รหัสโครงการ SUT1-102-54-12-14

รายงานการวิจัย

การพัฒนาอุปกรณ์ตรวจวัดอิมมูโนโลยีเพื่อตรวจหาเชื้อไวรัสตับอักเสบ บี และ ซี

Development of immunosensor for Hepatitis B and C detection

ได้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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คณะผู้วิจัย

หัวหน้าโครงการ

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รหัสโครงการ SUT1-102-54-12-14

ได้รับทุนอุดหนุนการวิจัยจากมหาวิทยาลัยเทคโนโลยีสุรนารี ปีงบประมาณ 2554-2555

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19 สิงหาคม 2555

กิตติกรรมประกาศ

ข้าพเจ้าขอขอบคุณมหาวิทยาลัยเทคโนโลยีสุรนารีที่ให้ทุนสนับสนุนงานวิจัยในครั้งนี้ และขอขอบคุณศูนย์เครื่องมือ มหาวิทยาลัยเทคโนโลยีสุรนารี ที่ให้ใช้อุปกรณ์และเครื่องมือในห้องปฏิบัติการชีวเคมีเพื่อการวิจัย ทำให้งานวิจัยสำเร็จลุล่วงไปด้วยดี

รองศาสตราจารย์ ดร. อัลแบร์ต ชูลเทอ
อาจารย์ประจำสาขาวิชาเคมี สำนักวิชาวิทยาศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
19 สิงหาคม พ.ศ. 2555

บทคัดย่อ

ในการศึกษานี้ได้พัฒนาห้องปฏิบัติการเคมีไฟฟ้าที่เหมาะกับอิมมูโนเซนเซอร์สำหรับการวิเคราะห์หาตัวบ่งชี้ทางชีวภาพที่ทำงานได้ดี โดยทำการทดสอบประสิทธิภาพของการวัดแบบ flow-based capacitive electrochemical assay กับ YKL-40 ซึ่งเป็นตัวบ่งชี้โรคมะเร็งและการติดเชื้ออักเสบ การเตรียมอิมมิโนเซนเซอร์ของ YKL-40 ทำได้ด้วยการตรึงแอนติเจน YKL-40 บนผิวของอิเลคโทรดทองคำที่มีรูปร่างเป็นแผ่นดิสค์เล็ก ๆ แล้วทำการวิเคราะห์หาระดับ capacitive current และ detection limit ของ YKL-40 ที่ความเข้มข้นต่าง ๆ แล้วสร้างเป็นกราฟมาตรฐานขึ้น โดยพบว่าทั้ง detection limit และค่า linear range ของตัวบ่งชี้ YKL-40 อยู่ในช่วงที่เปรียบเทียบได้กับอิมมิโนเซนเซอร์ของตัวตรวจวัดอื่น ๆ ที่มีการรายงานมาก่อน นอกจากนี้ค่า detection limit ของอิมมิโนเซนเซอร์ที่วัดมีความไวสูงกว่าการวัดด้วยวิธีทาง ELISA อยู่ 10 เท่า ซึ่งแสดงว่าอิมมูโนเซนเซอร์ที่พัฒนาขึ้นน่าจะมีความเหมะสมกับการตรวจหามะเร็งระยะเริ่มต้นได้ดีถ้ามีการปรับตัวแปรให้เหมาะสมกับการใช้งานในระดับการค้าและห้องปฏิบัติการทางคลินิกมากยิ่งขึ้น

ABSTRACT

In this study, a fully functional electrochemical workstation for the sensitive immunosensing of clinical disease markers has been established. The functionality and performance of the flow-based capacitive electrochemical assay was evaluated via the sensitive detection of YKL-40, a protein that recently has been claimed to be a cancer and inflammation disease marker. Electrochemical YKL-40 immunosensors have been prepared by immobilizing the antibody for YKL-40 onto the surface of disk-shaped gold electrodes. Calibration plots for the prepared immunosensors have been measured markers by means of capacitive current recordings and the detection limits evaluated. Both the detection limit and linear range of the approach for the YKL-40 biomarker protein were comparable to values reported previously for the quantification of other protein disease with the same technique. The detection limit of the established electrochemical detection is about 10 times more sensitive than the one listed for the commercialized YKL-40 ELISA kit, which may help to get an earlier detection of the cancer disease state of patient, if the technology is further optimized for an implementation in instrumentation that is suitable for commercialization and easy use in clinical laboratory settings.

สารบัญ

สารบัญภาพ

บทที่ 1
บทนำ

1.1. ความสำคัญและที่มาของปัญหาการวิจัย (Rationale/Motivation)

Human disease diagnosis [1] through the identification and quantification of specific biomarker molecules in, for instance, blood, serum or urine samples is an important clinical health issue as it provides the obligatory information for the doctor's judgments on the medical treatment needed of patient's problems. Among the modern strategies to diagnose diseases such as hepatitis, cancer, cardiovascular problems, viral infections or body inflammations of various sorts are immunochemical assays that use antibodies (Ab's) and antigens (Ag's) associated with the pathogenic status for an effective detection of the manifestation of the disease in a patient. Utilized for the measurement is the very specific Ab-Ag conjugate formation, which becomes well measurable when the antibody is immobilized on the surface of a physicochemical transducer device (e.g. an electrode, photon-detector, quartz-crystal microbalance or thermistor) and the binding event is transformed by the sensor into a proportional electrical signal. Currently the enzyme-linked immunosorbent assay (ELISA) [2, 3] with a chemiluminescence-based quantification is the main analytical approach that takes advantage of antibody/antigen-based disease marker detection in clinical samples. A shortcoming of existing ELISA assays with optical signal generation and readout is, however, their relatively poor sensitivity and the associated inability to offer early disease identification. An as early as possible disease recognition is on the other hand beneficial for facilitating a timely initiation of medication close to the onset of syndromes. In the case of delayed treatment viruses, for instance, may spread uncontrolled or cancer cells grow untreated longer than actually necessary and good for the patient. Methodologies with a better sensitivity and lower detection limits than offered by ELISA are therefore sought after and antibody/antigen-based electrochemical biosensing became thus an interesting theme of current research activity in the area of analytical and clinical sciences.

Because of their superb sensitivity of electroanalytical measuring schemes, electrochemical immunosensors [4, 5] got into the focus as a promising option to the conventional optical ELISA assays. It is commonly accepted that electrochemical immunosensing has in general a good potential to be used as an alternative point-of-care diagnostic tool in hospital laboratory units. The

methodology not only offers without prior fluorescent labeling a high sensitivity and reliability but also is associated with an instrumentation that is simple, non-bulky and comparably cheap and easy to set up and use. In 1997 the acquisition of changes in the interfacial electrode/electrolyte capacitance that occur in response to an interaction between immobilized Ab and dissolved Ag was reported as a sensitive electrochemical transduction scheme for immunosensors and low-level disease marker detection [6]. In the pioneering work the method was applied for the detection of human chorionic gonadotropin hormone but later the procedure got further optimized and tailored for the analysis of a variety of other clinically relevant antigens. Examples of successfully approached analytes include the endotoxin [7], carcinoembryonic antigen [8], HIV-1 p24 antigen [9], and the staphylococcal enterotoxin B [10]. The published success cases demonstrated well that capacitive immunosensing is simple and cheap in terms of instrumentation, relatively easy to establish for new applicants and, most importantly, capable of the detection of extreme low levels of antigens in sample fluids. The cholera toxin, for instance, has been measured with capacitive immunosensing at a sub-attomolar concentration [11], which is more very much below the level that is quantifiable via ELISA-based screening. At the time of the creation of this project there were still a few important disease markers that had not yet been tested for an ultra low-level detection with the attractive capacitive immunosensing, even though the performance of the scheme is ideal suited for attempts to improve their analysis, and hepatitis virus antigens [12] and YKL-40 [13, 14], a 40-kDa secreted glycoprotein and potential indicator molecule for cardiovascular disease [13], cancer [14] and osteoarthritis [15] are just representative cases. The development of a technically simple device for a capacitance-based reliable and sensitive electrochemical hepatitis and YKL-40 immunosensing thus made a certain sense and became therefore a target of joint biochemical, immunological and analytical efforts for establishment in the applicants SUT analytical laboratory setting.

1.2. วัตถุประสงค์ของโครงการวิจัย (Objectives)

1. Establishment of a device for the sensitive electrochemical immunodetection of human disease markers.
2. Establishment of the tracing of the immunosensors electrical capacitance via a potential step technique as basis for antigen quantification.
3. Performance test and system evaluation; proof of the functionality of the methodology for selected disease markers such as the hepatitis antigen and others.

1.3. ขอบเขตของการวิจัย (Framework)

The project is set in an area at the borderline between medical and analytical sciences. With an efficient disease detection and optimal patient treatment worldwide claimed to be a central target of the efforts of governmenta health schemes, advancements of the methods available for clinical laboratory analysis became an important issue and the quest for sensing strategies with optimal sensitivity, selectivity, detection limits and linear range is accordingly the subject of an intense global research activity. Electrochemical immunosensors, these are electrodes with surface-immobilized antibody entities that provide highly sensitive detection capability for the corresponding antigens, are a competitive alternative to optical (spectroscopic) assays for disease marker screening. Various types of electrochemical immunosensors have been proposed including the ones that use amperometry, potentiometry, electrochemical impedance spectroscopy or potential step-supported electrode capacitance assessments for disease marker analysis. However, though the methods have been demonstrated as successful selected antibody/antigen systems they have not yet been optimized for the recognition and quantification for the complete reservoir of relevant disease markers. There is thus still a lot of adaptation work left to be done and this project defined the establishment of an electrochemical immunosensing device and its adaptation to disease marker screening as its task.

1.4. ประโยชน์ที่ได้รับจากการวิจัย (Expected output/results)

ประโยชน์ที่คาดว่าจะได้รับจากงานวิจัยนี้มี 4 ประการหลักคือ

1. One publication in a peer-reviewed international journal with a good impact factor

2. Generation of local human resources (graduate students, Research assistants) with skills in electrochemical human disease marker detection
3. Setting up the foundation for the establishment of an alternative analytical scheme for clinical laboratories around the countries

หน่วยงานที่จะนำผลงานวิจัยไปใช้ประโยชน์

1. Public and private hospitals and their medical laboratories
2. The drug and laboratory equipment industry
3. National and international academic research institutions

บทที่ 2
วิธีดำเนินการวิจัย (Methodology)

The measurement of disease markers (antigens) in this study uses the analysis of the current response of antibody-modified gold working electrodes towards the application of 50 mV high and 6 ms long voltage pulses (see Figure 1).

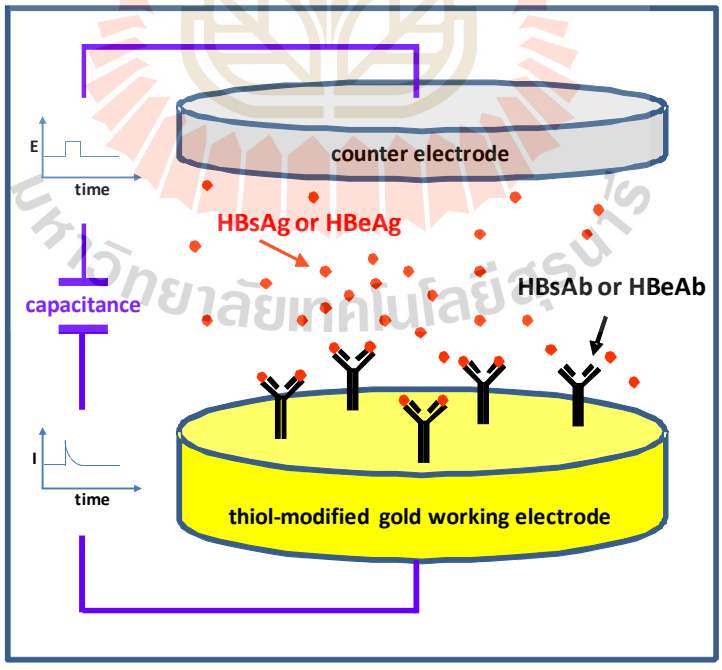

Figure 1: The principle of capacitive immunosensing with antibody-modified gold disc electrodes.

Current recordings were carried out in a specially established flow stream of running buffer with a Gilson peristaltic pump in use for generating the flow-stream and an Edaq potentiostat (the "EA163") in three-electrode configuration in charge of the potential application and current measurements. Used were:

1. A gold working electrode

Prior to measurements, the surface of the 3 mm-disc gold working electrode was covalently modified with antibody molecules that can bind later in the measurements to the corresponding antigen, which is a protein-based disease marker. Self-assembling monolayers made of thiols with remaining chemically reactive groups were used to attach the antibodies to the gold interface.

2. A silver/silver chloride reference electrode

3. A steel counter electrode

Used as counter electrode was the thin steel tubing that guided the running buffer out of the electrochemical flow cell.

4. Running buffer

Used was, if not otherwise mentioned, a 10 mM phosphate running buffer (pH 7) that was pumped with the aid of the peristaltic pump at a flow rate of, for instance, 100 µl/min through the electrochemical cell; injected samples volumes were 200 µl and the acidic regeneration buffer used to break antibody-antigen bonds and re-establish the state of a fresh sensor surface was a 25 mM HCL (pH 2.5).

5. The antibody/antigen system

The commercial hepatitis antibody/antigen couple turned out to be too expensive to be suitable as proposed for the required measurements for method establishment and validation. The target and main delivery of the project, a sensitive analytical scheme for the immunosensing of human disease markers, was thus adapted with the home-made YKL-40 antibody/antigen system, available at sufficiently large quantities from other projects in the Research Unit, used as a affordable hepatitis antibody/antigen replacement for sensing tests and performance trials.

บทที่ 3
ผลการทดลองและข้อวิจารณ์ (Results and Discussion)

Main target of the work was the development of an electrochemical immunosensor system that is capable of the sensitive detection of biological marker molecules for critical human diseases. Hepatitis B/C immunosensing was thought of as model application, however, the use of the device for the recognition of other diseases was considered, too. The initial steps focused on establishment of the suitable electrochemical workstation with which the capacitive (and, if desired, amperometric) measuring schemes could be executed. Purchased were a potentiostat with the electrochemical software for all conventional electroanalytical methods, an electrochemical flow-cell with a 3 mm gold disk working electrode, a peristaltic pump and a three-way injection valve for sample delivery to the flow line feeding the flow cell. The assembly of the individual components followed instructions available in the literature and a functional setup has been completed in course of the project work. Figure 2 is an illustration of the system was constructed available for the tasks of disease marker immunosensing in the project work.

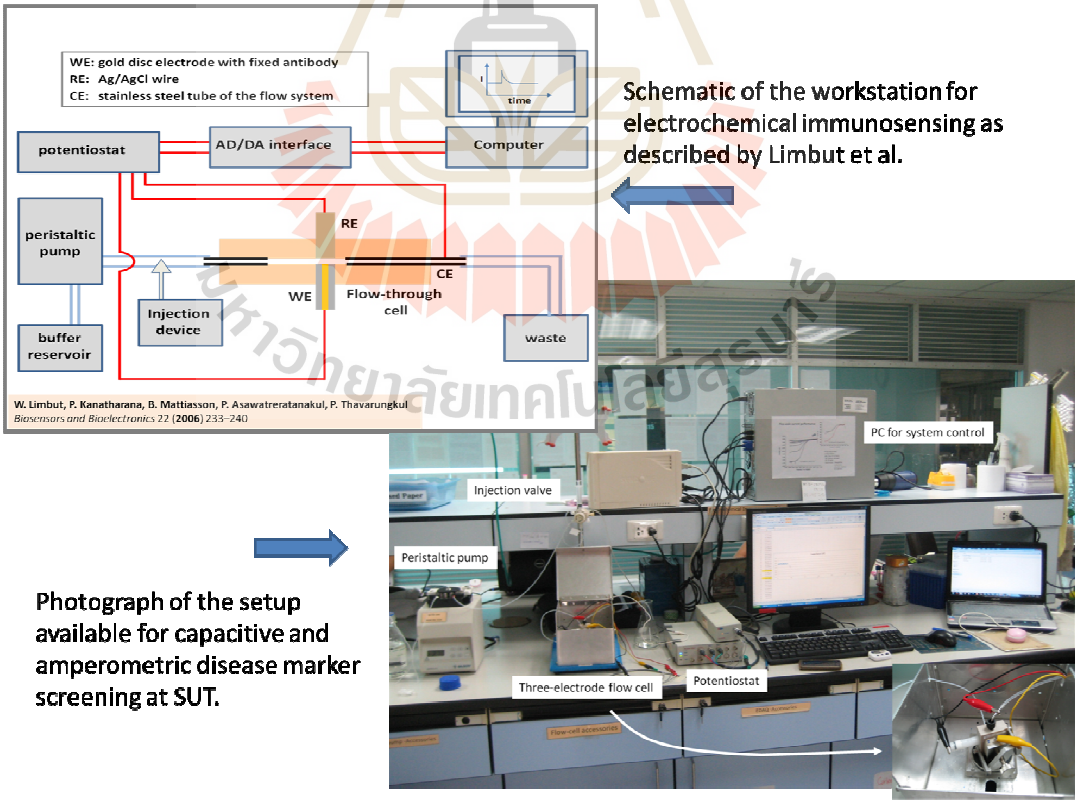

Figure 2: The workstation that was constructed within the immunosensor project in the Biochemistry –Electrochemistry Research Unit at Chalermprakiat (F9) building.

First voltammetric test measurements with the new device revealed severe problems related to unacceptably high electrical apparatus noise that apparently was introduced into the system through a poor building grounding. To be able to resolve nano- or even picoampere electrode currents and nano-Farad electrode capacitance changes, disconnection of the whole setup from the building was necessary and gained by an own direct earth grounding. Figure 3 is displaying a representative set of cyclic voltammograms (electrode current vs. electrode potential curves) that were obtained in a 0.1 M KCl-based electrolyte with ruthenium hexamine chloride as reducible redox species at concentrations between 1 mM and 15.125 µM and an about 7-µm-diameter carbon fiber disc microelectrode. The measured limiting currents (equal to the "height" of the S-shaped traces!) were about 1.6 nA and 0.8 nA for 1 and 0.5 mM concentrations of dissolved Ru3+, respectively. When, on the other hand, the level of the redox active ion approached rather low 30 and 15 µM, the observed limiting currents were found to be only a few tenth of pA (see inset Figure 3).

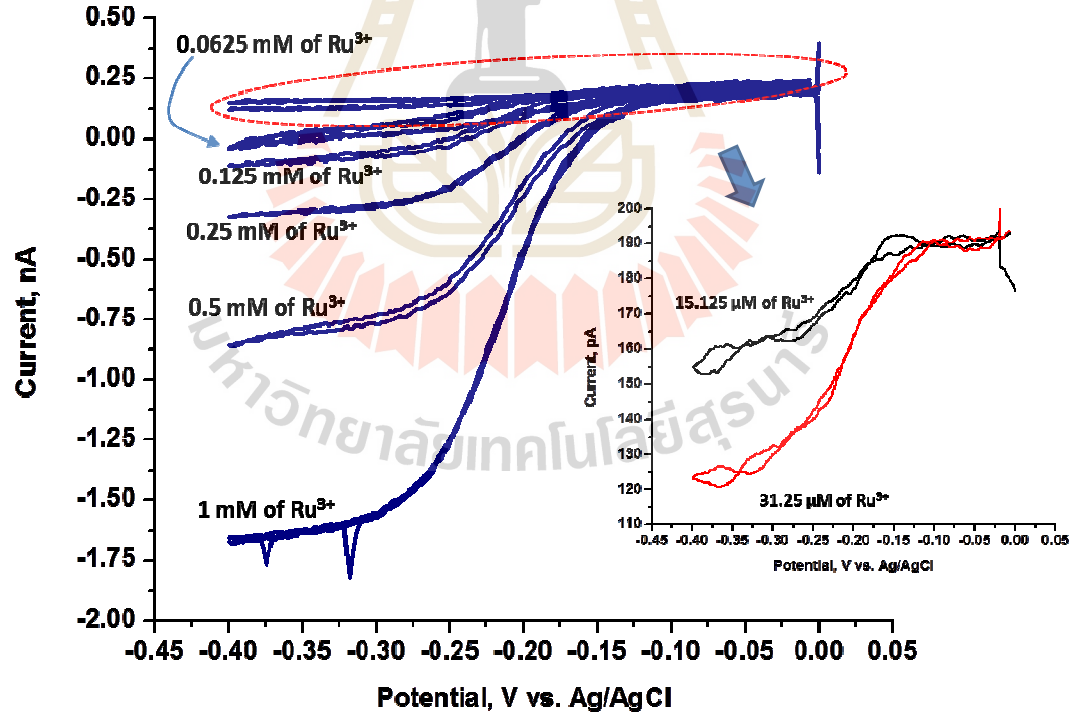

Figure 3: Cyclic voltammograms of ruthenium (III) in 0.1 M KCl measured with an about 7-µm-diameter carbon fiber disc microelectrode. The concentrations of the redox-active ion are indicated as numbers below or above individual I/E traces.

The ability of the electrochemical workstation to resolve nicely pA-scaled voltammograms was also well confirmed by recordings of well-looking differential pulse voltammograms in 0.25 mM solutions of Ru3+ (see Figure 4). The appearance of the cyclic and the differential pulse voltammogram with good pA current resolution clearly proved the established well satisfactory noise properties of the designated immunosensor setup and the possible high quality of the electrochemical current recordings.

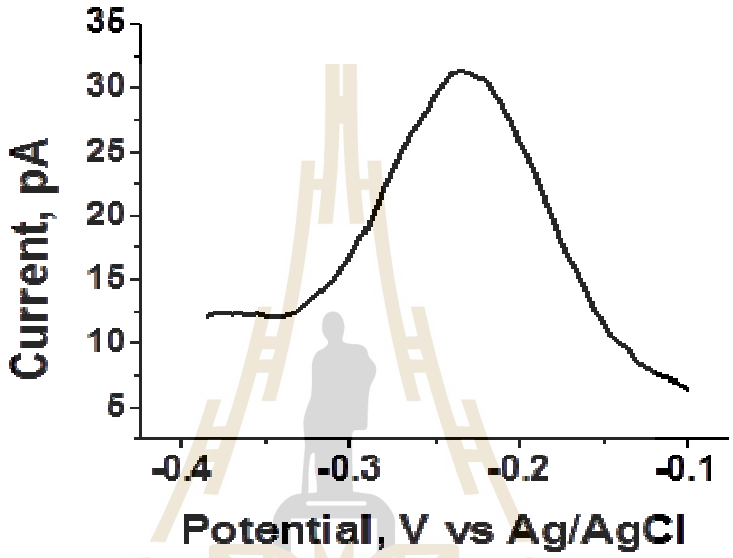

Figure 4: pA-level differential pulse voltammogram of 0.25 mM ruthenium (III) in 0.1 M KCl measured with an about 7-µm-diameter carbon fiber disc microelectrode.

To test the performance of the flow system with the peristaltic pump, injection valve and flow-through electrochemical cell, buffer solution was pumped through the tubings while subjecting the gold disc electrode to 1 s long pulses from +400 to 0 mV vs. Ag/AgCl. 400 mV is a potential where ferricyanide ($[Fe(CN)6]^{3-}$) is stable, while 0 mV is a potential cathodic enough to reduce ferricyanide to ferrocyanide ($[Fe(CN)6]^{4-}$). As long as pure buffer solution is passing through the flow cell, the response to the short potential steps is a sharp spike representing the charging current for the electrical double layer which then decays exponentially towards zero. Injection of ferricyanide will change the situation.

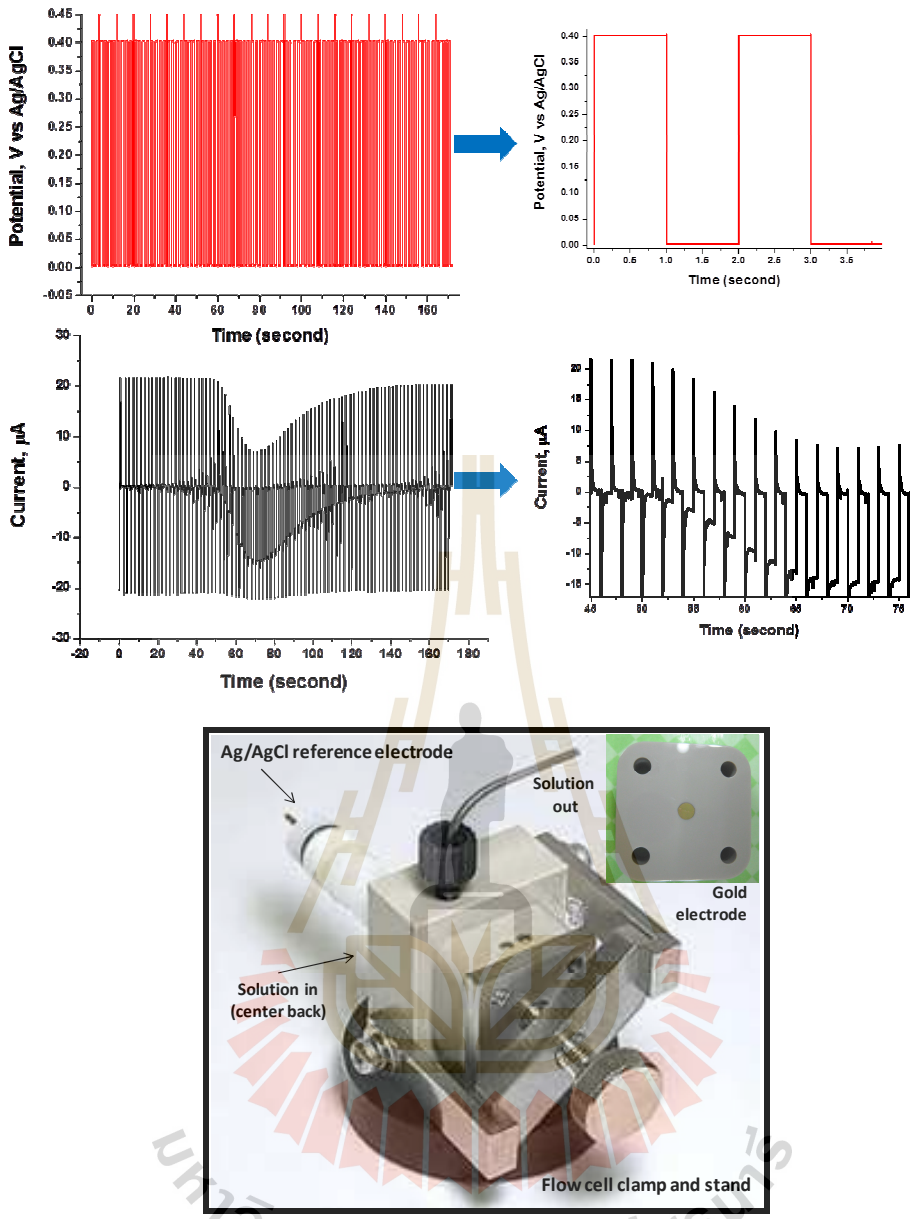

Figure 5: Test of the commercial three-electrode flow cell as shown to the bottom. An injection of 200 µL of 1 mM reducible ferricyanide ions into the tubing triggered the transport of the redox species through the tubing to the gold disk electrode of the flow cell by the action of the peristaltic pump at a flow rate 370 µL/min. Arrival and full passage of the Fe (III) species became visible via the appearance and disappearance of a cathodic current (a downward deflection in the current vs. time trace. Shown to the top left and, at higher time resolution, top right are the applied potential steps bringing the gold electrode from resting (+0.4 V vs. ref.) to reducing (0 V vs. ref.) state. The matching current traces are shown in the bottom two graphs.

Injection of ferricyanide will change the situation. Again there is the spike for the charging current but the current does not return to zero but to a certain level that depends on the concentration of the reducible ferricyanide ions in the vicinity of the electrode surface. Figure 5 shows the outcome of such an experiment. Injection of 200 µL ferricyanide took place at around 25 s after the recording was started. As expected the injection of the iron species increased markedly the current through the flow cell, which then reached a peak and later returned to baseline level. Apparently the flow system and sample injection were well functional and the setup ready for an immunosensing trial.

Gold disc electrodes for the flow cell were modified with antibody as explained in literature in order to tailor them for capacitive electrochemical immunosensing experiments. An electrode cleaning via repetitive potential scans in sulfuric acid-based electrolyte was followed by an exposure to (1) thiourea, (2) glutaraldehyde as crosslinking agent for chemical antibody bonding, (3) antibody solution and (4) dodecanethiol as small thiol covering any gold surface that still was uncovered. Figure 6 shows a set of cyclic voltammograms that were measured at the end of the different steps for gold electrode modification. As expected and in good agreement with literature, the anodic and cathodic peak currents in the voltammograms were highest for the bare gold electrode and decreased in amplitude with the applied surface coating getting in place.

In fact, after the dodecanethiol placement the sensor was ready for antigen screening, which then has been attempted. As mentioned earlier, commercial antigen and antibody preparations are usually highly priced and the need for enough protein for a large set of test trials for the establishment and optimization of a novel electrochemical immunosensing platform was a heavy economic burden on the limited project budget. Proposed was originally to work with Hepatitis B and C antibodies and antigens. However, for the reason of budget limitation the initial plan was changed and all experiments for system establishment and optimization performed with the self-made protein YKL-40, a human chitinase like protein and only recently identified cancer and tissue inflammation disease marker, and the corresponding antibody anti-YKL-40. Both biomolecules, the YKL-40 antigen and antibody, were available in significant quantities through other complementary projects and test trials with a capacitive immunosensing could be carried out with YKL-antibody modified gold electrodes and the YKL protein as analyte without a limitation in terms of access to relevant protein quantities.

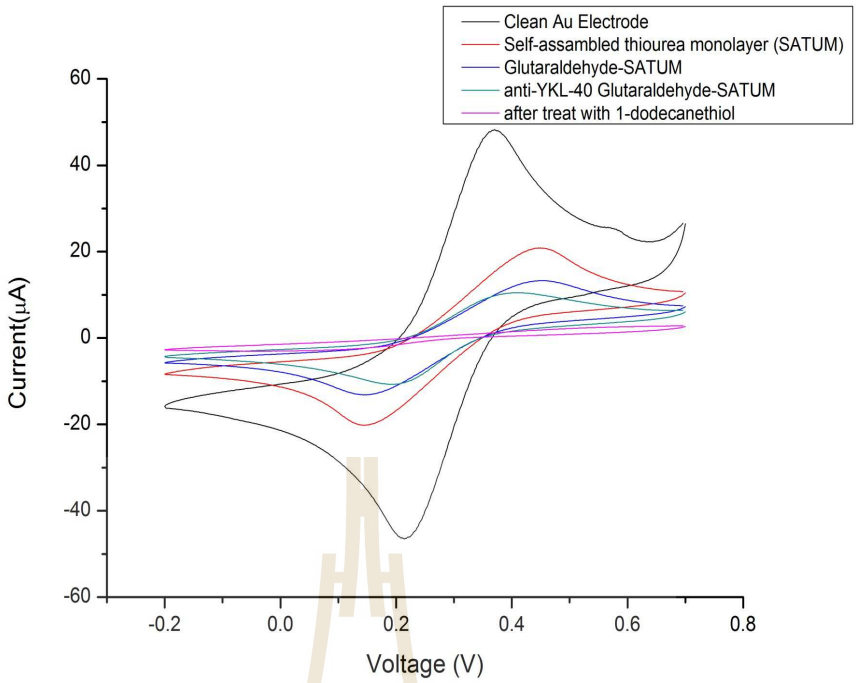

Figure 6: Set of five cyclic voltammograms (CVs) as acquired at different stages of the capacitive immunosensor preparation. **(Black)** CV for 5 mM ferricyande/0.1 M KCl at 100 mV/s. Visible are the cathodic (down) and anodic (up) current peaks for the reduction of ferricyanide to ferrocyanide and the oxidation of the ferrocyanide back to ferricyanide. **(Red)** The placement of a self-assembling thiourea monolayer is blocking the surface of the gold disk and smaller peak currents are the result. **(Blue)** A glutaraldehyde activation of the thiourea for antibody chemical bonding does not change the CV much. **(Green)** antibody immobilization of the (here self-made cheap anti-YKL-40) further blocks electrode activity and suppresses the peak currents. **(Purple)** Final electrode treatment with dodecanethiol is supposed to block remaining active surface.

Figure 7 and 8 allow a comparison of the first electrode capacitance trace that was measured in a beaker-type prototype measurement with the system built in this project (Figure 8) and the YKL antibody/antigen couple and a representative trace from a literature study (Figure 7). In either case, our own or the published trials from others, did the injection of the antigen cause the gold electrode capacitance to decrease by a few nF. The agreement of data of the opening YKL-40 immunosensing test with published analogue studies confirmed the functionality of the electrode antibody modification and

the correctness of the overall immunosensor preparation. Measurements then followed that were supposed to prove the reproducibility of the flow-based capacitive detection of anti-YKL-40/YKL-40 interaction and validate the analytical procedure. Important features to assess were the linear range, sensitivity and detection limit for the approach, initially with the antibody/antigen couples relevant for the YKL-40 disease marker and later for the Hepatitis analogue. However, in course of the attempts problems came up with the capacitive detection scheme in the commercial flow cell as shown in Figure 5. Despite of a lot of variations of the experimental parameters (flow rate, composition and strength of the flow buffer, concentration and type of regeneration buffer etc.), the capacitance change caused by antibody injection was not stably appearing and reproducible in magnitude. Also an injection of an acidic buffer solution in the the flow stream could not - as usual and required - regenerate the sensor surface for repeated measurements by breaking the antigen-antibody bond and reestablishing the unoccupied state for the surface-immobilized antibody entities. Apparently, the geometry and design of the electrolyte compartment of the flow cell – it was a kind of thin-layer arrangement - was not compatible with the technical requirements of a capacitive detection of antibody/antigen interactions in a flow-based approach.

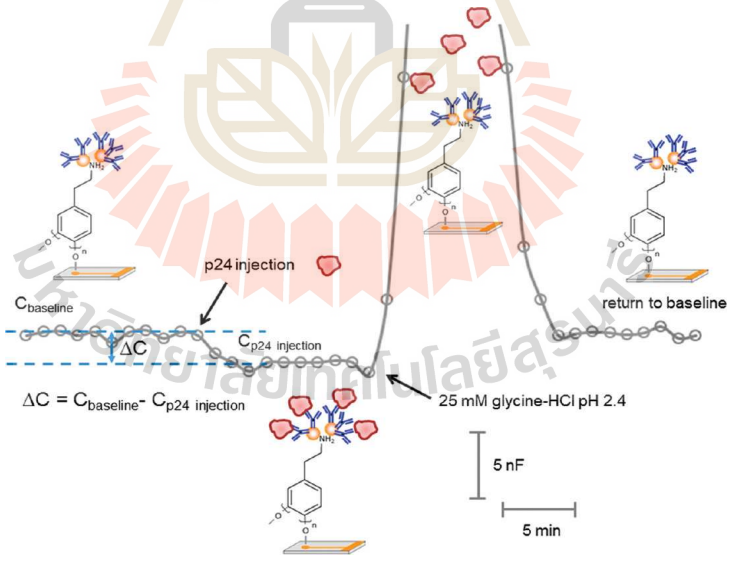

Figure 7: Time course diagram showing the decreases in capacitance after injection of antigen (here: standard HIV-1 p24 antigen). Average capacitances, $C_{baseline}$ and $C_{p24\ injection}$ are used for the calculation of a capacitance change (ΔC). After sample injection, the sensor was regenerated via an exposure to 25 mM glycine-HCl, pH 2.4. (from Teeparuksapun et al., Anal. Chem. 82 (2010) 8406).

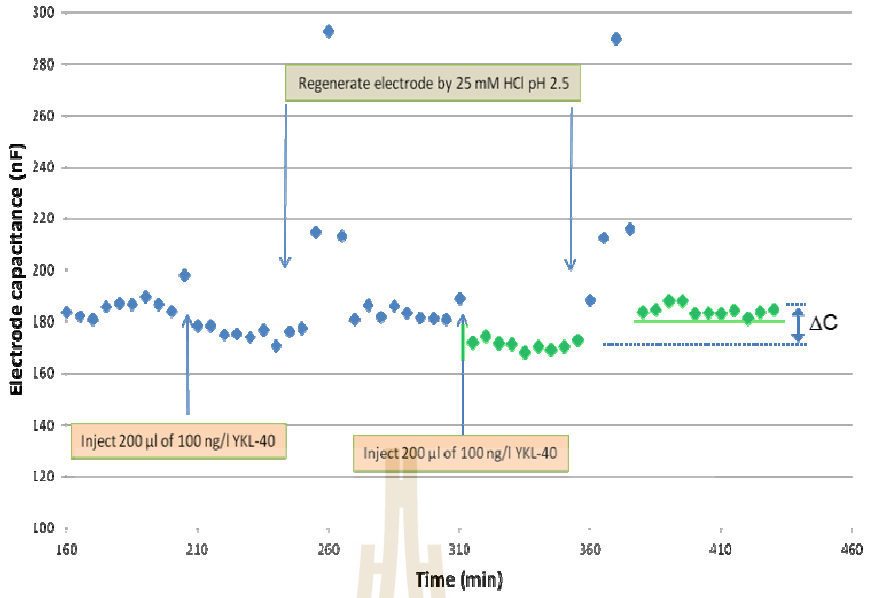

Figure 8: Capacitive immunosensing with YKL-40 antibody on a gold electrode and the YKL-40 applied via sample injection. Injection of YKL-40 antigen is followed by a drop in electrode capacitance due to affinity interaction. The difference in the capacitance before (~185 nF) and after (~175 nF) antigen injection is ~10 nF and compares well with responses in literature (see Figure 7).

Supported by input from collaborators from the Faculty of Science, Prince of Songkla University Hatyai, Songkhla, Thailand, namely Associate Professor Dr. Panote Thavarungkul, Associate Professor Dr. Proespichaya Kanatharana and Dr. Warakorn Limbut, the purchased commercial flow cell with its gold electrode was modified to make the desired capacitive disease marker detection possible and the novel construction adapted for successful operation.

Reused from the components of the commercial flow cell in the novel design were actually the polishable gold electrodes, which functioned as flow-cell bottom and the Ag/AgCl/1 M KCl reference electrode. Custom-made from a transparent hard polymer was, on the other hand, a new flow-cell top that had two drilled tubes of thinner diameter from opposite sides for the entrance and departure of the buffer carrier solution and another tube of adjusted diameter from the top to hold the reference electrode.

Underneath the tip of the glass tube of the reference electrode was the small reservoir kept free which was confining the volume of the measuring buffer in front of the gold disc working electrode to a few hundred of µl's. Shown in Figure 9, 10 and 11 are photographs of the individual components of the newly constructed custom-made flow cell for capacitive electrochemical immunosensing of human disease markers, an illustration of how to assemble the device for operation and a photo of the fully assembled cell plus a top-view photograph of the gold disc electrodes after polishing to requisite cleanness, respectively.

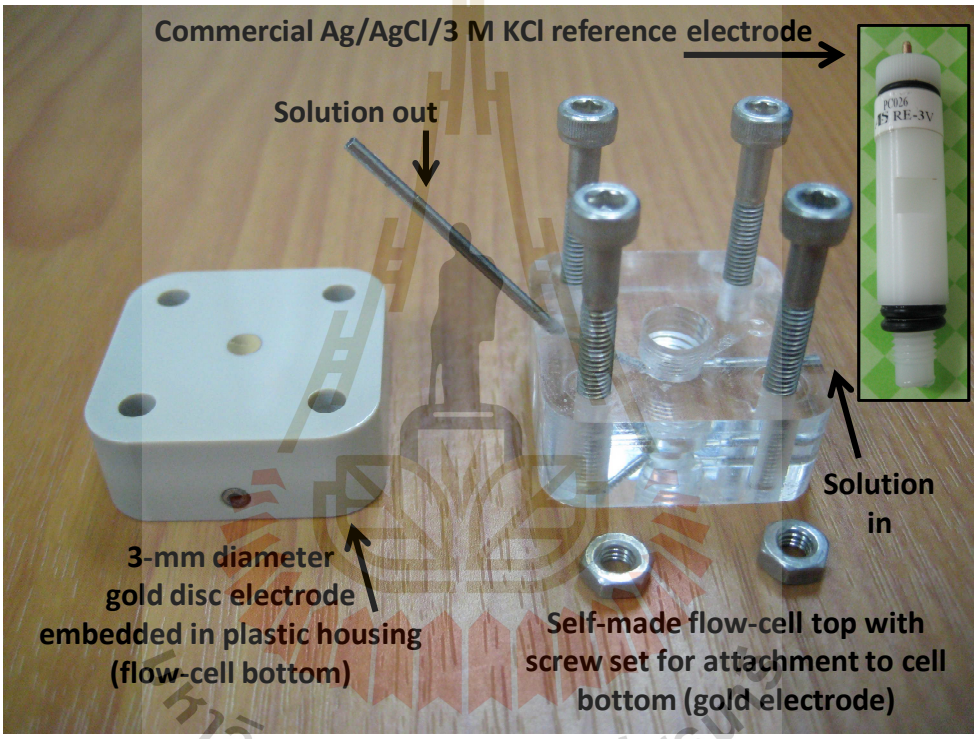

Figure 9: Components of the newly constructed custom-made flow cell for capacitive electrochemical immunosensing of human disease markers.

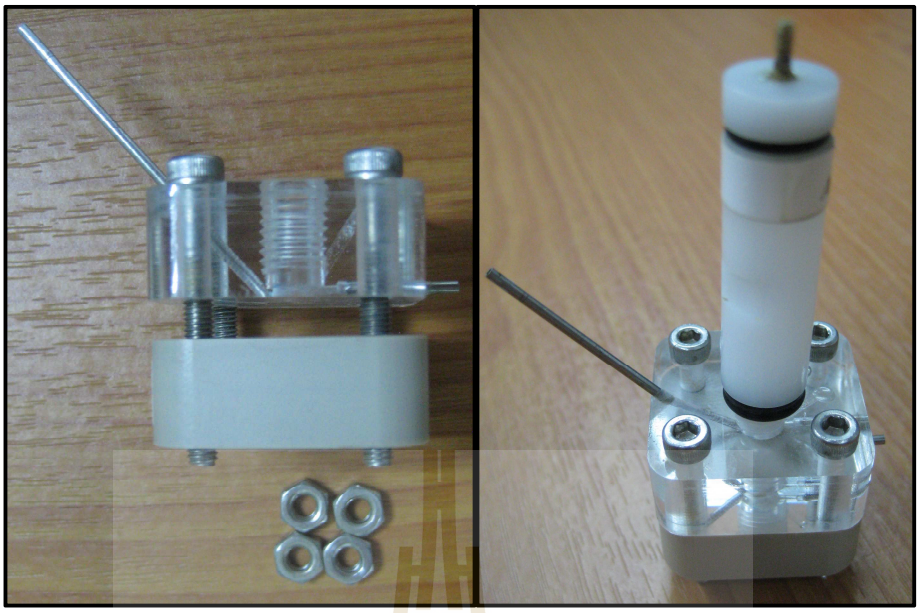

Figure 10: Assembly of the newly constructed custom-made flow cell for capacitive electrochemical immunosensing of human disease markers.

Figure 11: The fully assembled newly constructed custom-made flow cell for capacitive electrochemical immunosensing of human disease markers. The inset to the top-right displays a top-view to the 3 mm-diameter gold electrode that is prior to the biomarker measurements surface-modified with immobilized antibody for the target marker protein. A 10 Baht coin is shown to allow an estimation of the actual size of the whole system.

The new flow cell was subjected to calibration measurements for the quantification of YKL-40 antigen and an adaptation of procedure parameters. Figure 12 is a schematic representation of the whole system for capacitive immunosensing of disease markers which is helpful to describe the analytical procedure employed for the detection of antigen levels in calibration or sample solutions.

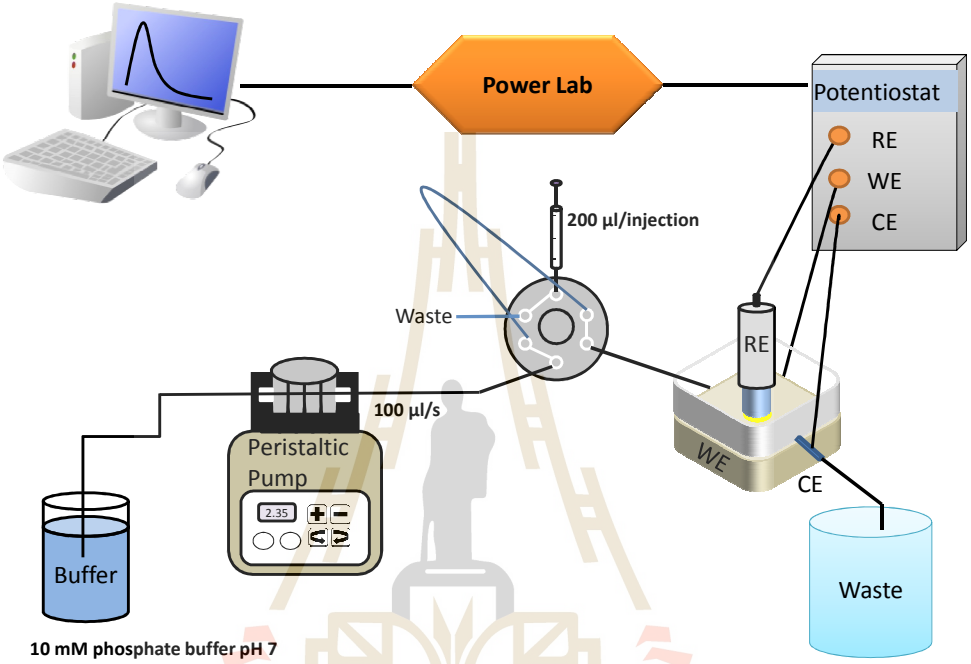

Figure 12: A schematic representation of the established flow-based setup for the electrochemical detection of disease markers in clinical samples.

In an optimized trial, the measuring buffer, actually 10 mM phosphate buffer solution at pH 7, was pumped at a flow rate of 100 µl/s through the system's channels. Continuously throughout the flow load of the electrochemical cell small voltage pulses (height: 50 mV; length: 6 ms) were applied to the gold working electrode and the current response to this agitation was monitored at a frequency of 1/min as function of time. The raw data were collections of I vs. t traces as displayed in Figure 13. An analysis of the linear log plot of the first about half a millisecond of the original I/t curves (see inset Figure 13) allows calculating the electrode capacitance, C, from the slope and y-axes intersection of the graph. C levels are different for the electrode with unoccupied antibody entities and the electrode with the antibody conjugated to its corresponding antigen and the magnitude of ΔC scales with antigen concentration in injected calibration or sample solutions. As expected, the injection of the same volume of the same solution of the YKL antigen lead

to identical electrode capacitance changes ΔC (see Figure 14). And for different YKL-40 injections ΔC scaled in good agreement with the literature, linearly with the log of concentration within the range 1 x 10^{-07} to 1 x 10^{-03} g /L (or 2.5 pM to 25 nM) (see Figure 15). Also in good accordance with reported values for other protein-based disease markers, the detection limit for the presence of YKL-40 protein in a sample solution was found to be about 10-07 g/L (or 2.5 pM).

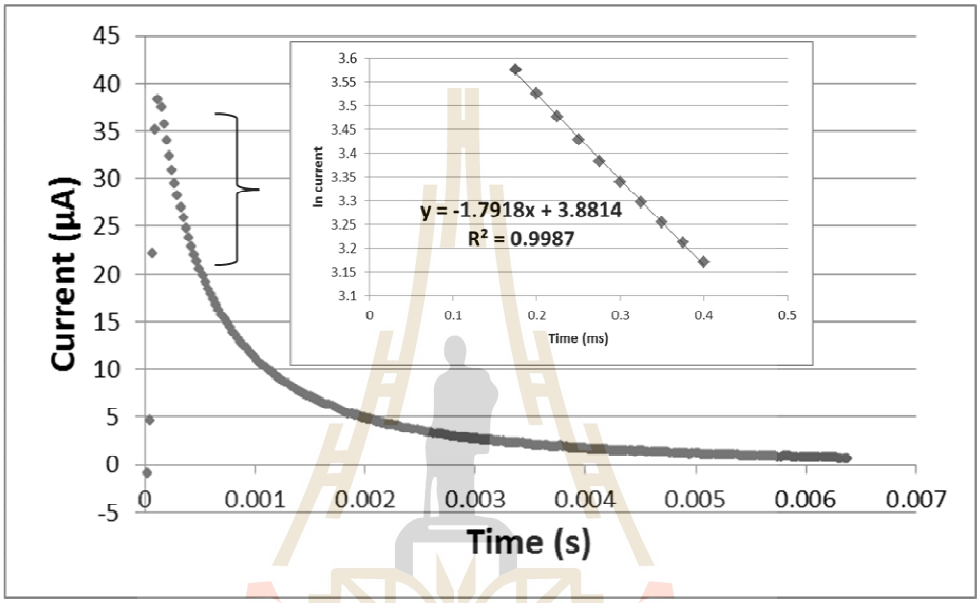

Figure 13: Typical current response of a gold disc working electrode to the application of a 50-mV-high and 6-ms-long potential pulse in 10 mM phosphate buffer at pH 6. The exponentially decaying curve is representative of the capacitive current that is flowing through the electrochemical cell in order to charge the electrodes Helmholtz double layer in accordance to the applied voltage. The inset is a log plot of the current, however, only for the first about half of a millisecond. From the slope and y-axes intersection of the linear plot the electrode capacitance can be computed.

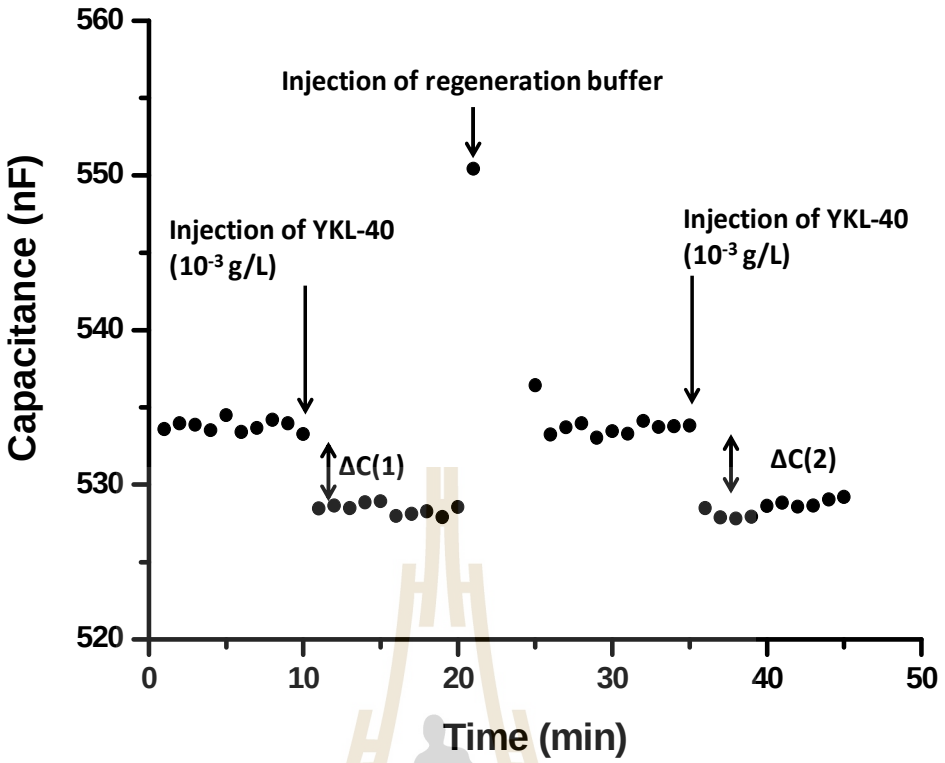

Figure 14: Plot of the measured working electrode capacitance in 10 mM phosphate buffer (pH 7) as a function of time. An injection of 200 µl of a solution containing XX g/l YKL-40 protein was followed by an electrode regeneration (= breaking of YKL-40 to anti YKL-40 conjugation and re-establishment of the anti-YKL-40-modified sensor surface in full unoccupied state) and an injection of 200 µl of a solution containing XX g/l YKL-40 protein. Regeneration brings the electrode capacitance back to the same baseline level while exposure to equal injections of YKL-40 solutions caused reproducibly identical capacitance changes ($\Delta C(1) \approx \Delta C(1)$). For different YKL-40 injections ΔC scaled linearly with the log of concentration (see Figure 15).

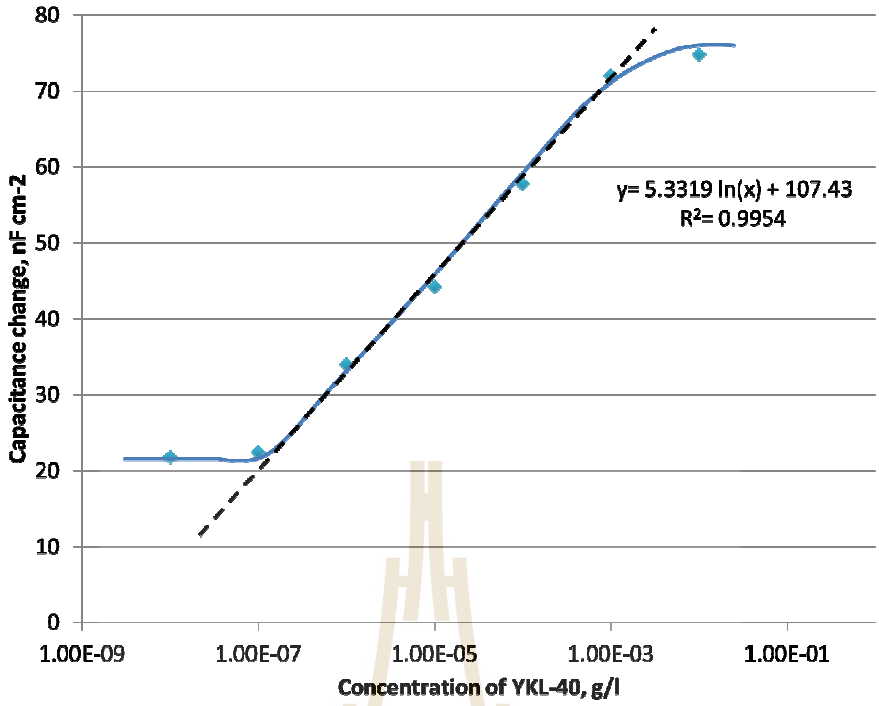

Figure 15: Calibration plot of the YKL-40 protein as obtained with flow injection-based capacitive immunosensing with a anti-YKL-40-modified gold disc electrode under the following conditions: flow rate 100µl/min; injected volume: 200 µl; regeneration buffer: 25 mM HCL (pH 2.5); carrying buffer: 10 mM phosphate buffer (pH 7); capacitance detection pulse: 50 mV/6 ms.

Figure 16 on the next page is a schematic summary of the procedures to be applied for the preparation of the gold electrode-based immunosensor for the YKL-40 marker protein including an overview of individual steps and a timeline of the procedure from start (gold electrode polishing) till finish (immunosensor stabilization in a stream of 10 mM phosphate buffer, pH 7). The completion of the YKL-40 immunosensor including priming/stabilization occupies about 3 days. If stabilization of the immunosensor is carried out overnight, on the morning of Day 4 work can be started on the assessment of the calibration curve. A first injection of a YKL-40 sample with known concentration sample is carried out and the capacitive assay used to measure a first capacitance change value for the raise to the particular YKL level. Regeneration with an acidic buffer breaks the antibody/antigen bonds and "cleans" a used immunosensor from bound analyte. The capacitance measurement and regeneration for a single YKL-40 concentration takes about 120 minutes. Usually, seven to ten data points are used to make up the calibration curve

and each level is assessed three times to get an averaged values with an n equal to 3. This means that the acquisition of a complete calibration curve needs about 3-5 days (8 am – 11 pm).

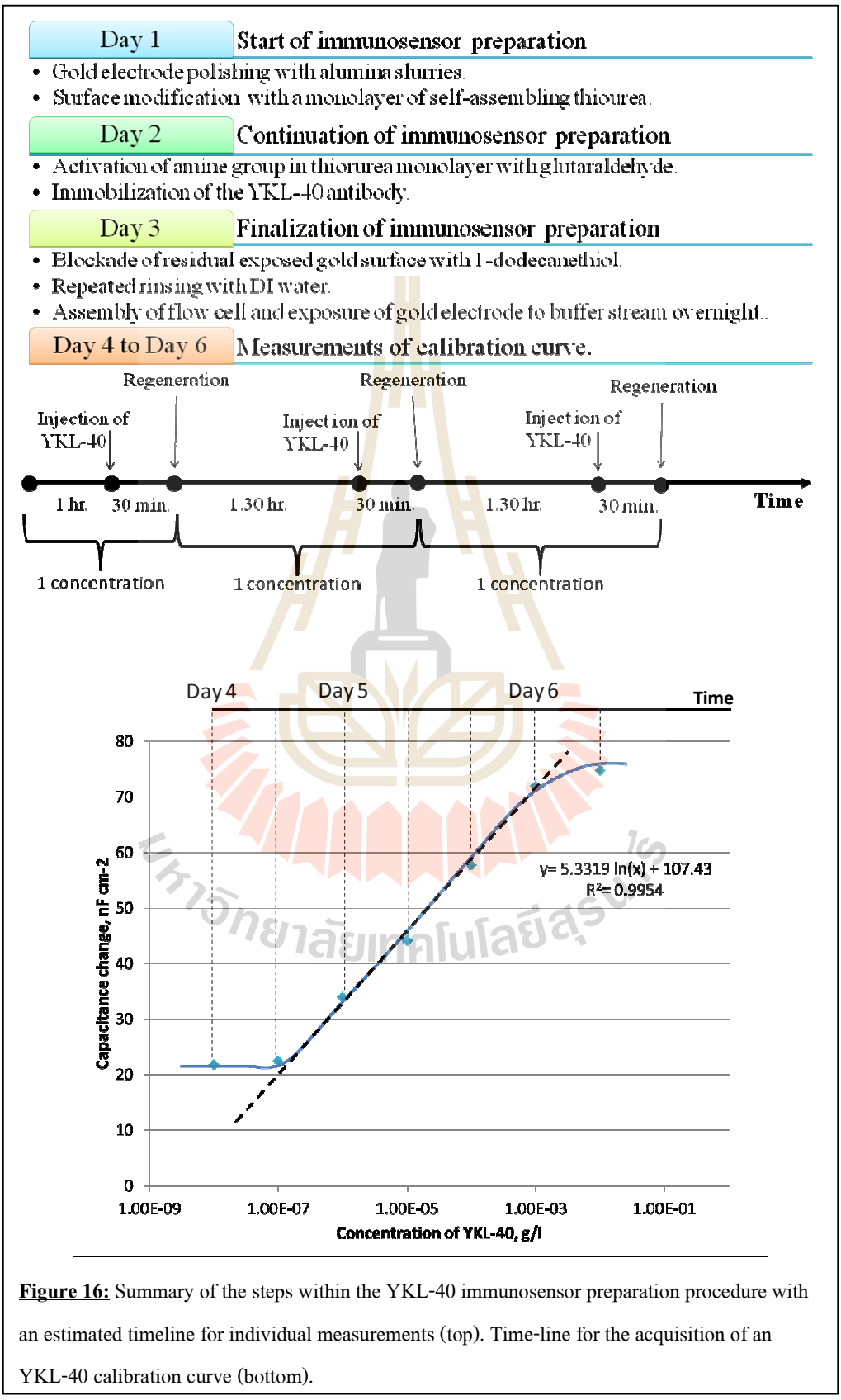

Figure 16: Summary of the steps within the YKL-40 immunosensor preparation procedure with an estimated timeline for individual measurements (top). Time-line for the acquisition of an YKL-40 calibration curve (bottom).

บทที่ 4

บทสรุป (Conclusion/Summary)

A fully functional electrochemical setup for the sensitive immunosensing of clinical disease markers has been established through the work within the project. The functionality and performance level of the flow-based capacitive electrochemical assay was evaluated via the detection of YKL-40, a protein that recently has been claimed to be a cancer and inflammation disease marker with potential to be used for clinical analysis. Calibration plots and the detection limits have been determined for the YKL-40 case. The detection limit and linear range of the approach for the YKL-40 biomarker protein was comparable to what was reported previously for the quantification of other protein disease markers by means of capacitive current recordings. Important to mention is that the detection limit is about an order of magnitude lower than the one listed for the commercialized YKL-40 ELISA kit, which probably could favor of an earlier detection of the cancer disease state of patient with the electrochemical assay. The potential to be a competitive analytical option for e.g. clinical laboratories, however, has to be proven in further detailed studies through thorough statistical trials with model and real clinical samples and the assessment of, for instance, recovery rates and accuracies of the particular determinations.

บรรณานุกรม (References)

1. Durner, J. (2010) Clinical Chemistry: Challenges for analytical chemistry and the nanosciences from medicine. Angew. Chem. Int. Ed. 49, 1026 – 1051.
2. Premjeet, S., Deepika, G., Sudeep, B., Sonam, J., Sahil, K., Devashish, K., Sunil K. (2011) Enzyme-Linked Immuno-Sorbent Assay (ELISA), basics and it's application : A comprehensive review. J Pharm. Res. 4, 4581-4583.
3. Lequin, R.M. (2005) Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). Clin. Chem. 51, 2415-2418.
4. Skladal, P. (1997) Advances in Electrochemical Immunosensors. Electroanalysis 9, 737-745.
5. Ricci, F., Adornetto, G., Palleschi, G. (2012) A review of experimental aspects of electrochemical immunosensors. Electrochim. Acta, In press (DOI 10.1016/j.electacta.2012.06.033).

6. Berggren, C., Johansson, G. (1997) Capacitance measurements of antibody–antigen interactions in a flow system. Anal. Chem. 68, 3651–3657.
7. Limbut, W., Hedström, M., Thavarungkul, P., Kanatharana, P., Mattiasson, B. (2007) Capacitive biosensor for detection of endotoxin. Anal. Bioanal. Chem. 389, 517-525.
8. Limbut, W., Kanatharana, P., Mattiasson, B., Asawatreratanakul, P., Thavarungkul, P. (2006) A reusable capacitive immunosensor for carcinoembryonic antigen (CEA) detection using thiourea modified gold electrode. Anal. Chim. Acta 561, 55-61.
9. Teeparuksapun, K., Hedström, M., Wong, E.Y., Tang, S., Hewlett, I.K., Mattiasson, B. (2010) Ultrasensitive detection of HIV-1 p24 antigen using nanofunctionalized surfaces in a capacitive immunosensor.. Anal. Chem. 82, 8406-8411.
10. Labib, M., Hedström, M., Amin, M., Mattiasson, B. A capacitive biosensor for detection of staphylococcal enterotoxin B Anal. Bioanal. Chem. 393, 1539-1544.
11. Loyprasert, C., Hedström, M., Thavarungkul, P., Kanatharana, P., Mattiasson, B.. (2010) Sub-attomolar detection of cholera toxin using a label-free capacitive immunosensor. Biosens. Bioelectron. 25, 1977–1983.
12. Pawlotsky JM (2002) Molecular diagnosis of viral hepatitis. Gastroenterol. 122, 1554-1568.
13. Kastrup, J. (2012) Can YKL-40 be a new inflammatory biomarker in cardiovascular disease? Immunobiol. 217, 483-491.
14. Johansen, J. S., Schultz, N.A., Jensen, B.V. (2009) Plasma YKL-40: a potential new cancer biomarker?. Fut. Oncol.. 5, 1065-1082.
15. Huang, K., Wu L.D. (2009) YKL-40: a Potential Biomarker for Osteoarthritis. J. Internat. Med. Res. 37, 18–24.

ภาคผนวก ก Appendix 1
ผลงานตีพิมพ์และการเผยแพร่

1. ผลงานตีพิมพ์ในวารสารนานาชาติ 1 ผลงาน (Output/Publication)
   Suginta W., Khunkaewla, P., Schulte, A.*, Electrochemical Biosensor Applications of the Polysaccharides Chitin and Chitosan. *Chemical Reviews* **2013**, 113 (7), pp 5458–5479 DOI: 10.1021/cr300325r. (JIF2012 = 41.298)

2. In addition to the above publication that acknowledges the provision of the funds, the following publication activities of the grant holders are strongly related to the project theme:

   - Grant holder A. Schulte contributed to a book chapter entitled "Amperometric biosensors" which is published as part of the book "*Advances in Electrochemical Science and Engineering, Volume 13: Bioelectrochemistry*", Alkire, R. C., Kolb, D. M. and Lipkowski, J. (eds), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. (2011) doi: 10.1002/9783527644117.ch1. One subject is on reviewing the state of the art of electrochemical immunosensors, the subject of the research of this work.

   - A manuscript entitled "Electrochemical Immunosensing of Human YKL-40, a Mammalian Chitinase-Like Protein and Disease Marker" has been submitted to the Journal "*Bioelectrochemistry*" (JIF2012 = 3.947) and is currently under review.

ภาคผนวก ข Appendix 2

ประวัตินักวิจัย (Personal Resume/CV)

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Research Interest

1. Electrochemical biosensing
   Enzyme biosensors, Immunosensors
2. Automated electroanalysis in microtiter plates
   Food analysis, Drug analysis, Environmental analysis
3. Electroanalysis in ultrasmall volumes
4. Electrochemical scanning probe microscopy
   Scanning electrochemical microscopy, Electrochemical scanning tunneling microscopy

List of publications (2007-2012)

1. Schulte A. *, Khunkaewla P., Suginta W. Chem. Rev. Biosensor Applications of the Polysaccharides Chitin and Chitosan. Chemical Reviews. Submitted and under review. (JIF2011 = 40.2)
2. Intarakamhang S., Schulte A. * Automated electrochemical free radical scavenger screening in dietary samples. Anal. Chem. 84 (2012) 6767–6774. (IF2011 = 5.9)

3. SCHULTE A.*, NEBEL M., SCHUHMANN W. Single live cell topography and activity imaging with the shearforce-based constant-distance scanning electrochemical microscope. Methods Enzymol. 2012 (IF2011 = 1.97)
4. BORGMANN S., SCHULTE A., NEUGEBAUER S., SCHUHMANN W. Amperometric biosensors. Book chapter in: Advances in Electrochemical Sciences and Engineering, Vol. 13 “Frontiers in Bioelectrochemistry” (Eds. Alkire, Kolb, Lipkowski, Ross); Wiley-VCH Verlag GmbH, Weinheim, Germany. 2012, p.1. doi: 10.1002/9783527644117.ch1 (Book series, no IF)
5. SHKIL H., SCHULTE A., GUSCHIN D.A., SCHUHMANN W. Electron transfer between genetically modified Hansenula polymorpha yeast cells and electrode surfaces via Os-complex modified redox polymers. Chem. Phys. Chem. 12 (2011) 806–813. (IF2011 = 3.4)
6. SUGINTA W.*, MAHENDRAN K.R., CHUMJAN W., HAJJAR E., SCHULTE A., WINTERHALTER M., WEINGART H.*. Molecular analysis of antimicrobial agent translocation through the membrane porin BpsOmp38 from an ultraresistant Burkholderia pseudomallei strain. BBA-Biomembr. 1808 (2011) 1552-1559. (IF2011 = 4.0)
7. SRIPIROM J., NOOR S., KÖHLER U., SCHULTE A.* Easily made and handled carbon nanocones for scanning tunneling microscopy and electroanalysis. Carbon 49 (2011), 2402-2412. (IF2011 = 5.4)
8. INTARAKAMHANG S., LESON C., SCHUHMANN W., SCHULTE A.* A novel automated electrochemical ascorbic acid assay in the 24-well microtiter plate format. Anal. Chim. Acta 687 (2011) 1-6. (IF2011 = 4.6)
9. NEBEL M., ECKHARD K., SCHULTE A., ERICHSEN T., SCHUHMANN W.* 4D Shearforce-based constant distance mode scanning electrochemical microscopy (SECM). Anal. Chem. (2010) 7842–7848. (IF2011 = 5.9)
10. HUSSEIN E. M., SCHUHMANN W., SCHULTE A.* The shearforce constant-distance scanning electrochemical microscope as fabrication tool for needle-like carbon-fiber nanoelectrodes. Anal. Chem. 82 (2010) 5900. (IF2011 = 5.9)
11. SCHULTE A.*, NEBEL M., SCHUHMANN W.* Scanning electrochemical microscopy in neuroscience. Ann. Rev. Anal. Chem. 3 (2011) 299. (IF 2011 = 9.0)
12. SCHULTE A., RUAMCHAN S., KHUNKAEWLA P., SUGINTA W.* The outer membrane protein VhOmp of Vibrio harveyi: pore-forming properties in black lipid membranes. J. Membr. Biol. 230 (2009) 101. (IF2009 = 1.8)
13. RUHLIG D., GUGEL H., SCHULTE A., THEISEN W., SCHUHMANN W.* Visualization of local electrochemical activity and local nickel ion release on laser-welded NiTi/steel joints using combined alternating-current mode and stripping-mode SECM. Analyst 133 (2008) 1700-1706. (IF2011 = 4.2)

14. ECKHARD K., ETIENNE M., SCHULTE A., SCHUHMANN W.* Constant distance AC_SECM for the visualization of corrosion pits. Electrochem. Comm. 9 (2007) 1793-1797. (IF2011 = 4.9)
15. SCHULTE A.*, SCHUHMANN W.* Single cell microelectrochemistry. Angew. Chem. Int. Ed. 46 (2007) 8760-8777. (IF2011 = 13.5)

Selected Oral Conference Contributions

1. KULLAWONG P., SCHULTE A. * Untainted carbon nanotube networks as competitive immobilization matrix for amperometric enzyme biosensors. Regional Electrochemistry Meeting of South-East Asia 2010 (REMSEA 2010); 16th – 19th November 2010, Bangkok, Thailand.
2. SCHULTE A. * Electrochemistry and Nanoscience: The world of ultrasmall electrodes, nanoscale sensor modifiers, and electrochemical microscopy schemes. (Invited). German-Thai Symposium on Nanoscience and Nanotechnology (GTSNN2011); 13th – 16th September, 2011, Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, Thailand.
3. SRIPIROM J., KUHN S., JUNG U., MANGUSSEN O., SCHULTE A. * Pointed carbon microelectrodes with nanometer tip radii: Potential tools for EC-STM and voltammetry. 12th International Fischer Symposium on "Frontiers in Nanoelectrochemistry"; 3rd – 7th June 2012, Luebeck Germany.
4. SCHULTE A.*, SCHUHMANN W., INTARAKAMHANG S. Robotic stripping voltammetry of trace lead and cadmium in water and soil samples. The 63rd Annual Meeting of the International Society of Electrochemistry: Electrochemistry for Advanced Materials, Technologies and Instrumentation, 19-24 August, 2012, Prague, Czech Republic. ()