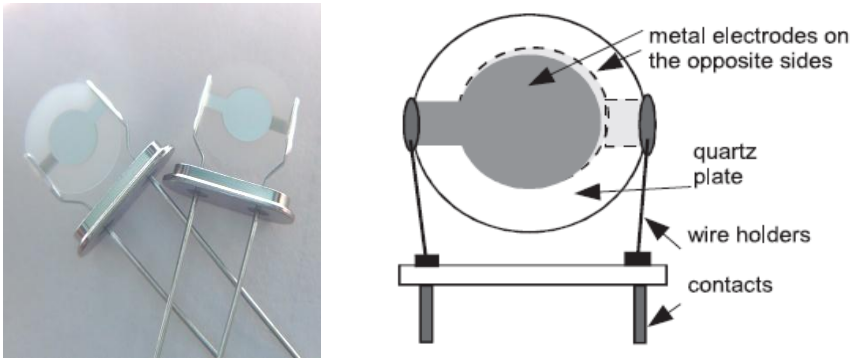

Figure 3.8 Piezoelectric quartz crystal structures; metal electrodes, quartz plate, wire holders, and contacts modified from Petr (70).

The piezoelectric device is a mass sensing, when the mass on the crystal surface changes thus the resonant frequency of QCM device changes. Therefore, the relationship between the resonant frequency shift and surface mass change of this device can be described by the Sauerbrey's equation. This equation discovered by Sauerbrey which is as a principle of QCM that is shown the increasing of mass on the sensor surface resulting in the frequency of vibration decreases.

In gas phase, the relationship of resonant frequency shift and the mass loading on the surface of the crystal is given by Sauerbrey's equation as following below (68):

$$\Delta F = -2F_0^2 \Delta m / A(\rho_q \mu_q)^{1/2}$$

Where;

ΔF = measured frequency shift in hertz

F_o^2 = the fundamental resonant frequency of the crystal in hertz

Δm = mass change in gram

A = piezoelectric active area (area of electrode surface) in cm^2

μ_q = shear modulus of quartz = 2.947×10^{11} g/cm^2

ρ_q = density of quartz = 2.648 g/cm^3

For an AT-cut crystal, the equation can be simplified as:

$$\Delta F = -2.27 \times 10^{-6} F_0^2 \Delta m / A$$

Where;

ΔF = measured frequency shift in hertz

F_o^2 = the fundamental resonant frequency of the crystal in hertz

Δm = mass change in gram

A = piezoelectric active area (area of electrode surface) in cm^2

This Sauerbrey's equation is capable to only gas phase mass deposition. In addition, the QCM in liquid phase has been developed. In liquid phase has been proposed a new theory to interpret the resonant frequency shifts due to liquid properties such as conductivity, viscosity, density, and dielectric constant.

In liquid phase, the relationship between the frequency change and the mass change of quartz in solution has been reported by the Kanazawa and Gordon's equation as shown below (68, 73):

$$\Delta F = -F_0^{3/2} (\rho_L \eta_L / \pi \rho_q \mu_q)^{1/2}$$

Where;

ΔF = measured frequency shift

f_o^2 = the fundamental resonant frequency of the crystal

ρ_L = density of the liquid

η_L = viscosity of the liquid

μ_q = shear modulus of quartz

ρ_q = density of quartz

3.4.2 Immobilization techniques

In biosensor devices, the immobilization plays an important role step for biosensor fabrication. This step has been used to immobilize the biological element onto the sensor surface. There are many immobilization techniques in biosensor that can be classified into main groups as shown in Figure 3.9.

3.4.2.1 Adsorption

This technique is the simplest non-covalent method and easy to prepare but the bonding of the immobilization is weak (74). The physical absorption is involved the formation of Van der Waals bonds, hydrophobic, and hydrogen bonding (75).

3.4.2.2 Microencapsulation

This method is performed by the biological molecules that are held in place behind a membrane, giving close contact between the biological molecules and the transducer. It can be permeable to small molecules, gas molecules, and electrons (76).

3.4.2.3 Entrapment

The movement of target molecule is restricted by the polymeric gel. The gel is prepared in a solution which containing the biological molecules and trapped within in the gel matrix. It can be loss of bioactivity through pores in the gel (76).

3.4.2.4 Cross-linking

The biological molecules in this method can link to supporting materials by cross-linking agents. The common cross-linking agents are

glutaraldehyde, hexamethylenediisocyanate, disuccinyl substrate, and N-gamma-melaimidobutyroxy succinimide ester (74). It can damage the biological molecules.

3.4.2.5 Covalent bonding

The nucleophilic groups such as NH_2, COOH, OH_3, C_6H_4OH, and imidazole are utilized for coupling by covalently bonded to the transducer. Carbodiimide, glutaraldehyde, and succinimide derivatives are general used for covalent binding method. This method can improve uniformity, density, distribution of bound molecules, and reproducibility of surface (77).

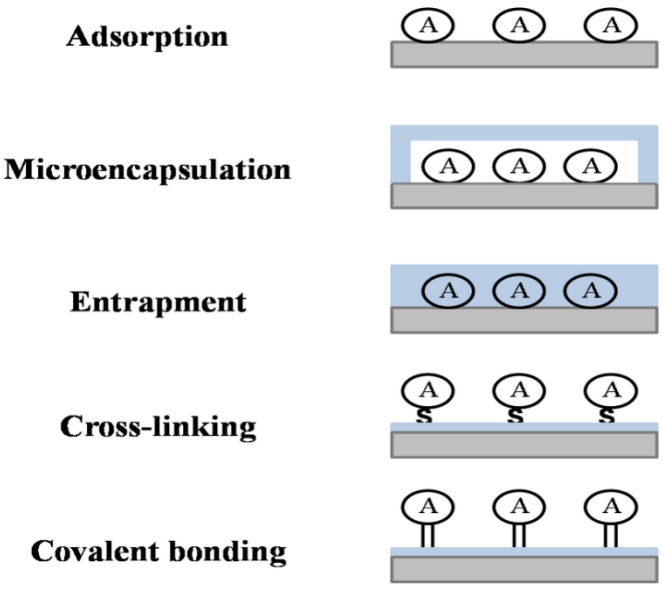

Figure 3.9 Schematic diagram of immobilization method in biosensor modified from Brian (76).

3.4.3 Self-assembled monolayer

The self-assembled monolayer (SAM) is one of immobilization technique by covalent binding. This method is commonly used in the biosensor devices for inducing the SAM formation by the strong chemisorptions between the substrate and head group of selected organic molecule. It provides one of the most elegant approaches towards making ultrathin organic films of controlled thickness. In addition, SAM is a molecular assembly method that immerses an appropriate substrate into a solution for specific with the surface. SAM is distinguished from ordinary surfactant

monolayer. In fact, one end of the molecule is designed to have a favorable and specific interaction with the solid surface of the substrate. And the one end of molecule is also designed the specific binding for the target molecule. It is easy formation of ordered, pinhole free and stable monolayer. This method is easy and compatibility with metal substrate such as gold or silver provided benefits for biosensor application. Moreover, disulfides, sulfides, or thiols bond are occurred very strongly onto the metal substrate. Thiols on gold are formed very well-assembled monolayer which is highly resistant to wash due to the strong chemisorptions of the surface atoms. It is a rapid reaction between –SH group and gold atom with formation of S-Au bond. For the various alkanethiol agents are performed in SAM system such as mercaptopropionic acid (MPA) and mercaptoundecanoic acid (78-80).

This SAM method is a successfully used in many previous studies. For example, Zhou et al. (81) compared different immobilization methods and different immobilization architectures by self-assembling monolayer. Caruso et al. (82) immobilized oligonucleotides directly on the gold quartz crystal by forming self-assembled monolayer and also used biotin-oligonucleotide to immobilize them on avidin-modifide gold quartz crystal.

CHAPTER IV
MATERIALS AND METHODS

This chapter demonstrated the materials and methods that were used in this study. The materials section consisted of instruments, chemical reagents, and reagent preparation. The methods section included oxidative modification of LDL (OxLDL) preparation, the measurement system and OxLDL immunosensor preparation, OxLDL immunosensor system optimization, and OxLDL immunosensor performance evaluation.

4.1 Materials

This section described the details of instruments, chemicals, and reagents preparation that were used in this study.

4.1.1 Instruments, chemicals, and reagents

The detail of instruments, chemicals, and reagents was shown in the Table 4.1: The list of instruments and Table 4.2: The list of chemicals and reagents.

Table 4.1 List of instruments

Instrument Name	Company	Country
Analytical balance - XT 220A - XT 2200C	Precisa Instruments Ltd.	Switzerland
Autoclave, Hiclave HV-85	Hirayama	Japan
Automatic pipettes - 1-10 µl - 2-20 µl - 20-200 µl - 100-1,000 µl	Gilson	France
Centrifuge, 420101	Clay-Adams Dynac	USA
Desiccators	Nikko	Japan
Disposable syringes - 10 ml - 20 ml	Nipro (Thailand)	Thailand
Fume hood, Ascent™ Max	Esco Ductless	USA
Incubators	Binder	USA
pH meter, 420A	Thermo	USA
Quartz crystal silver electrodes (10 MHz AT-cut) with 9 mm in diameter	Kyocera-Kinseki	Thailand
Research Quartz Crystal Microbalance (RQCM)	MAXTEK Inc.	USA
Spectrophotometer, Genesys 20	Thermo	USA
Syringe pump, Multi-Phaser™ NE-1000	New Era Pump Systems Inc.	USA
Vortex mixer, VX 100	Labnet International Inc.	USA
Water bath	Memmert	Germany

Table 4.2 List of chemicals and reagents

Reagent Name	Formula	MW	Company	Country
Absolute ethanol	C_2H_6O	46.07	Merck	Germany
Bovine serum albumin (BSA)	-	-	Sigma	USA
1-Butanol	$C_4H_{10}O$	74.12	Sigma	USA
Copper(II) sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	249.69	Sigma	USA
Ethylenediaminetetra acetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	292.24	Sigma	USA
1-Ethyl-3-(3-dimethylaminopropril) carbodiimide (EDC)	$C_8H_{17}N_3$	155.24	Sigma	USA
Human high density lipoprotein (HDL)	-	175-360 kDa	Millipore	USA
Human low density lipoprotein (LDL)	-	2300 kDa	Millipore	USA
Human very low density lipoprotein (VLDL)	-	-	Millipore	USA
Hydrochloric acid	HCl	36.36	Merck	Germany
3-Mercapto propionic acid (MPA)	$C_3H_6O_2S$	106.14	Sigma	USA
N-hydroxysulfo succinimide (NHS)	$C_4H_5NO_3$	115.09	Fluka	Switzerland
Polyclonal Anti-Cu^{2+}-oxidized low density lipoprotein (anti-OxLDL)	-	-	Millipore	USA

Table 4.2 List of chemicals and reagents (cont.)

Reagent Name	Formula	MW	Company	Country
Potassium chloride	KCl	74.56	Merck	Germany
Potassium dihydrogen phosphate	KH_2PO_4	136.09	Fluka	Switzerland
Sodium chloride	NaCl	58.44	Merck	Germany
Sodium hydroxide	NaOH	40.00	Merck	Germany
Sodium phosphate dibasic heptahydrate	$Na_2HPO_4.7H_2O$	268.07	Merck	Germany
1,1,3,3-Tetramethoxypropane (TMP)	$C_7H_{16}O_4$	164.20	Sigma	USA
Thichloroacetic acid (TCA)	$C_2HCl_3O_2$	163.39	Sigma	USA
2-Thiobarbituric acid (TBA)	$C_4H_4N_2O_2S$	144.15	Sigma	USA

4.1.2 Reagent preparation

All of reagents that were used in this study were described below.

1% Bovine Serum Albumin (BSA) Solution

One g of BSA was completely dissolved in 100 ml of 10 mM phosphate buffer saline (PBS) pH 7.4. This solution was kept at 4^{o}C until used.

50 µM Copper(II) Sulfate ($CuSO_4$) Solution

The stock solution of copper sulfate solution was prepared by dissolving 0.002 g of $CuSO_4.5H_2O$ in 2 ml of distilled water (DW). Then, five µl of stock solution was added in the DW to obtain the final volume of 500 µl.

0.25 mM Ethylenediaminetetraacetic Acid (EDTA) Solution

This EDTA solution was prepared by adding 0.0018 g of EDTA into 25 ml of 10 mM PBS pH 7.4. This solution was kept at 4^{o}C.

200 mM 1-Ethyl-3-(3-dimethylaminopropril) Carbodiimide (EDC) Solution

The EDC at the amount of 7.35 µl was added in DW to get the total volume of 200 µl. This solution should be fleshly prepared, placed away from direct light source and immediately used after preparation.

Human Low Density Lipoprotein (LDL) solution

The preparation of human LDL at different concentrations (25, 50, 100, 200, 300, 400, and 500 µg/ml) were performed by pipetting 98 µl of human LDL and adding 10 mM PBS buffer pH 7.4 to the total volume of 1 ml. This stock solution was diluted to obtain the final concentration of 25 µg/ml and then kept in 4°C.

1 M Hydrochloric Acid (HCl) Solution

One M HCl solution can be obtained by adding 8.29 ml of Conc. HCl into DW to get the total volume of 100 ml. This solution was kept at room temperature (RT).

10 mM 3-Mercaptopropionic Acid (MPA) Solution

The MPA at the amount of 8.8 µl of was added in the absolute ethanol to get the total volume of 10 ml. This solution should be kept away from light.

50 mM *N*-Hydroxysulfosuccinimide (NHS) Solution

This NHS solution was 0.0012 g of was dissolved in 200 µl of DW. It should be prepared shortly before use and kept away from light.

0.1 M Phosphate Buffer Saline (PBS) pH 7.4

One-tenth M PBS solution pH 7.4 was prepared by dissolving 80 g of sodium chloride (NaCl), 2 g of potassium chloride (KCl), 11.5 g of sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$), and 2 g of potassium dihydrogen phosphate (KH_2PO_4) in 900 ml of DW. This buffer was adjusted to pH 7.4 with 1 M HCl. The total volume of solution was adjusted to 1 l with DW.

10 mM Phosphate Buffer Saline (PBS) pH 7.4

One hundred ml of 0.1 M PBS pH 7.4 was mixed in 900 ml of DW to the final volume of 1 l. Then, it was adjusted to pH 7.4 with 1M HCl solution.

Polyclonal Anti-Cu^{2+}-Oxidized Low Density Lipoprotein (anti-OxLDL) Solution

The different concentrations of anti-OxLDL (10, 20, 50, 100 and 200 µg/ml) were prepared by pipetting 5 µl of anti-OxLDL to the 450 µl of DW. Two fold serial dilutions were carried out to obtain the final concentration at 10 µg/ml. This solution was kept in freezer at -20°C.

1 M Sodium Hydroxide (NaOH) Solution

One g of NaOH was dissolved in 25 ml of DW. This solution was kept at room temperature.

Standard 1,1,3,3-Tetramethoxypropane (TMP) Solution

The stock standard TMP solution was prepared by pipetting 10 µl of standard TMP to the 990 µl of DW and performed 10 times dilution to get the final concentration of 60 µmole/l. Then, the working standard solution at the concentration 1, 2, 4, 6, 8, and 10 µmole/l were prepared by two fold serial dilution.

1.22 M Thichloroacetic Acid (TCA) Solution

The 19.92 g TCA was dissolved in DW. Then, it was mixed with 60 ml of 1 M HCl and adjusted to the total volume of 100 ml with DW. This solution was kept at 4^{o}C.

4.6 mM Thiobarbituric Acid (TBA) Solution

A quarter g of TBA was dissolved in 1 M NaOH 3 ml. Then, it was added into DW to get the final volume of 37.5 ml. This solution was kept at 4^{o}C.

4.2 Methods

This section consisted of the experimental processes in this study. The first experiment was the oxidative modification of LDL (OxLDL) preparation by using copper ion. The second experiment was aimed to prepare the measurement system and OxLDL immunosensor. The third experiment was the optimization condition of OxLDL immunosensor in liquid phase. The evaluation the performance of OxLDL immunosensor in liquid phase was the last experiment in this study.

4.2.1 The oxidative modification of LDL (OxLDL) preparation

The OxLDL particles as target antigen of OxLDL immunosensor were prepared by the oxidation of LDL using copper ion. One hundred µg/ml human LDL was oxidized by incubation with 50 µM $CuSO_4$ at 37°C for 24 h and the reaction was terminated by adding 0.25 mM EDTA at RT for 15 min. The amount of oxidative modification of LDL was determined by thiobarbituric acid reactive substance (TBARS) assay (11) as shown in Figure 4.1. The pink-colored product of this reaction was measured with spectrophotometer at the wavelength of 532 nm as shown in Figure 4.2.

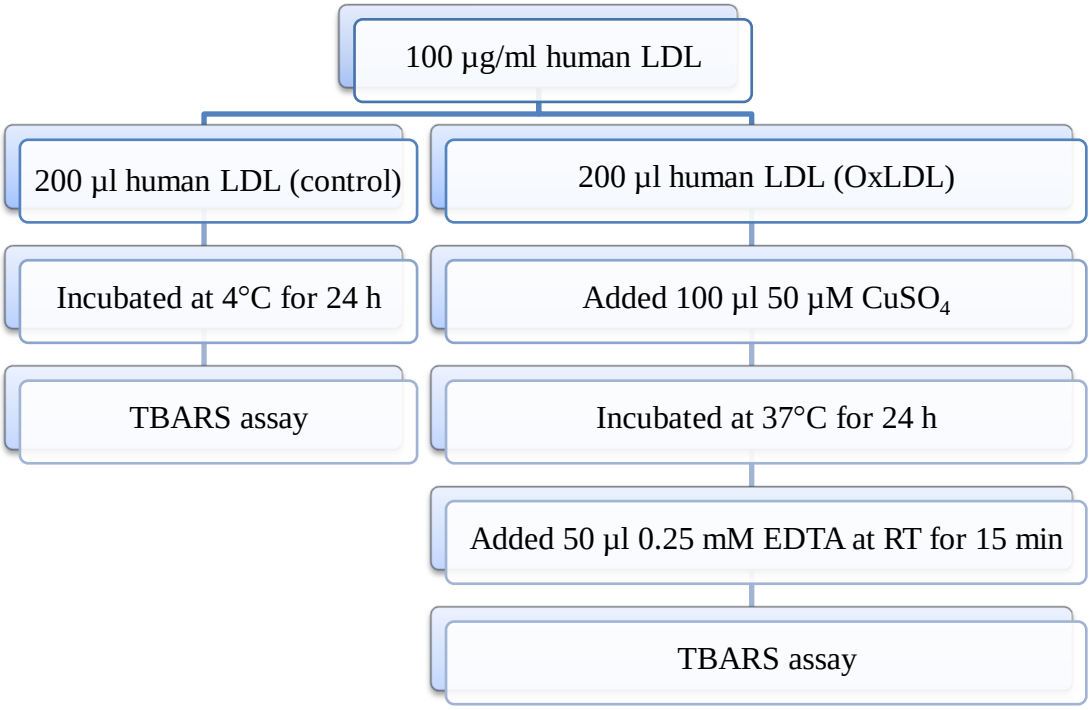

Figure 4.1 A schematic diagram of OxLDL preparation

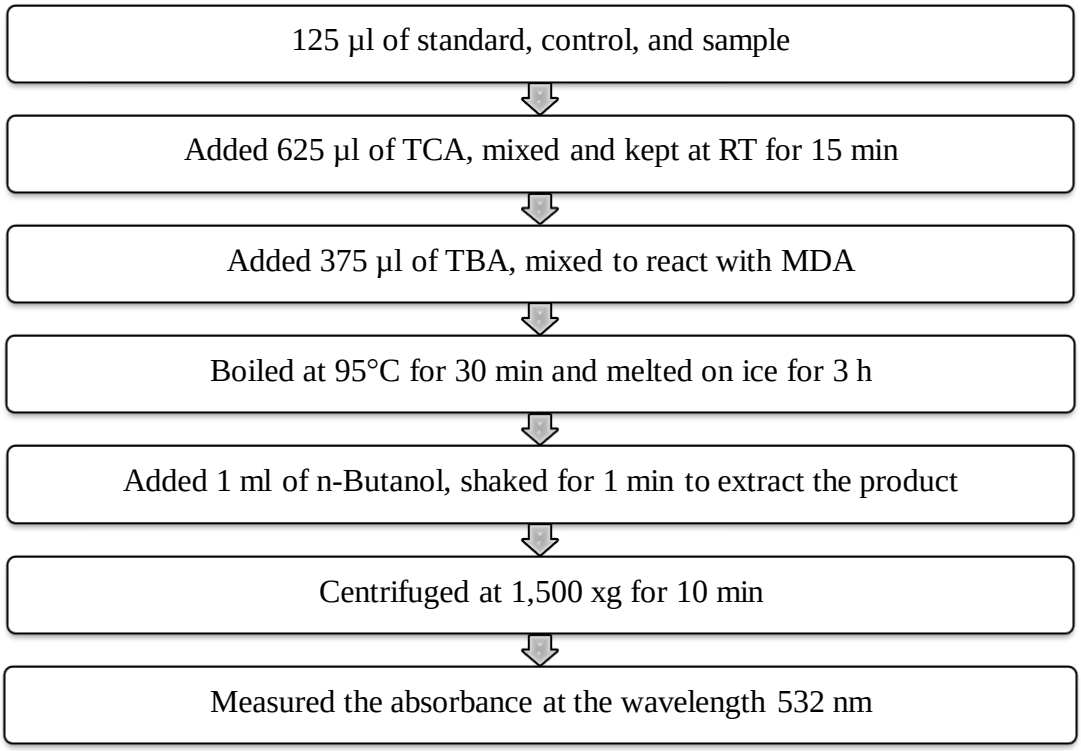

Figure 4.2 A schematic diagram of TBARS assay

4.2.2 The measurement system and OxLDL immunosensor preparation

The silver-electrode quartz crystal (AT-cut, 10 MHz, 9 mm in diameter) was embedded into flow cell as shown in Figure 4.3 and connected to the syringe pump system. Quartz crystal in each experiment was monitored it's resonant frequency shift using research quartz crystal microbalance (RQCM) resonant frequency counter from Maxtek Inc., USA as shown in Figure 4.4. The optimal flow rate of this flow-through system was performed by applying 10 mM PBS buffer pH 7.4 for 30 min at different flow rate of 30, 60, 120, 180, 300, and 500 µl/min. The resonant frequency shift of quartz crystal was recorded by a RQCM.

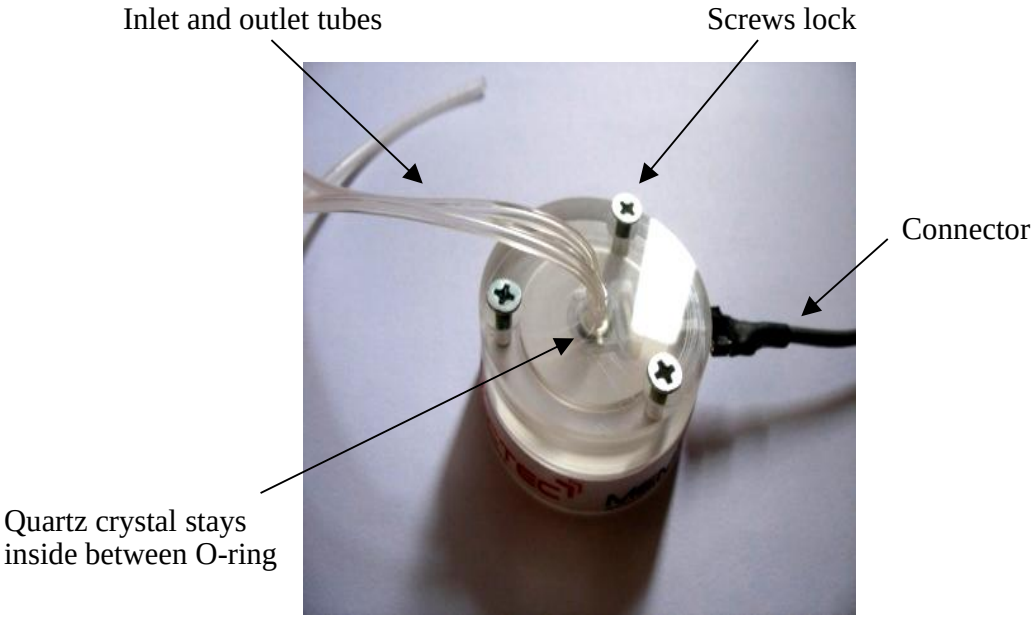

Figure 4.3 The construction of flow cell

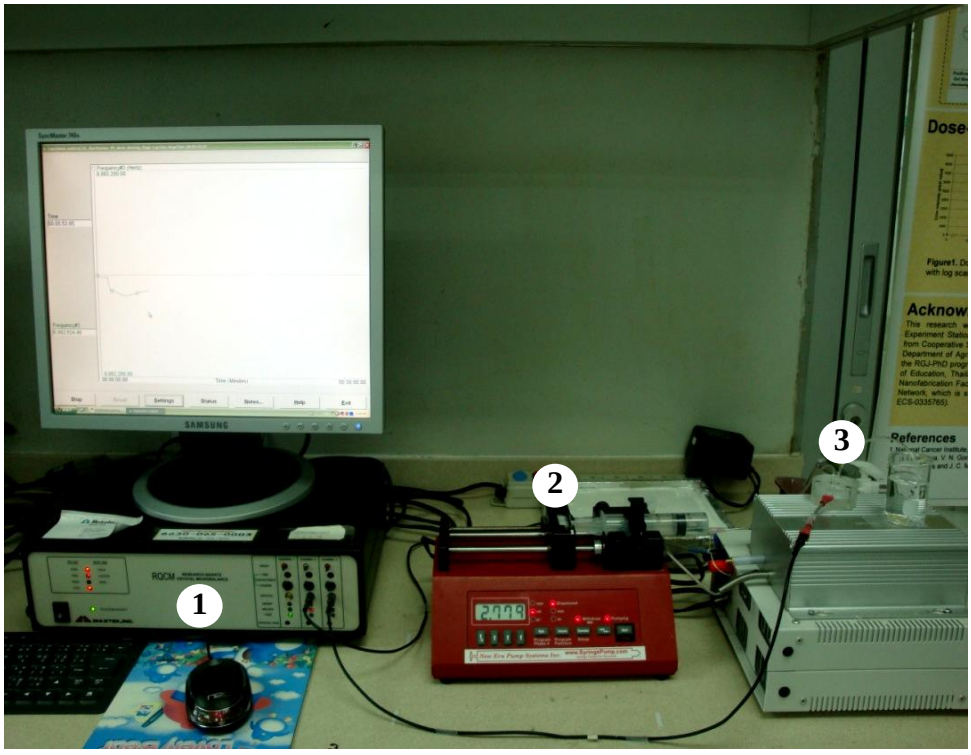

Figure 4.4 A schematic of measuring system setup (1 = RQCM, 2 = syringe pump, and 3 = flow cell)

Anti-OxLDL was immobilized by using covalent binding method via self-assembled monolayer (SAM) as shown in Figure 4.5. The electrode surface modification method was previously described by Chunta, S. (83). The cleaned silver electrode surface of 10 MHz quartz crystal was immersed into 10 mM 3-mercaptopropionic acid (MPA) at RT for 30 mins for SAM formation. Then, both sides of the electrode surface were rinsed with absolute ethanol and DW to remove some excess thiol. Monolayer surface was activated the carboxylic group of MPA and interacted with antibody by adding 5 µl of 200 mM 1-Ethyl-3-(3-dimethylaminopropril) carbodiimide (EDC) solution and 50 mM *N*-hydroxysulfosuccinimide (NHS) solution for 30 mins at RT. These solutions were placed over the only one side of surface electrode and kept in the dark moist chamber. This modified quartz surface was then rinsed with DW and embedded into flow cell of RQCM system. In addition, the modified quartz surface was finished in gas phase and anti-OxLDL immobilization was ready to operate in liquid phase. The performance of piezoelectric immunosensor in liquid phase was represented the real time detection system by RQCM.

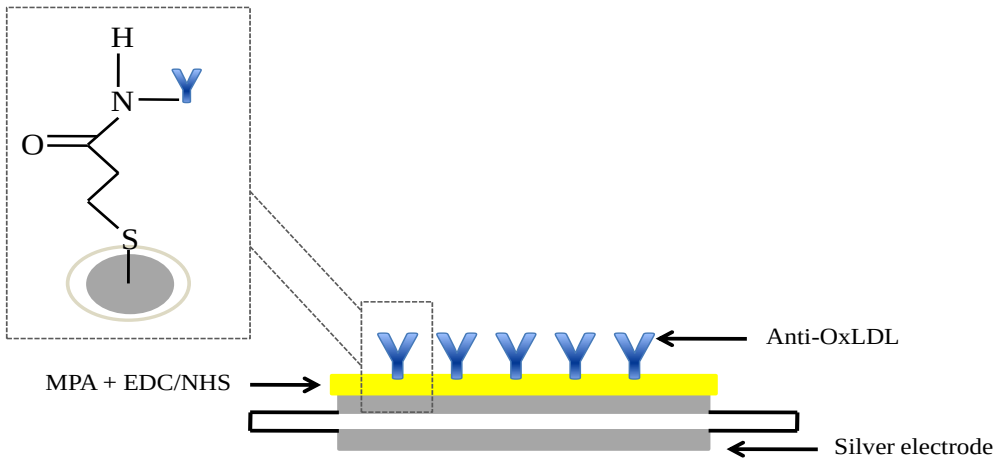

Figure 4.5 A schematic model for anti-OxLDL immobilization on silver electrode of piezoelectric quartz crystal

After the electrode surface of quartz crystal was modified with MPA and embedded into flow cell as shown in Figure 4.3. The one side of the modified crystal surface should be contacted the solution during assay. The flow-through system was started after warming up the device for 30 to 60 mins. Then, 10 mM PBS buffer pH 7.4 was flown through flow cell by syringe pump for 30 mins under flow rate at 60 µl/min. The PBS buffer was used as a baseline solution. Next, anti-OxLDL was injected into flow cell by syringe pump. The injected antibody was stopped and then stood for 30 mins. The excess antibody was washed out with 10 mM PBS buffer pH 7.4. The immobilization of anti-OxLDL was applied with 1% BSA for 15 mins to block the remaining potential reactive surface and then washed out with 10 mM PBS buffer pH 7.4 for 15 mins. After washing out the blocking agent with 10 mM PBS buffer pH 7.4, the frequency shift of quartz crystal (F_0) was measured as baseline signal. Next, OxLDL solution was injected into flow cell and incubated for 30 mins without flow. The excess OxLDL was washed out with 10 mM PBS buffer pH 7.4. After washing out the excess OxLDL, the frequency shift of quartz crystal was measured (F_1). Finally, the association between anti-OxLDL with OxLDL solution was evaluated by subtraction the frequency shift from baseline. The schematic diagram of OxLDL immunosensor preparation was shown in Figure 4.6.

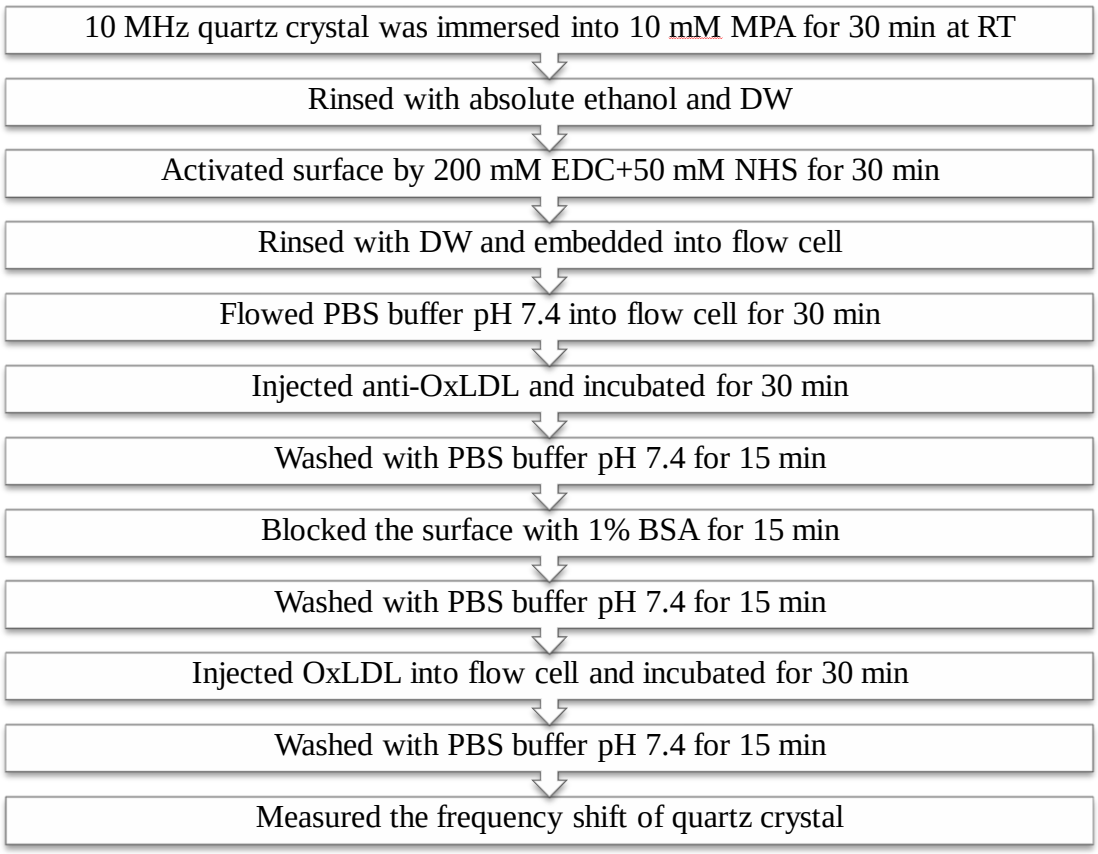

Figure 4.6 A schematic diagram of OxLDL immunosensor preparation

4.2.3 OxLDL immunosensor system optimization

This experiment was aimed to perform the optimal condition of liquid phase system including optimal concentration, incubation time, and binding ability of antibody in the OxLDL immunosensor.

4.2.3.1 Immobilization of anti-OxLDL

The anti-OxLDL at 10, 20, 50, 100, and 200 µg/ml was injected into flow cell by syringe pump. The flow-through system of each anti-OxLDL solution was stopped and incubated for 30 mins. The frequency shift of anti-OxLDL immobilization was monitored and recorded by RQCM system after washed out the excess anti-OxLDL with 10 mM PBS buffer pH 7.4.

The optimal incubation time of anti-OxLDL immobilization was performed by injection of 100 µg/ml anti-OxLDL solution into flow cell. The

flow-through system was stopped and then stood for 15, 30, 45, and 60 mins, respectively. The frequency shift was monitored by RQCM system.

4.2.3.2 Binding ability of anti-OxLDL on immunosensor surface

The binding ability of immobilized anti-OxLDL was demonstrated by the resonant frequency shift during the interaction between anti-OxLDL and OxLDL. Each immobilized anti-OxLDL at 20, 50, 100, and 150 µg/ml was applied with 1% BSA solution for 15 mins to prevent the interaction ability of possible vacant space on sensor surface. The resonant frequency shift of modified quartz crystal was measured as baseline signal after washed out the blocking agent. Four µmole/L OxLDL was injected into flow cell by syringe pump after washing out the excess BSA solution with 10 mM PBS buffer pH 7.4 for 30 mins without flow. The resonant frequency shift was recorded after washed out the excess OxLDL with PBS buffer pH 7.4.

4.2.4 OxLDL immunosensor performance evaluation

This experiment was purposed to evaluate the performance of OxLDL immunosensor in term of detection range, accuracy, precision, sensitivity, and specificity.

4.2.4.1 Dose response curve

One hundred µg/ml anti-OxLDL was used as an immunological recognition element of this OxLDL immunosensor. Each concentration of OxLDL solution (0.52, 1.12, 1.88, and 3.11 µmole/l) was injected into flow cell over the immobilized quartz crystal surface. The dose response curve and limit of detection of this system was generated by plotting the resonant frequency shift of various OxLDL concentrations.

4.2.4.2 Accuracy

The expectation test was performed to estimate the accuracy of OxLDL immunosensor by mixing technique. OxLDL solutions at the concentrations of 0.67 and 1.45 µmole/l were prepared by mixing 0.57 µmole/l OxLDL solution into 0.79 and 2.36 µmole/l OxLDL solution with a ratio 1:1, respectively. Two groups of OxLDL immunosensor were fabricated for measuring the amount of OxLDL solution

in both samples. The accuracy of this assay was calculated from found value and expected concentration of OxLDL solution.

4.2.4.3 Precision

The precision test was performed in two experiments; within-run precision test (intra-assay) and between-day precision test (inter-assay).

4.2.4.3.1 Within-run precision test

Within-run precision test was performed by performing 3 times testing in 1 day under the repeatability conditions including same measuring instrument, same measurement procedure, same observer, same conditions, and same location. Three pieces of OxLDL immunosensor were prepared for measuring the OxLDL solution at the concentration of 3 µmole/l. Each resonant frequency shift was converted to the OxLDL concentration by comparing with the frequency shift obtained from standard curve of OxLDL solution. Mean, standard deviation (S.D.), and coefficient of variation (C.V.) were calculated.

4.2.4.3.2 Between-day precision test

The one piece of OxLDL immunosensor was fabricated for measuring the OxLDL solution at the concentration of 3 µmole/l in the same manner on 20 consecutive days. The resonant frequency shift of each test was compared with the frequency shift obtained from standard curve of OxLDL solution to get the OxLDL concentration. Mean, S.D., and C.V. were calculated.

4.2.4.4 Interference of the OxLDL immunosensor

High density lipoprotein (HDL), very low density lipoprotein (VLDL), and low density lipoprotein (LDL) as the suspected interference were commonly found in serum. Moreover, it is possibly interfered the OxLDL detection. Therefore, these lipoproteins were used to evaluate the specificity of OxLDL immunosensor. The suspected interferences were measured by using OxLDL immunosenser under the same condition.

CHAPTER V
RESULTS

This chapter presented the results from the experiments that were conducted based on materials and methods described in chapter IV. The result consisted of four parts. The first part showed the result of oxidative modification of LDL preparation. The second part explained the measurement system and OxLDL immunosensor preparation. The third part showed the result of the optimal condition of OxLDL immunosensor. And the last part described the performance of OxLDL immunosensor.

5.1 The Oxidative Modification of LDL (OxLDL) Preparation

The objective of this experiment was to prepare OxLDL particles that were referred as the target antigen of OxLDL immunosensor. The oxidative modification of LDL was generated by inducing LDL oxidation with copper ion and measured its product with thiobarbituric acid reactive substance (TBARS) assay. Malondialdehyde (MDA) is a secondary product from lipid peroxidation that refers to OxLDL in this experiment. MDA can be reacted with thiobarbituric acid (TBA) to form MDA-TBA complex which is the product of lipid peroxidation. 1,1,3,3-Tetramethoxypropane (TMP) was used as a standard solution for establishing the standard curve of TBARS assay. In addition, the amount of OxLDL from oxidative modification of LDL could be figured out from standard curve of 1,1,3,3-Tetramethoxypropane (TMP). The results from standard curve of TMP were shown in Figure 5.1.

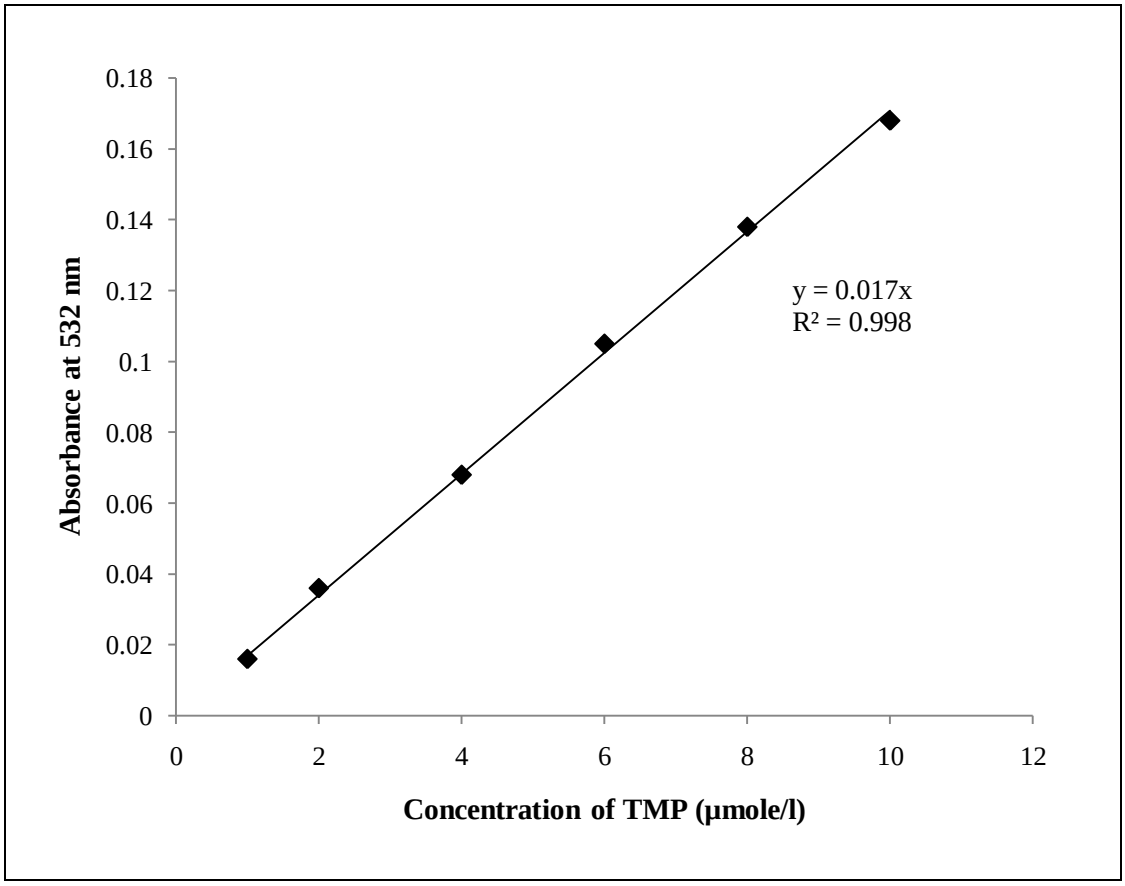

Figure 5.1 The relationship between 1,1,3,3-Tetramethoxypropane (TMP) concentration (µmole/l) and the absorbance at 532 nm were measured by TBARS assay for calculating the concentration of OxLDL (n = 3).

From Figure 5.1, standard curve of TMP was utilized to calculate the amount of OxLDL after the oxidative modification of LDL. This result was reliable for determinating of OxLDL concentration due to the Coefficient of Determination (R^2) of this experiment was 0. 9988.

Next, the human LDL at different concentration was incubated with copper ion at 37°C for 24 h and measured the amount of OxLDL concentration by TBARS assay with spectrophotometry. The result showed that 25 to 500 µg/ml human LDL were successfully oxidized by inducing with copper ion to yield OxLDL. The application at higher concentration tended to produce the same amount of OxLDL concentration that was less than the concentration at 200 µg/ml as shown in Figure 5.2.

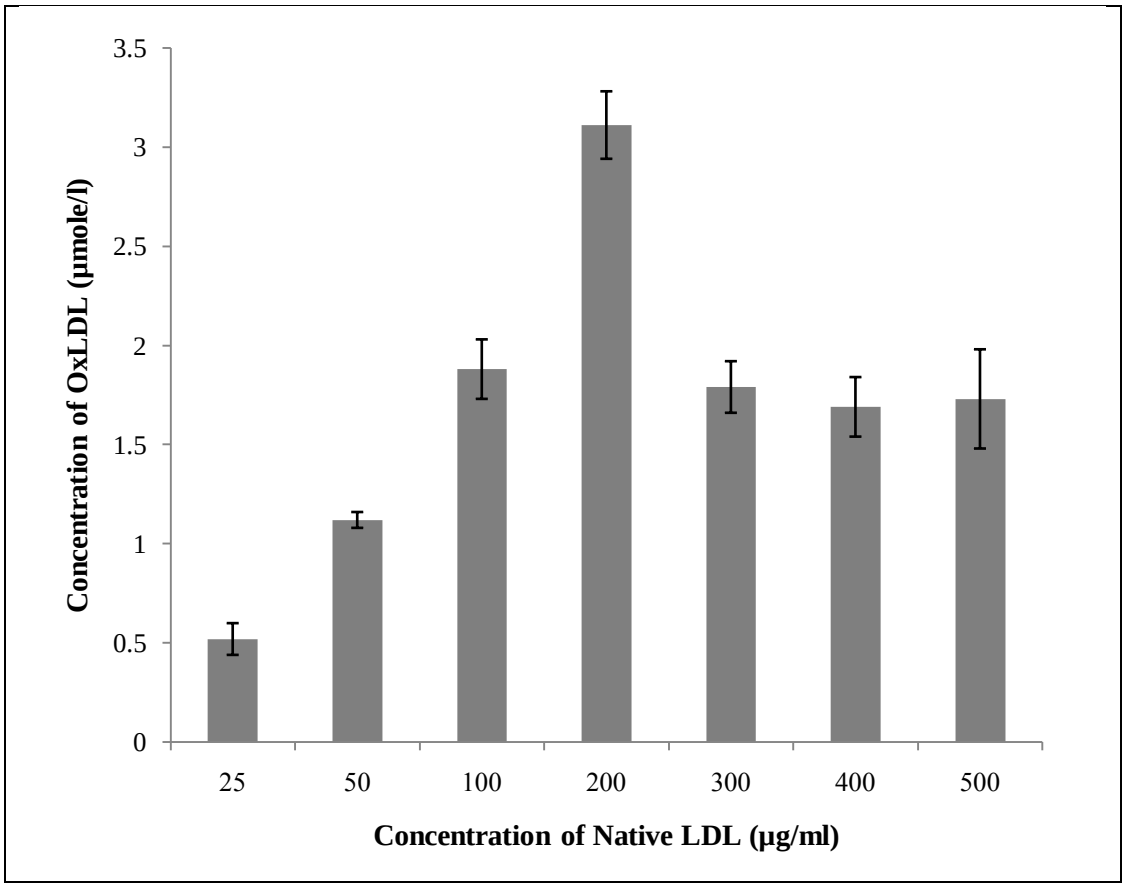

Figure 5.2 The relationship between concentration of human LDL inducing with $CuSO_4$ at 37°C for 24 h and the concentration of OxLDL from the oxidative modification measured by TBARS assay (n = 3).

5.2 The Measurement System and OxLDL Immunosensor Preparation

The optimal flow rate of this measurement system was performed by PBS buffer pH 7.4 which was driven through flow cell under the different flow rates with syringe pump. The frequency response of the different flow rates were monitored by the frequency counter (RQCM). The flow-through system gave the most stable resonant frequency signal at 60 µl/min flow rate (ΔF±1 Hz) as shown in Figure 5.3. The higher flow rate up to 300 µl/min also maintained acceptable signal stability (ΔF±10 Hz) after 10 mins lag time. Therefore, the flow rate at 60 µl/min was chosen to operate in this measuring system.

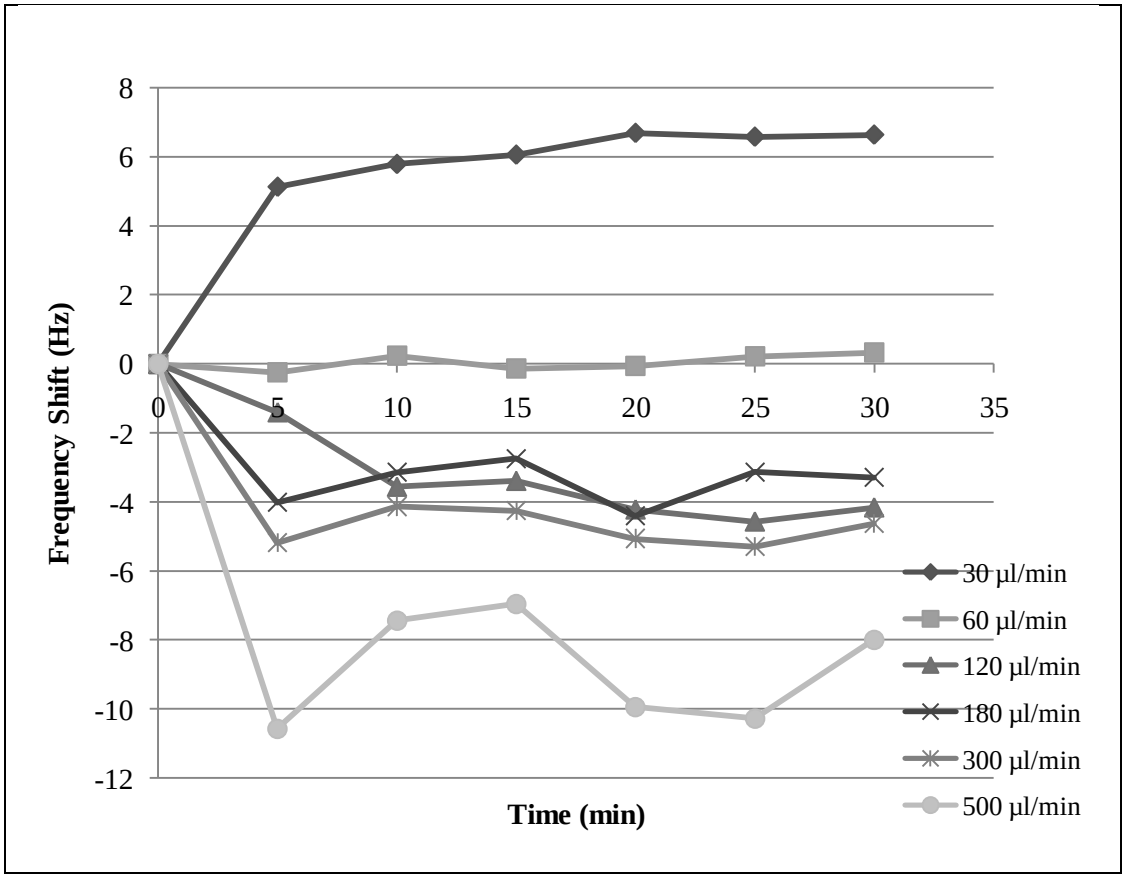

Figure 5.3 The signal stability of measurement system was performed by injecting PBS buffer pH 7.4 through the flow cell for 30 min at the different flow rates by syringe pump.

In this study, anti-OxLDL was immobilized by covalent binding method via self-assembled monolayer (SAM). Therefore, the cleaned silver electrode surface was immobilized by assembling MPA onto the quartz crystal surface before immobilizing with anti-OxLDL. The functional group of MPA was activated by EDC/NHS due to the carboxyl group of MPA was reacted with NHS in the coupling solution of EDC/NHS. The NHS ester formed covalent linkage for antibody binding. Then, anti-OxLDL was immobilized onto modified surface of MPA. The sensor surface was flown with 10 mM PBS buffer pH 7.4 to remove excess antibody and blocked with 1% BSA to cover the remaining vacant space on the silver surface. Finally, the sensor surface was washed out the excess of blocking agent with 10 mM PBS buffer pH 7.4. The quartz crystal was ready to use as the OxLDL immunosensor for measuring OxLDL as shown in Figure 5.4.

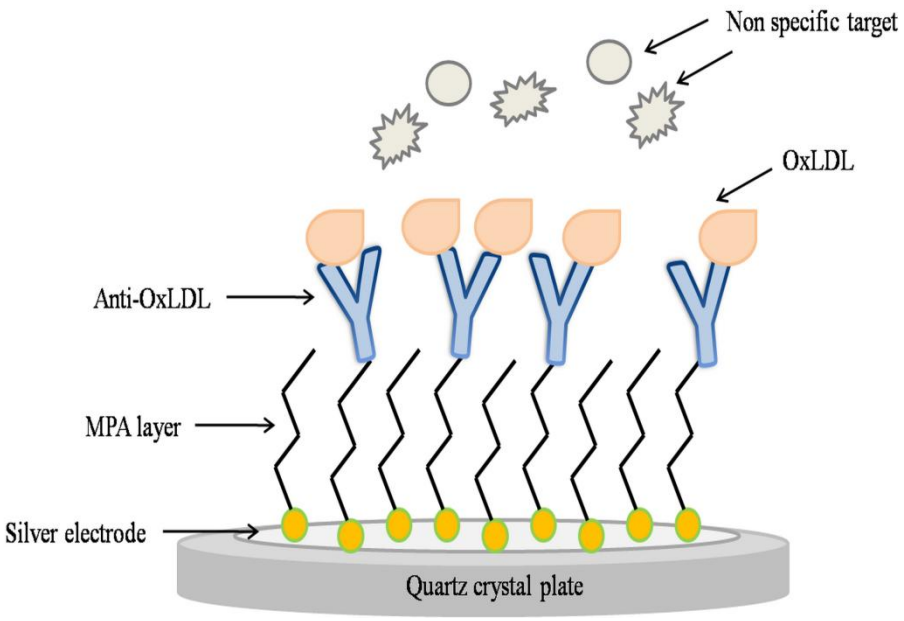

Figure 5.4 A schematic model of OxLDL immunosensor preparation showed the immobilization of anti-OxLDL via self-assembled monolayer (SAM) onto modified quartz crystal.

5.3 OxLDL Immunosensor System Optimization

5.3.1 Immobilization of anti-OxLDL

The silver electrode surface was successfully coated with 10 mM MPA. The functional group of MPA was activated by EDC/NHS, resulting in the functional group which formed covalent linkage with primary amine of antibody molecule. The different concentration of anti-OxLDL at 10, 20, 50, 100, and 200 µg/ml was immobilized on the modified quartz crystal surface. The different resonant frequency signal between the resonant frequency of the immobilized-antibody mass (f_1) in different concentration and the original resonant frequency of modified quartz crystal that was flown with 10 mM PBS buffer pH 7.4 (f_0) was obtained as the frequency shift ($\Delta F = f_1 - f_0$). The result showed that the increasing of resonant frequency shift when applying higher concentration of anti-OxLDL as shown in Figure 5.5. The highest resonant frequency shift ($\Delta F = 268 \pm 8.65$ Hz) was obtained from the anti-OxLDL at concentration of 100 µg/ml. The higher concentration did not present significant different frequency shift.

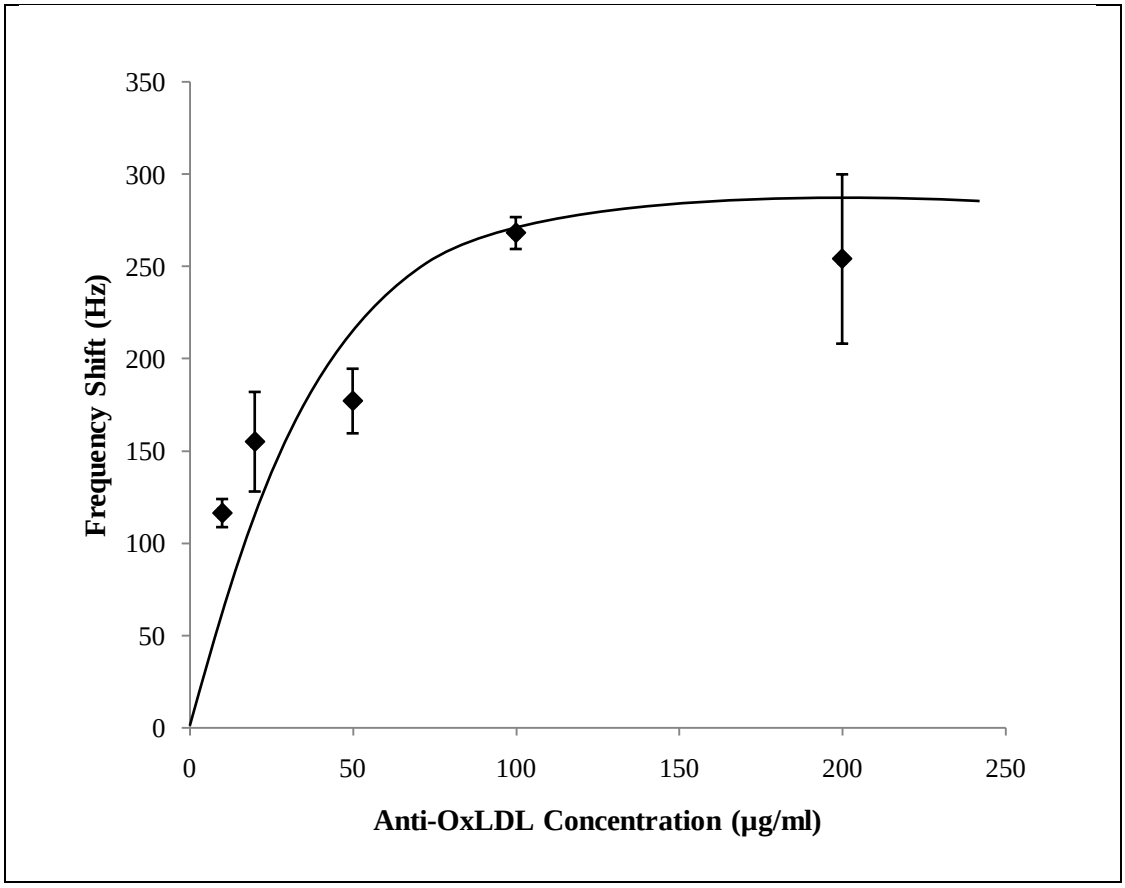

Figure 5.5 The relationship between the concentration of anti-OxLDL and the resonant frequency shift (Hz) of quartz crystal. Anti-OxLDL at concentration of 10-200 µg/ml were injected into flow cell over sensor surface by syringe pump and stood for 30 mins. The resonant frequency shift after immobilization was monitored by using RQCM (n = 3).

The optimal incubation time of anti-OxLDL immobilization was tested by immobilizing anti-OxLDL at the concentration of 100 µg/ml in different incubation times (15, 30, 45, and 60 mins). The increasing resonant frequency shift due to the higher incubation time of anti-OxLDL immobilization was shown in Figure 5.6. The incubation time longer than 30 mins did not significantly change the resonant frequency. Therefore, the optimum application time of anti-OxLDL immobilization was 30 mins.

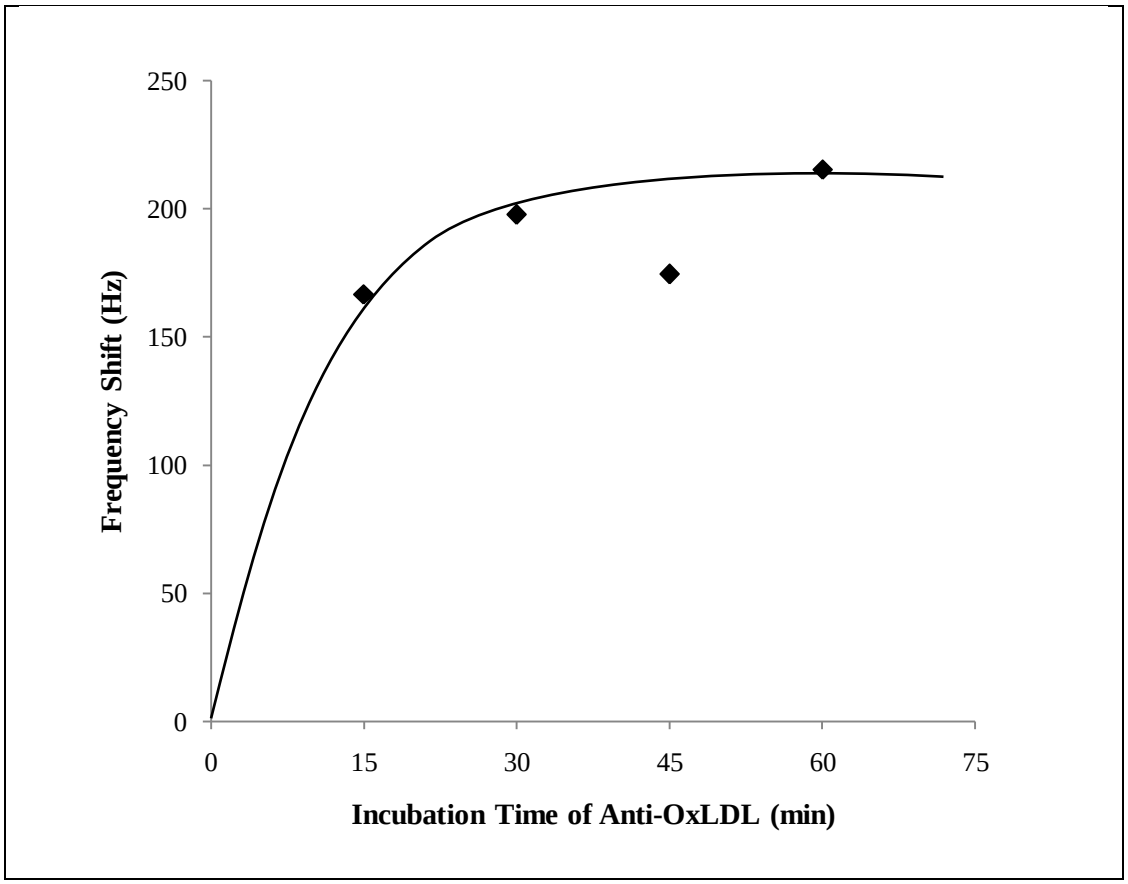

Figure 5.6 The relationship between the incubation time of anti-OxLDL immobilization and the resonant frequency shift (Hz) of quartz crystal. The 100 µg/ml anti-OxLDL was immobilized on the sensing surface for 15-60 mins and monitored the resonant frequency shift after immobilization by using RQCM.

5.3.2 Binding ability of anti-OxLDL on immunosenser surface

The binding ability of anti-OxLDL was investigated by the interaction between OxLDL particle in tested solution and immobilized anti-OxLDL on immunosenser surface. Four µmole/l OxLDL was applied on OxLDL immunosensor surface with 20, 50, 100, and 150 µg/ml anti-OxLDL for 30 mins at room temperature. The different resonant frequency shift was responded due to the anti-OxLDL concentration binding with OxLDL. The 100 µg/ml anti-OxLDL showed 250 Hz change on the sensor surface as shown in Figure 5.7. The higher anti-OxLDL concentration did not significantly represent the resonant frequency shift. Moreover, the lower amount of anti-OxLDL on the sensor surface gave less resonant frequency shift of interaction binding. Therefore, OxLDL immunosenser with 100 µg/ml anti-OxLDL was chosen to interact with OxLDL particle in this system.

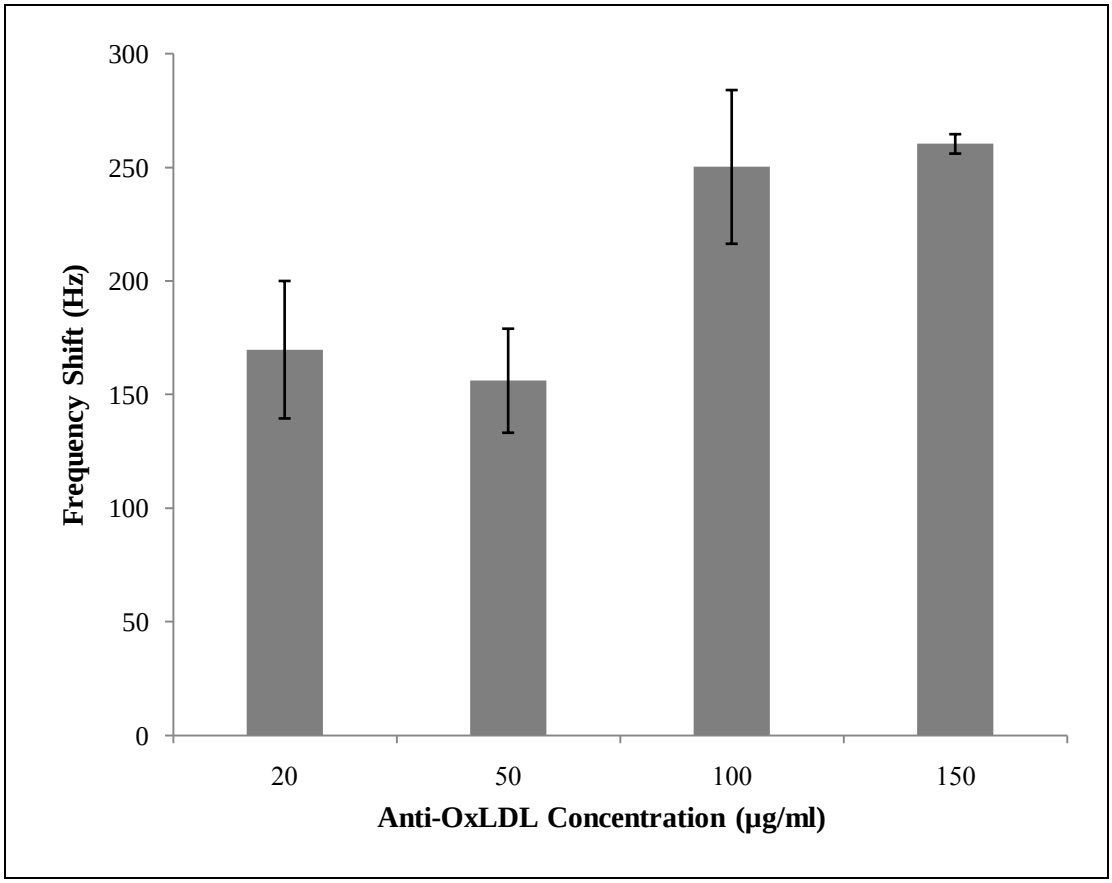

Figure 5.7 The relationship between the binding ability of anti-OxLDL on immunosensor surface and the resonant frequency shift (Hz) of quartz crystal. The different concentration of anti-OxLDL was injected into flow cell and stood for 30 mins to bind with OxLDL solution. The interaction between OxLDL and anti-OxLDL was monitored by using RQCM (n = 3).

5.4 OxLDL Immunosensor Performance Evaluation

The objective of this experiment was to demonstrate the performance of OxLDL immunosensor in term of detection range, accuracy, precision, and specificity.

5.4.1 Dose response curve

Under the optimal condition, OxLDL immunosensor was fabricated for measuring the amount of OxLDL particle at the concentration of 0.5-3.1 µmole/l. The concentration of OxLDL particle in µmole/l was calculated from the standard curve of TMP. A linear correlation that was observed OxLDL concentration as a function of resonant frequency shift was 0.5 µmole/l to 3.1 µmole/l as shown in Figure 5.8. The higher concentration of OxLDL (more than 3.1 µmole/l) did not show the resonant frequency shift due to the oxidation of LDL saturated under 50 µM $CuSO_4$ condition. The limit of detection of this assay was 0.5 µmole/l because the lower concentration of OxLDL (lesser than 0.5 µmole/l) did not show any the resonant frequency shift. Therefore, the detection range of this system was 0.5 µmole/l to 3.1 µmole/l.

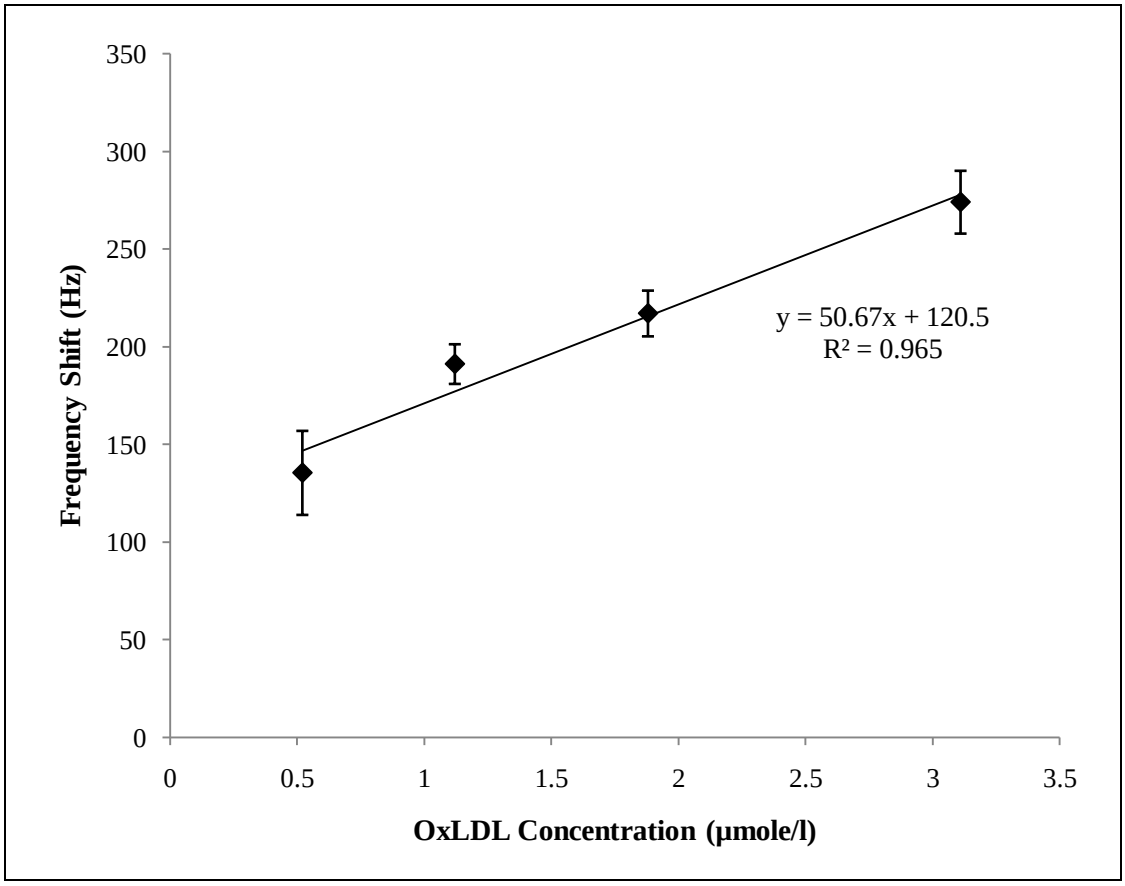

Figure 5.8 Dose response curve of OxLDL immunosensor was performed by measurement OxLDL at the concentration of 0.5-3.1 µmole/l under the optimal condition of OxLDL immunosensor (n = 3).

5.4.2 Accuracy of the assay

Expectation test was studied to evaluate the accuracy of OxLDL immunosensor. The expected values were 102.71% and 91.34% for 0.67 and 1.45 µmole/l OxLDL, respectively, as shown in Table 5.1. Moreover, the average % expected of this assay was 97.02%.

Table 5.1 Expected values of OxLDL detection by using OxLDL immunosensor

OxLDLmixing (µmole/l)	Frequency Shift (Hz)	OxLDLmeasured (µmole/l)	Expected value (%)
0.67	282.25	0.69	102.71
1.45	382.33	1.32	91.34
Average % Expected			**97.02**

5.4.3 Precision of the assay

The concentration of OxLDL solution at 3 µmole/l was chosen to determine in the precision of this assay. This concentration was measured three and twenty times for determination the intra-assay and inter-assay precision, respectively. The resonant frequency shifts of each test were converted to OxLDL concentration by using the linearity of dose response curve. Coefficients of variation (C.V.) for intra-assay and inter-assay test were 9.37% and 23.04%, respectively, as shown in Table 5.2.

Table 5.2 Precision of OxLDL detection by using OxLDL immunosensor

OxLDL(µmole/L)	Intra-assay (n = 3)		Inter-assay (n = 20)	
	Mean ± S.D. (µmole/l)	% C.V.	Mean ± S.D. (µmole/l)	% C.V.
3	3.39 ± 0.32	9.37	3.29 ± 0.8	23.04

5.4.4 Interference of the OxLDL immunosensor

The specificity of OxLDL immunosensor was investigated by using other classes of lipoprotein such as HDL, LDL, and VLDL as the suspected interferences. The HDL, LDL, VLDL, and OxLDL were determined this performance by using OxLDL immunosensor. The result showed that the applied HDL represented the resonant frequency shift lower than the resonant frequency shift of OxLDL. Thus, the applied HDL showed non-significant resonant frequency shift in OxLDL immunosensor as shown in Figure 5.9. However, results of the applied LDL and VLDL showed significantly increased resonant frequency shift as shown in Figure 5.9. In addition, bovine serum albumin (BSA) was applied to test the sensor specificity. It showed non-significant resonant frequency shift which was similar to the applied HDL but not in LDL and VLDL. Therefore, the results indicated that LDL and VLDL have significantly interfered to the OxLDL immunosenser system due to in these samples may contained lipoprotein component as same as OxLDL components.

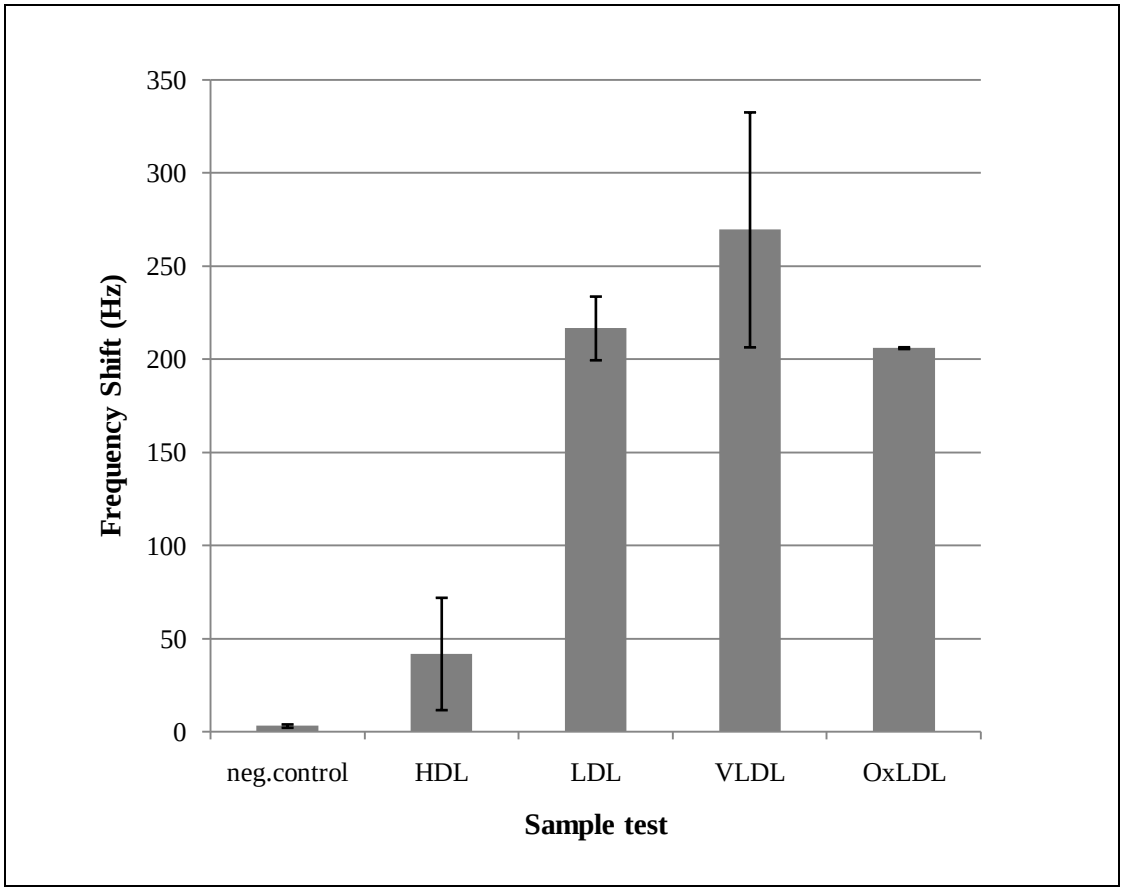

Figure 5.9 Specificity of OxLDL immunosensor was tested with the suspected interferences such as HDL, LDL, and VLDL by using OxLDL immunosensor under the same condition (n = 3).

CHAPTER VI
DISCUSSION

Oxidized low density lipoprotein (OxLDL) is a major risk factor in atherosclerosis. The increasing of plasma OxLDL level can be utilized as a biomarker for atherosclerosis to monitor and prevent the risk factor of atherosclerosis (6, 61). Commonly, the level of plasma OxLDL is very low in normal population due to it is rapidly removed from the circulation by reticuloendothelial system and anti-oxidants (40). The normal range of plasma OxLDL is not widely reported, however it has been reported in 0.58 ± 0.23 ng/5µg LDL protein (84). There are several detection methods to investigate the amount of plasma OxLDL such as TBARS assay, conjugated diene, HPLC, and NMR (8, 14, 56). These methods are indirect determination of plasma OxLDL by measuring the product from LDL oxidation. Recently, ELISA assay has been developed as the commercial kit for detection the specific structure of OxLDL (60, 61). This method has high complexity, costly, and time-consuming. Moreover, the previous described detection methods of plasma OxLDL are not standard method to assess the amount of OxLDL in clinical diagnosis.

Therefore, in this study, piezoelectric-based immunosensor was proposed to develop for measuring the amount of plasma OxLDL, so called, OxLDL immunosensor. The OxLDL immunosensor was fabricated on 10 MHz AT-cut quartz crystal with silver electrode (85, 86). Generally, the quartz crystal with gold elcectrode is frequently used for quartz crystal microbalance (QCM) system according to its stability and reusability. For the silver electrode, it has been applied in many previous studies such as in Salmonella species detection and malaria diagnosis (89, 90) because the silver electrode of QCM has higher reactivity with chemical, disposable, and low-cost of fabrication (87, 88). So, it was recommended in this study. However, silver electrode can be oxidized but it does not significantly effect to the resonant frequency shift of quartz crystal (91). In addition, silver electrode quartz crystal should be kept in closed cap to prevent the oxidation reaction on silver surface.

For oxidative modification of LDL, the incubation of human LDL with copper ion has been used as a standard method to produce OxLDL which is generated a variety of modification on apolipoprotein and lipid such as MDA, HNE, and amino acids residues (92). The copper ion have been extensively used for LDL oxidation. It is a better oxidant of LDL than ferric ion because copper can bind to apo B protein to form Cu-LDL complex that is essential in copper-mediated peroxidation of LDL during incubation of LDL with copper ion (93, 94-97). In this study, native LDL at concentration 200 µg/ml gave the highest product from LDL oxidation by inducing with 50 µM $CuSO_4$. The application at higher concentraion gave lower concentration of OxLDL than 200 µg/ml. Because the saturation concentration of LDL that was incubated with 50 µM $CuSO_4$ condition in this study was 200 µg/ml (Figure 5.2). This result showed the relationship with the previous study, the incubation of human LDL with $CuSO_4$ lead to the formation of Cu-LDL complexes which were depended on both LDL and copper ion concentrations in the range of 0.1-0.4 mg LDL protein/ml and 5-40 µM Cu^{2+}, respectively (97). So, in this study, human LDL at concentration 25-500 µg/ml can be induced to OxLDL by using 50 µM $CuSO_4$ at 37°C for 24 h. At 200 µg/ml of the human LDL was saturated concentration of Cu-induced LDL oxidation in this study due to the relation between Cu^{2+} binding to LDL. In addition, ethylenediaminetetraacetic acid (EDTA) was used in this study to stop the terminal reaction of LDL oxidation (41). Moreover, in some study, butylated hydroxytoluene (BHT) and nitrogen gas have been used to stop the oxidation similar to EDTA (14).

The measurement system of this study was performed by using RQCM device that was integrated with modified quartz crystal embedded into flow cell. The liquid-flow system may be developed to reduce the application time of washing and drying steps of sensor preparation. Moreover, this system can monitor the frequency change in real-time detection. Normally, the flow rate of liquid phase system depends on the diameter of quartz crystal, diameter of flow cell, and the pressure of pumping system. In this study, the appropiate flow rate for 10 MHz AT-cut quartz crystal by syringe pump was 60 µl/min. This flow rate gave more stable output signal than other flow rates. The lower or higher of flow rate than 60 µl/min, the signals were stable after 10 min in lag time. The increasing of flow rate can induce the high pressure of flow-through system lead to unstable signal of the frequency response. Therefore, the

flow rate at 60 µl/min was chosen to operate in this measurement system due to it is suitable in the diameter of 10 MHz quartz crystal and flow cell for this study. In addition, the application of QCM in liquid phase, the resonant frequency shift is related to the surface mass-loading as well as the properties of the liquid such as viscosity, density, conductivity, and permittivity (98). Therefore, all of these factors was controlled by using same designated conditions to avoid possible influence factors to the result output.

In this study, polyclonal anti-Cu^{2+}-oxidized low density lipoprotein antibody (anti-OxLDL) that obtained from Millipore was used as biological recognition element of this system. The self-assembled monolayer (SAM) of MPA was selected to immobilize antibody in this study due to its robustness and reproducibility. From previous study, Park et al. (99) presented that mercaptopropionic acid (MPA) was the best reagent for monolayer formation due to the strongest cross-linker-antibody complex binding to the gold electrode of the quartz crystal. In this study, silver electrode of quartz crystal was dipped into 10 mM MPA for 30 min to modified crystal surface (83). The functional group of MPA was activated by EDC/NHS to form bonding with the primary amine ($-NH_2$) of antibody on the electrode surface (85, 100). In principle, amine (NH_2) group presents in the lysine amino acid side-chain on the antibody surface can be used for covalent bonding in a random immobilization (101). Therefore, anti-OxLDL immobilization of this study was formed the covalent linkage with MPA on modified electrode surface by using random orientation.

The performance of OxLDL immunosensor is strongly dependent on the density of antibody on the sensor surface and its availability to react with OxLDL. The effective sensor preparation in term of an optimal anti-OxLDL concentration and application time were studied. This study presents that the resonant frequency shift of quartz crystal was related to the amount of antibody immobilization on the electrode surface from 10-200 µg/ml. The optimal concentration of antibody immobilization was 100 µg/ml due to its gave the highest resonant frequency shift and represented the higher saturation of antibody immobilization. The higher concentration did not show significant different frequency shift due to the binding of antibody on the sensor surface. For the optimal application time of antibody immobilization was 30 mins

which was given the saturation of antibody immobilization in the shortest time. The longer incubation time of antibody immobilization did not produced the increasing resonant frequency response. This study showed result in the line with previous study, Brogan KL et al. (102) which spent 30 mins to immobilize anti-calf alkaline phosphatase-immunoglobulin G (RACAP-IgG) onto gold surface. Therefore, the optimal condition of antibody immobilization that gave the binding of antibody on the sensor surface at the saturation state were 100 µg/ml and 30 mins, respectively (Figure 5.5 and Figure 5.6). This condition of antibody immobilization can save cost and time for immunosensor preparation.

The result obviously showed ability of anti-OxLDL on immunosensor surface that reacted with OxLDL particle by the changing of sensor resonant frequency shift. The result showed that at the concentration of anti-OxLDL 100 µg/ml represented the function ability to bind with OxLDL. The frequency change was 250 Hz. The higher concentration showed the frequency change of antigen-antibody reaction was nearly anti-OxLDL 100 µg/ml immobilized. In addition, the incubation time of antigen-antibody interaction was 30 mins (data not shown). The reaction time would be increased with the decreasing of the molecular concentration. The changing of resonant frequency was resulted from mass of each immunocomplexes that deposited on the surface. Therefore, all of data was indicated that the 100 µg/ml anti-OxLDL immobilized on the electrode surface has a suitiable ability to bind with the antigen. However, the frequency response of this sensor is also dependent on both the density and viscosity of the solution which passed over the QCM crystal surface (103).

Dose response curve of this assay was studied by measuring the amount of OxLDL that used as a target antigen of OxLDL immunosensor. The detection range of this assay was 0.5-3.10 µmole/l of OxLDL concentration that had the sensitivity performance for detection the concentration of OxLDL. The limit of detection was 0.5µmole/l OxLDL concentration. Each concentration of OxLDL was depended on the concentration of native LDL that was incubated with copper ion. So, the concentrations of OxLDL that were used in this study were between 0.5 to 3.10 µmole/l. In addition, the higher concentration of OxLDL which is more than 0.5 µmole/l should be diluted to lower concentration before measurement due to sensitivity and limit of detection of this assay less than 0.5 µmole/l. However, the

binding limit of antibody immobilization on the surface was associated with OxLDL concentration and its application antibody concentration that affected to the frequency response. Furthermore, the result in this study was comparable to the previous study, Chunta S. (83) demonstrated the detection limit of HDL-P immunosensor from 0.21 to 2.5 mg protein/ml. In addition, the normal range of OxLDL, in each laboratory was recommended to develop its own reference range which is different in the unit. The detection range in this study was represented in the micromole/l (µmole/l) unit. So, It has sensitivity higher than the previous study.

To determine the performance of this immunosensor, expectation test that gave the average expected value was 97.02%. It was implied that the accuracy of this immunosensor is satisfactory. The precision of this assay was shown in term of coefficients of variation (C.V.) of intra-assay and inter-assay experiment. The CVs of intra-assay and inter-assay experiment were 9.37% and 23.04%, respectively. In fact, the % C.V. of inter-assay was higher than intra-assay due to the variation among days. In addition, the intra-assay of this study was measured 3 times a day because the interval time of this assay was 3 hours per each experiment. In the immunoassay, the C.V. acceptable level is typically below 20% (104). Moreover, the C.V. acceptable level of inter-assay is recommended as less than or equal to 30% (105). Therefore, this assay provides an acceptable precision for predicting the performance of OxLDL immunosensor. In addition, the results of precision (Table 5.2) represented the mean of OxLDL concentration that was converted from resonant frequency shift rather than the control sample due to the immunoreaction of this system and interference factors from liquid phase system.

The interference test of OxLDL immunosensor was performed to determine the specificity of anti-OxLDL immobilization on the modified electrode surface. HDL, LDL, and VLDL were measured in OxLDL immunosensor as the suspected interference. The result showed significantly interference from cross-reactions that presented in the frequency response of each sample. The cross-reaction of this assay may be occurred from the specificity of antibody and interference of OxLDL such as LDL and VLDL.

The anti-Cu^{2+}-oxidized low density lipoprotein (anti-OxLDL) was immobilized on the modified electrode surface to react with OxLDL solution. This

antibody is a polyclonal antibody from rabbit which is strongly specific to LDL oxidation including Cu^{2+}-oxidized LDL, MDA-LDL, and HOCl-LDL (data from company). From the result (Figure 5.9), anti-OxLDL can be reacted with LDL and VLDL that gave the resonant frequency shift as same as OxLDL but HDL did not present significantly resonant frequency shift. Therefore, the anti-OxLDL immobilization of this immunosensor is not only specific with OxLDL because it can bind with the other molecules, especially molecules that have structure similarly to OxLDL result in reducing of specificity. In addition, polyclonal antibodies are produced by using traditional immunization procedures such as in rabbits, goats, sheep, and pigs so it is not possible to produce identical antibody specificity even in two animals of the same species and also lack of specificity (106). Polyclonal antibody can recognize multiple epitopes on any one antigen.

The interference of OxLDL occurred from the other class lipoprotein. All classes of lipoprotein have the same structure; triglyceride (TG), cholesteryl ester (CE), phospholipid (PL), and unesterified cholesterol (UC). The different type of apolipoprotein (apo) on the outer surface can be identified lipoprotein classes. LDL and VLDL contain apo B100 on the outer surface but the amounts of molecules are different. The oxidation of LDL can be generated a variety product by many mechanisms in LDL particle such as metal ions and enzymatic activity. In addition, oxidation of lipid in LDL by copper-induced oxidation generates the reactive aldehydes which can interact with the lysine residue of apo B100. The lysine residue can be substituted or modified by aldehydes resulting in OxLDL. The structures of LDL are changed after modification and could not identify its structure. Therefore, LDL and VLDL can be interfered in OxLDL immunosensor due to LDL oxidation in apo B100. Moreover, the enzymatic activity can be oxidized both the lipid and the protein moiety of LDL. All these pathways result in aldehyde-type modification of the protein moiety of LDL. The underlying mechanisms of oxidation of LDL may be different in different sample. However, the oxidation of LDL by inducing with copper ion can be oxidized LDL to OxLDL but could not know the exactly structure of OxLDL after modification. Therefore, OxLDL immunosensor has cross-reaction with LDL and VLDL due to the same structure of these lipoproteins. In addition, the resonant frequency shift of applied LDL and VLDL decreased more than OxLDL may

be it cause from the molecular weight of LDL and VLDL are higher than OxLDL. So, this immunosensor absorbed the non-specific from LDL and VLDL lead to the resonant frequency shift of LDL and VLDL has significantly increased.

Therefore, this problem can be solved by using monoclonal antibody that recognized to specific structure of OxLDL lead to the specificity of this system increased. Due to monoclonal antibody has specificity higher than polyclonal antibody and recognize only one epitope on an antigen (107). In addition, the OxLDL immunosensor should be used two antibodies to measure OxLDL. The procedure of two antibodies is set up similar to the sandwich ELISA. For example, OxLDL-DLH3 assay, anti-OxPC monoclonal antibody (DLH3) and anti-human apo B polyclonal antibody are used, thus an antigen is caught between two antibodies (5, 16, 108). The antigen of this assay should be contained apo B that have been modified by OxPC. This application may be increased the specificity of this system.

Moreover, OxLDL is generated from oxidative modification of LDL so this particle may contain the combination of OxLDL and LDL. This interference can solve by pretreatment assay to purify OxLDL before measurement. LDL, VLDL, and OxLDL can be isolated by ultracentrifugation that depended on their densitybefore measuring (109). In some study,ion-exchange HPLC and affinity chromatography have been used to isolated lipoproteins to purify sample. Thus, pretreatment assay can be proposed to purify and isolate OxLDL and the lipoprotein interference resulting in specificity increased and interference decreased.

Furthermore, molecular imprinted polymer (MIP) may be proposed to solve this problem by creating the template-shape of OxLDL in polymer that used as molecular recognition of OxLDL. The OxLDL that has a similar shape to this site is recognized in the recognition site. However, structure of OxLDL in each state of oxidation is not similar because it can be modified in both lipid and protein. Hence, the structure of OxLDL that is changed after undergoes oxidation is not exactly known. Therefore, MIP can be used to solve this problem if the exactly structure of OxLDL is clarify.

All of these assays were proposed to solve the problem of specificity result in the increasing specificity of OxLDL immunosensor. However, the interference of OxLDL immunosensoris not only from specific of antibody and cross-reaction of

immunosensor but also the interference from liquid phase system such as density, viscosity, and conductivity of liquid. Therefore, this immunosensor should be developed the specificity in the future.

In this study, OxLDL immunosensor was successfully developed to detect OxLDL. There are many advantages offered by OxLDL immunosensor such as the minimum amount of antibody and the labeled-free of this system. However, this immunosensor would be proposed to develop for the clinical usage in the future. The suggestion for development this immunosensor are composed of study the reliability of OxLDL immunosensor by comparision with ELISA method, measurement OxLDL by using OxLDL immunosensor in serum sample, and study regeneration ability of OxLDL immunosensor. These suggestions can indicate the efficiency of OxLDL immunosensor for clinical diagnostic. In addition, the OxLDL immunosensor optimization is an important point for developing this immunosensor resulting in reliability of OxLDL immunosensor. Altogether, this immunosensor would be developed in term of specificity and clinical usage for application in clinical diagnostic.

CHAPTER VII
CONCLUSION

The high circulating oxidized low density lipoprotein (OxLDL) plays an important role in atherosclerosis. Therefore, OxLDL particle is one of biomarker for assessing risk factor of atherosclerosis. This study is aimed to develop piezoelectric-based immunosensor system for OxLDL detection. The results of this study were summarized as following.

1. Human LDL at the concentration of 25-500 µg/ml were oxidized by incubation with 50 µM $CuSO_4$. The saturation concentration of LDL that oxidized to OxLDL was 200 µg/ml.

2. OxLDL immunosensor was created by an optimal condition of anti-OxLDL at the concentration of 100 µg/ml which is immobilized on silver electrode surface of quartz crystal via self-assembled monolayer (SAM) method for 30 mins.

3. The opimal flow rate of this measurement system was at flow rate of 60 µl/min.

4. The detection range of this immunosensor was 0.5–3.10 µmole/l and the limit of detection of OxLDL immunosensor was 0.5 µmole/l.

5. The analytical accuracy of this sensing was 97.02% and the intra-assay and inter-assay precision (CVs) were 9.37 and 23.04%, respectively.

From all of results, OxLDL immunosensor was successfully developed for OxLDL detection. This system demonstrated a direct analytical process to detect OxLDL. It is rapid and simple to use without multi labeling processes. However, this immunosensor would be proposed to develop for assessment of atherosclerotic risk in clinical diagnostic.

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APPENDIX

PIEZOELECTRIC-BASED IMMUNOSENSOR FOR OXIDIZED LOW DENSITY LIPOPROTEIN DETERMINATION

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ABSTRACT

Oxidized Low Density Lipoprotein (OxLDL) is a crucial contributor of atherosclerosis by inducing tissue damage and inflammation of the arterial wall. The present of atherosclerosis inside the blood vessel wall directly affects to blood flow rate which closely relates to the risk of heart attack and stroke. The elevation of OxLDL in plasma is also associated with pathogenesis of atherosclerosis. Therefore, OxLDL has been recognized as the biomarker for atherosclerosis events. The reliable detection method for this biomarker is recently essential for atherosclerosis risk assessment. The piezoelectric immunosensor with modified quartz crystal surface was developed as a detection system to determine OxLDL concentration upon the binding interaction between OxLDL and anti-OxLDL. The anti-OxLDL was immobilized on silver electrode surface of 10 MHz AT-cut piezoelectric quartz crystal via self-assembled monolayer (SAM) of mercaptopropionic acid (MPA). The optimal anti-OxLDL immobilization was investigated to achieve the binding capability of this antibody on the immunosensor surface. The optimized immobilization of anti-OxLDL was at the concentration of 100 µg/ml and 30 min for its immobilization time, respectively. The anti-OxLDL and OxLDL particle interaction creates surface mass deposition which has ability to change the resonance frequency of quartz crystal upto 250 Hz. with linear relationship between amount of OxLDL and frequency shift. This OxLDL immunosensor was successfully developed to determine the amount of OxLDL. Therefore, this immunosensor would be a promising device for assessment of atherosclerotic risk.

Keywords: Atherosclerosis, Oxidized low density lipoprotein (OxLDL), Biosensor, Immunosensor.

INTRODUCTION

Atherosclerosis is a disease of the arterial wall which undergoes the formation of plaque in the intima layer or subendothelial space leading to cardiovascular disease (CVD) that causes the death of the world's population [1]. There are many risk factors associated with atherosclerosis such as low density lipoprotein (LDL) level, high density lipoprotein (HDL) level, obesity, hypertension, diabetes mellitus, and smoking. The high circulating LDL in plasma can be trapped into intima layer of arterial wall and these LDL molecules become susceptible to oxidation by free radicals, metal ions, lipoxygenase and myeloperoxidase, called oxidized LDL (OxLDL) [2, 3]. The OxLDL is, then taken up by macrophages through scavenger receptors, leading to accumulate of foam cell in atherosclerosis plaque [4, 5]. In addition, the OxLDL is an important contributor and major risk factor of atherosclerosis progression. Therefore, the OxLDL would be one of biomarker for predicting and monitoring atherosclerosis events [6, 7].

There are several methods to measure plasma OxLDL level such as thiobarbituric acid reactive substances (TBARS) assay and conjugated diene methods. These methods are general used for measurement the plasma OxLDL [8, 9]. The principle of TBARS assay is the reaction

between thiobarbituric acid and malondialdehyde (MDA) to yield secondary fluorescent product of lipid peroxidation. However, malondialdehyde can be generated from the other sources. The final reaction product of TBARS assay is not only come lipid peroxidation [10]. The conjugated diene method measures the rearrangement of double bonds in polyunsaturated fatty acid [9]. Both methods are simple and rapid, but they are non-specific and low limit detection for LDL oxidation [11, 12]. The OxLDL can be definitely determined by nuclear magnetic resonance spectroscopy (NMR), high-performance liquid chromatography (HPLC) and mass spectrometry (MS). These methods are inconvenience for routine operation regarding to the requirement of sophisticate analytical equipment, the complexity of assay technique, and the running cost of analysis. Recently, enzyme-linked immunoabsorbent assay (ELISA) has been developed for measuring OxLDL using monoclonal antibodies (mAbs) such as E06, FOH1a/DLH3 and 4E6 antibodies [13, 14]. However, this method offers only medium sensitivity and specificity. Moreover, it still has unpreferable time-consuming and expensive analytical cost. Therefore, the alternative detection methods of plasma OxLDL should be developed to overcome the disadvantages of those methods.

Several cutting-edge technologies including the biosensor based approach has been developed by integrating the immunoassay to relevant sensing device, so call ed immunosensor. Recently, utilizing of piezoelectric quartz crystal device has more attractive due to the unique properties of labeled-free method. The piezoelectric-based immunosensor is based on antigen-antibody interaction which causes mass deposition on the surface of sensing device. This mass change can be directly measured the corresponding frequency change. This detection system has been developed for environmental, chemical analysis and medical diagnosis applications [15]. In addition, it is also applied for direct measurement of lipoprotein particle such as LDL and HDL [16, 17].

OxLDL immunosensor based on piezoelectric quartz crystal was developed by immobilization of anti-OxLDL on the modified quartz crystal surface via covalent linkage. The anti-OxLDL on immunosensor plays the key role of binding interaction between OxLDL particles and anti-OxLDL. This interaction produces accumulation of mass on the surface of device which can be monitored using the resonant frequency shift of quartz crystal.

OBJECTIVE/ RESEARCH QUESTION

The key objective of this study is to create an appropriate analytical method for quantification of OxLDL using piezoelectric-based quartz crystal immunosensor system.

RESEARCH METHODS

Preparation of oxidative modification of LDL (OxLDL)

The OxLDL particle was prepared by the oxidation of LDL using copper ions. The 100 µg/ml human LDL (Sigma-Aldrich, USA) was oxidized by incubation with 50 µM $CuSO_4$ at 37°C for 24 h and the reaction was terminated by adding 0.25 mM EDTA for 15 min. The amount of oxidative modification of LDL was determined by thiobarbituric acid reactive substance (TBARS) assay method. The colored product of this reaction was measured with spectrophotometry at the wavelength of 532 nm. This OxLDL solution was adjusted to 4 µmole/L for testing of developed immunosensor binding ability.

The measuring system and OxLDL immunosensor preparation

The silver-fabricated quartz crystal (AT-cut, 10 MHz, 8 mm in diameter) was obtained from Kyocera-Kinseki, Thailand. This crystal was embedded into flow cell which was connected to syringe pump (New Era, USA). The optimal flow rate of this flow system was tested for 30 min at 30, 60, 120, 180, 300, 500 µl/min, respectively. The resonance frequency (Hz) of quartz crystal in all experiment was followed and recorded using a RQCM resonant frequency counter (Maxtek, USA).

Anti-OxLDL (Millipore, USA) was immobilized by covalent binding method via self-assembled monolayer (SAM). The cleaned silver electrode surface of quartz crystal was immersed into 10 mM 3-mercaptopropionic acid (MPA) (Sigma-Aldrich, USA) at room temperature for 1 hour. Then, the functional group of MPA was activated by adding 10 µl of 200 mM 1-Ethyl-3-(3-dimethylaminopropril) carbodiimide (EDC) (Sigma-Aldrich, USA) solution and 10 µl of 50 mM N-hydroxysulfosuccinimide (NHS) (Sigma-Aldrich, USA) solution for 30 min at room temperature. This modified quartz surface was then rinsed with distilled water and embedded into flow cell of RQCM system.

Immobilization of anti-OxLDL

The anti-OxLDL at 10, 20, 50, 100 and 200 µg/ml was injected into flow cell by syringe pump. The flow of each antibody solution was stopped and then stood for 15, 30, 45 and 60 min, respectively. The resonant frequency shift of anti-OxLDL immobilization was monitored by RQCM system.

Binding ability of anti-OxLDL on immunosensor surface

The binding ability of immobilized anti-OxLDL was demonstrated by the resonant frequency shift during the OxLDL and anti-OxLDL interaction. Each OxLDL immunosensor from immobilization of 20, 50, 100 and 150 µg/ml anti-OxLDL was applied with 1% BSA (Sigma-Aldrich, USA) solution to block the remaining potential reactive surface. Four µmole/L OxLDL was injected into flow cell after washing out the excess BSA with PBS buffer pH 7.4 and then incubated for 30 min. The response frequency shift was recorded.

RESULTS

Preparation of oxidative modification of LDL (OxLDL)

The oxidative modification of LDL was generated by inducing LDL oxidation with copper ion and measured its product with TBARS assay. The secondary product of lipid peroxidation, MDA, refers to OxLDL in this experiment. The concentration of OxLDL can be figured out from the standard curve of 1,1,3,3-Tetramethoxypropane (TMP) (Figure 1.).

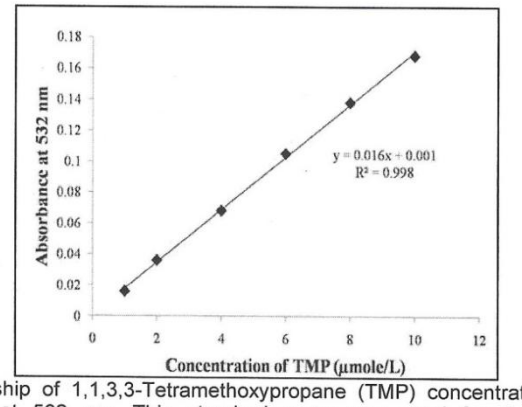

Figure 1 The relationship of 1,1,3,3-Tetramethoxypropane (TMP) concentration (µmole/L) and the absorbance at 532 nm. This standard curve was used for determination of OxLDL concentration by TBARS assay.

The human LDL at different concentration was incubated with copper ions at 37°C for 24 h and measured the amount concentration of OxLDL by TBARS assay. The result showed that 50 to 500 µg/ml human LDL successfully yielded OxLDL. The application at higher concentration tends to produce more amount of OxLDL as shown in Figure 2.

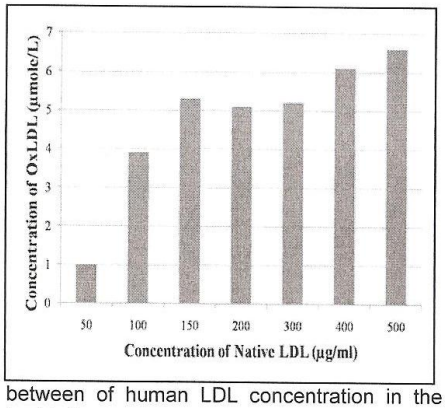

Figure 2 The relationship between of human LDL concentration in the reaction and the product, OxLDL, of the lipid oxidation.

The measuring system and OxLDL immunosensor preparation

The flow rate of this measuring system was tested by driving PBS pH 7.4 solution through the flow cell embedded with 10 MHz AT cut quartz for 30 min. The system gave the most stable resonant frequency signal at 60 µl/min flow rate (ΔF±1 Hz) as presented in Figure 3. The higher flow rate up to 300 µl/min also maintained acceptable signal stability (ΔF±10 Hz) after 10 min lag time. Therefore, the 60 µl/min flow rate was chosen to conduct throughout this experiment.

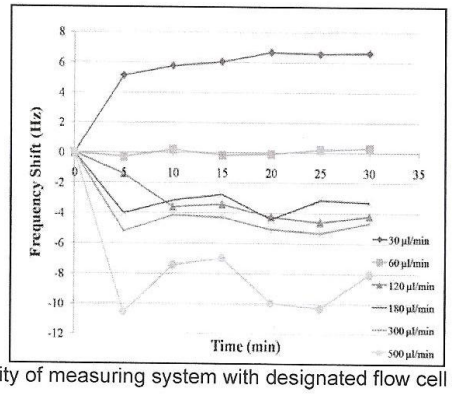

Figure 3 The signal stability of measuring system with designated flow cell at the different flow rates.

Immobilization of anti-OxLDL

The silver electrode surface was successfully coated with 10 mM MPA. The functional group of MPA was activated by EDC/NHS generating active functional group to form covalent linkage with primary amine of antibody molecule. The anti-OxLDL was immobilized onto this modified surface. The anti-OxLDL at 10, 20, 50, 100 and 200 µg/ml was immobilized onto the modified quartz crystal surface.

The result in Figure 4 showed increasing of resonance frequency change when applying higher concentration of anti-OxLDL.

The highest resonant frequency shift (ΔF = 268 Hz) was obtained from the anti-OxLDL at concentration of 100 µg/ml. The concentration higher than this value did not present significant different frequency shift.

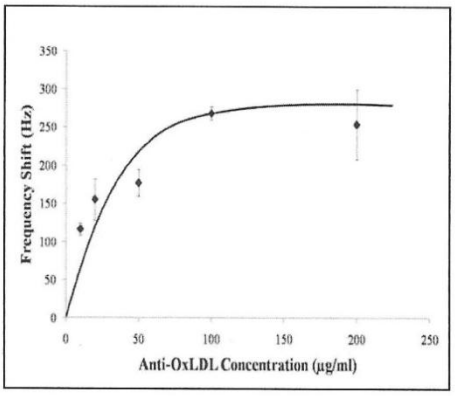

Figure 4 Optimization of anti-OxLDL immobilization on silver electrode surface of 10 MHz AT-cut quartz crystal.

In addition, Figure 5 suggested the appropriate incubation time of anti-OxLDL immobilization for 30 min at room temperature.

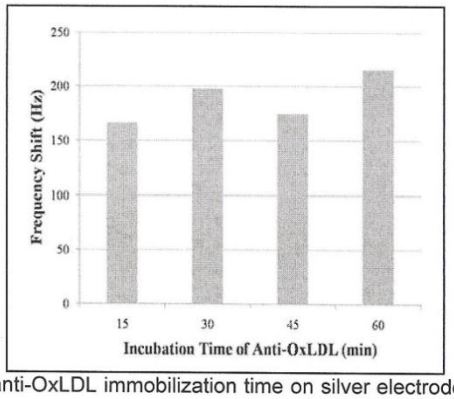

Figure 5 Optimization of anti-OxLDL immobilization time on silver electrode surface of 10 MHz AT-cut quartz crystal.

Binding ability of anti-OxLDL on immunosensor surface

The binding ability of anti-OxLDL was investigated by the interaction between OxLDL particle in tested solution and immobilized anti-OxLDL on immunosensor surface. Four µmole/L of OxLDL was applied on OxLDL immunosensor surface with 20, 50, 100 and 150 µg/ml anti-OxLDL for 1 h at room temperature. The resonant frequency shift from the immunosensor without anti-OxLDL illustrated the binding activity of anti-OxLDL on the sensor surface. The result in Figure 6 showed 250 Hz change on the sensor surface with 100 µg/ml anti-OxLDL or higher. The lower amount of anti-OxLDL on the sensor surface gave less resonant frequency shift (binding ability).

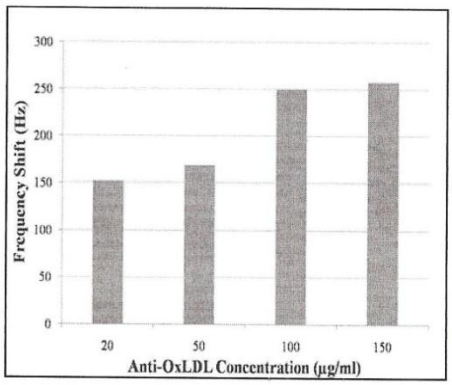

Figure 6 The binding ability of anti-OxLDL on OxLDL immunosensor surface.

DISCUSSION AND CONCLUSION

Oxidized low density lipoprotein (OxLDL) is a major risk factor in atherosclerosis. The increasing of plasma OxLDL level can be applied as a biomarker for atherosclerosis to monitor and prevent the risk factor of atherosclerosis [18, 19]. Commonly, the level of OxLDL circulation is very low in normal population (0.58 ± 0.23 ng/5 µg LDL proteins) [20]. There are many methods to determine the amount of plasma OxLDL [10, 21-23]. Recently, ELISA assay has been developed as the commercial kit [18, 24]. However, this technique has high complexity, costly and time-consuming. Therefore, piezoelectric-based biosensor was proposed to develop for measuring the amount of plasma OxLDL (OxLDL immunosensor). The OxLDL immunosensor was fabricated on 10 MHz AT-cut quartz crystal with silver electrode [25, 26]. Generally, the quartz crystal with gold electrode is frequently used for quartz crystal microbalance system (QCM) regarding to their stability and reusability. The silver electrode was recommended in this study due to the lower cost of fabricated electrode [27, 28]. However, the silver electrode can be oxidized but it do not significant effect to the frequency shift of quartz crystal [29].

The incubation of human LDL with copper ion has been used as standard method to produce OxLDL which is generated a variety of modification on apolipoprotein and lipid such as MDA, HNE and amino acids residues [30]. The copper ion has been extensively used for LDL oxidation. It is a better oxidant of LDL than ferric ion because copper can bind to apoB protein to form Cu-LDL complex that essential in copper-mediated peroxidation of LDL during incubation with copper ion [8, 31-34]. However, the formation of Cu-LDL complexes were dependent on both LDL and copper ion concentrations in the range of 0.1-0.4 mg LDL protein/ml and 5-40 µM Cu^{2+}, respectively [34].

The measuring system of this study was performed by using RQCM device integrated with quartz crystal embedded flow cell system. Normally, the flow rate of liquid phase depends on the diameter of quartz crystal and pumping system. In this study, a suitable flow rate for 10 MHz AT-cut quartz crystal was 60 µl/min. The self-assembly monolayer (SAM) of MPA was selected to immobilize antibody due to its robustness and reproducibility. The silver electrode of quartz crystal was dipped into 10 mM MPA for 30 min. The functional group of MPA was activated by EDC/NHS to form bonding with the primary amine in antibody [35, 36]. Therefore, anti-OxLDL was formed the covalent linkage with MPA modified surface electrode.

The performance of OxLDL immunosensor is strongly dependent on the density of antibody on the surface and its availability to react with the OxLDL. The effective sensor preparation in terms of an optimal concentration and time incubation were studied. The study presented that

the resonant frequency shift of quartz crystal related to the amount of antibody immobilization (10-200 µg/ml). The optimal concentration and incubation time of anti-OxLDL are 100 µg/ml and 30 minutes, respectively. The result in this study was comparable to the previous study, Yang and Chen [25].

The result obviously showed the ability of anti-OxLDL to bind OxLDL particle by changing of sensor resonant frequency of 250 Hz when using anti-oxLDL at 100 µg/ml or higher. It was indicated that the immobilized antibody on the electrode surface had ability to bind with the antigen. It implied the successful OxLDL immunosensor preparation. The method validation should be figured out in term of limit of detection, analytical accuracy, precision, sensitivity, and specificity regarding to the ISO and the Clinical Laboratory Improvement Amendments of 1988 (CLIA'88) requirements.

In summary, the OxLDL immunosensor has been successfully developed for OxLDL particle detection and quantification. The anti-OxLDL can be immobilized onto surface electrode *via* self-assembly monolayer in the immunosensor. This system demonstrated a direct analytical process to detect OxLDL. It is simple to use without multiple labeling processes. It is also offers high sensitive response and possibility for real time detection which is suitable for diagnostics application.

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PRESENTATION & INTERNATIONAL MEETING ABSTRACTS

1. **Sripoorote, P.,** Khaowkhiew, T., & Promptmas, C. (2011). Development of Biosensor System for Quantification of Oxidized Low Density Lipoprotein. In Abstract of 9th Asian Conference on Chemical Sensors, November 14-17, 2011, Taipei, Taiwan. (Poster Presentation)
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