Application of a New Non-Invasive Skin Cholesterol Detection Technology in Atherosclerosis Screening

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Research

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Abstract

Background: Establishing a high-accuracy and non-invasive method is essential for evaluating cardiovascular disease. Skin cholesterol is a novel marker for assessing the risk of atherosclerosis and can be used as an independent risk factor for early assessment of atherosclerotic risk.

Methods: we propose a non-invasive skin cholesterol detection method based on absorption spectroscopy. Detection reagents specifically bind to skin cholesterol and react with indicator to produce colored products, the skin cholesterol content can be obtained through absorption spectrum information of colored products detected by noninvasive technology. Gas chromatography is used to measure cholesterol extracted from the skin to verify the accuracy of the noninvasive test method. A total of 163 subjects were divided into normal group(n=58), disease group (n=26) and risk group(n=79). All subjects underwent noninvasive skin cholesterol test. The diagnostic accuracy of the measured value was analyzed by receiver-operating characteristic (ROC) curve.

Results: The proposed method is able to identify porcine skin containing gradient concentration of cholesterol and the values measured by non-invasive detection method were significantly correlated with gas chromatography measured results ($r=0.9074$, $n=73$, $p<0.001$). We further evaluated the method on patients with atherosclerosis and high risk population as well as normal group, patients and high risk atherosclerosis group exhibited higher skin cholesterol content than normal group (all $P<0.001$). The area under the ROC curve for distinguishing Normal/Disease group was 0.8243(95% confidence interval, 0.7165 to 0.9321), however, the area under the ROC curve for distinguishing Normal/Risk group was 0.8488(95% confidence interval, 0.7793 to 0.9182).

Conclusions: The method demonstrated its capability of detecting different concentration of skin cholesterol. This non-invasive skin cholesterol detection system may potentially be used as a risk assessment tool for atherosclerosis screening, especially in a large population.

Background

Cardiovascular disease is the leading cause of death worldwide, and atherosclerosis constitutes the main pattern of cardiovascular disease. Effective control at an early stage will delay or prevent the development of asymptomatic atherosclerosis into cardiovascular disease[1, 2]. At present, for the detection of atherosclerosis, examinations such as angiography and ultrasound can detect abnormalities only after the lesions have appeared in the arteries, thus there are limitations in the early screening of atherosclerosis. Other risk factors such as blood lipids, blood sugar and other indicators are invasive tests, and are affected by factors such as diet and fluctuation. There is an urgent need for clinical non-invasive and convenient atherosclerosis early screening methods.

Skin contains about 11% of the human body's cholesterol. It is estimated that epidermis contains seven times as much cholesterol by weight as the dermis[3, 4]. For most of the previous studies, Cholesterol synthesis is essential for skin barrier homeostasis[5, 6], a recent research has reported the impact of

Cholesterol depletion on the permeability properties and microstructure of model membranes and human, they speculate that the stratum corneum cholesterol domains may have a more complex role in the skin, other than a barrier limiting water loss and the entry of chemicals[7]. Numerous studies have shown that skin cholesterol(SC) content is associated with deposition of cholesterol in the coronary arteries and aorta [8–10]. Thus, SC content has been suggested to be quantified as a marker for identifying patients with atherosclerotic arterial disease. Previous efforts towards larger studies of SC were hampered by methods that required a skin sample for cholesterol measurement, however, these concerns have now been resolved by a non-invasive method to determine SC for cardiovascular risk assessment[11].

Using above assay that measure SC content in epidermis, SC is proved to be associated with angiographic coronary artery disease, and the presence of myocardial ischemia in patients with positive stress test results[10]. In asymptomatic patients, there is an association between SC and coronary artery calcium and circulating inflammatory markers and with CIMT[12–14]. All these studies reveal that SC may be a marker of subclinical atherosclerosis, and SC content could become a useful tool for assessing cardiovascular risk, which means it is capable of discriminating among healthy individuals, those at risk of developing atherosclerosis, and those with overt disease. The above non-invasive measurement of SC content use a point-of-care test defined as Cholesterol 1,2,3™, which contains three different concentrations of HRP conjugate used to bind to cholesterol in the skin and then visual scoring. Cholesterol 1,2,3 uses quantitative interpretation and a single concentration of detector. One major drawback of this device is that the measurement is easily affected by the pressure change caused by hand-held instrument operated by different operators, besides, the chroma of different palm from different population differ from each other which may lead to measured deviation when situ measurement is performed.

A reasonable approach to tackle these issues is to perform No-Touch palm measurement utilizing absorption spectrum instead of diffuse reflection spectrum. Strategy of No-Touch palm measurement would make it overcome the pressure change caused by different operator; meanwhile, the method can be more accurate by absorption spectrum regardless of chroma difference of different population’s palm. To this end, we develop a No-Touch palm measurement device, which can non-invasively assess cholesterol in epidermis, and the following study is to introduce our non-invasive skin cholesterol detection system and prove the accuracy and stability of the device as well as exploring the initial clinical application in Chinese population.

Results

The hardware architecture and data processing algorithm of non-invasive skin cholesterol detection system

The hardware description of the system

The optical system of non-invasive skin cholesterol detection system, as depicted in Fig. 1A, consists of a constant current source module, a white LED light source(NSPL510DS,Nichia), a sample platform with a sample pool, a compact CCD device spectrometer（AM1280，OtO photonics）and an computer. In addition, there are other parts such as power supply units, control units, optical and mechanical parts.

The white LED was chosen as the light source. The digitally adjustable constant-current source can ensure the stability of the light source. Intensity variations of the LED were typically < 1% during the measurements in the present work. Light from the LED passed through the sample, and then focused into a fiber which connected with the spectrometer. The concentration of the sample to be tested can be obtained by measuring the change in light intensity before and after through the sample.

The stability of the light source is critical to the accuracy of the measurement. Figure 2 displays the flow chart of light source intensity control and spectra collection. Light intensity variation is within 1% by constant current source control. In order to determine the dynamic regulation performance of the constant current source, we tested whether the LED light source can finally reach the target light intensity under different initial light intensity, we set target light intensity as I_0, the initial intensity varies from $0.5I_0$ to $1.5I_0$($0.5I_0$, $0.75I_0$, I_0,$1.25I_0$ $1.5I_0$), the process of LED dynamic state response curve can be obtained by constant measurement of spectrum, As shown in Fig. 3A, different initial intensity can reach target intensity respectively, and the time reaching target intensity is no more than 4 S. Furthermore, we examined the stability of the light source after reaching target intensity, the result show that light-intensity variation is within 1%(Fig. 3B). The above experiment results indicate that the detection system is able to reach steady light source intensity state quickly, and the intensity can also be stable for a long period of time which is essential to the accurate measurement of skin cholesterol.

One character of our system is that we designed a sample platform for small-volume liquid measurement. The volume of the detection reagent is very small, only a few tens of microliters, which are very disadvantageous for concentration detection, based on absorption spectroscopy techniques. When measuring the concentration of the reaction solution, we need to obtain the absorption optical path of the liquid to be tested. Therefore, it is usually necessary to put it into the cuvette, which requires a large volume of liquid. As shown in Fig. 1B, the device consists of two transparent glass sheets. The distance between the glass sheets is fixed, which reflect the depth of the detected liquid samples. The light from the light source can pass through the glass sheet and the liquid directly, and then received by the detector. Since the distance between the two glass sheets is fixed, and the absorption optical path is determined, as long as the liquid can fill the entire detection path, there is no requirement for the liquid volume. Due to the adsorption effect, tens of microliters of liquid can fill the entire detection path. The advantage of the sample platform is that there is no need to know the volume of the liquid sample to be tested, and it can measure samples from a few microliters to several hundred microliters.

Data Processing

2048 discrete data points are collected by the spectrometer with wavelengths ranging from 380 nm to 780 nm, After interpolation, 400 integer wavelength data are obtained. According to the absorption characteristics of the reaction solution, 475 nm to 780 nm are chosen as the characteristic band. The relative concentrations of cholesterol were retrieved based on Beer-Lambert Law, as shown in the following equation:

$$I_1(\lambda) = I_0(\lambda) \cdot \exp(-\alpha(\lambda)cl) \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (1)$$

Where c is the relative concentration of the cholesterol, α is the absorption molar extinction coefficient, and l is the depth of the absorber (1 mm). I_0 is the Initial light spectrum, I_1 is the transmitted light spectrum, λ is the wavelength. Thus, c can be defined as:

$$c = \frac{1}{\alpha(\lambda)l} \ln\left[\frac{I_0(\lambda)}{I_1(\lambda)}\right] \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (2)$$

In the calculation process, the relative concentration of the cholesterol sample was obtained by least squares method in the characteristic band.

Non-invasive skin cholesterol detection system is capable of recognizing gradient color solution

The final variations in the color of blue chromogenic reagent catalyzed by detection reagent linked to skin cholesterol should be recognized by our device. The amount of colored products produced by the reaction between the substrate and the enzyme is non-linear, so we calibrate the standard curve of colored product with the instrument. Gradient concentration of detection reagent were reacted to an excess mount of TMB to simulate the reaction between cholesterol and detection reagents, As shown in Fig. 4, the larger value of reaction product detected by our device is as the detection reagent concentration increases. The fitted curve obtained by the experiment can be used as a calibration curve to calculate the concentration information of the cholesterol content in the skin sample to be tested.

Non-invasive skin cholesterol detection system can distinguish gradient skin cholesterol in pig skin

To mimic the measurement of skin cholesterol in different humans, pig skin extracted with the mixture of ethanol and ethyl ether with a proportion of 3:1 for different time course (0 min, 1 min, 2 min, 3 min, 4 min) was used to obtain skin containing gradient Cholesterol. As shown in Fig. 5A and B, with the increase of extraction time, the shape of absorption spectrum remained unchanged and the intensity gradually increased and the color of blue become lighter, meanwhile, the value measured by device is decreased with the prolongation of extraction time (Fig. 5B).

The correlation between Skin cholesterol content measured by Non-invasive skin cholesterol detection system and gas chromatography

To determine the accuracy of Non-invasive skin cholesterol detection system, we extract the cholesterol in epidermis after non-invasively measurement with our detection system, and the cholesterol in extractive liquid were measured by gas chromatography, As shown in Fig. 6, the correlation coefficient was 0.9074, and there is a prominently strong correlation between the non-invasive detection system measured value and gas chromatography measured value.

Non-invasive skin cholesterol detection system can distinguish subclinical atherosclerosis, atherosclerosis patients and healthy individuals

To examine whether No-Touch palm measurement device can recognize healthy individuals and atherosclerosis patients, as well as high risk atherosclerosis population, 26 atherosclerosis patients and 79 high risk populations were measured, meanwhile, 58 low risk individuals were also enrolled as normal groups. The result revealed that the shape of absorption spectrum remained the same, the intensity of the normal group is stronger than that of disease group and high risk group (Fig. 7A). Meanwhile, disease group and high risk group have a significant higher skin cholesterol value compared to normal group (Fig. 7A), however, the values between disease group and high risk group remained no significant difference. The area under the ROC curve was applied to evaluate the efficacy of skin cholesterol values on screening for atherosclerosis risk. As shown in Fig. 7C, the area under the ROC curve for distinguishing Normal/Disease group was 0.8243(95% confidence interval, 0.7165 to 0.9321), however, the area under the ROC curve for distinguishing Normal/Risk group was 0.8488(95% confidence interval, 0.7793 to 0.9182). The efficacy of skin cholesterol values for distinguishing Normal/High risk group was stronger than for distinguishing Normal/Disease group.

Discussion

In this study, we proposed a No-Touch palm measurement device for non-invasive skin cholesterol detection, by using absorption spectrum instead of diffuse reflection spectrum, the device demonstrates two advances compared with the previous method, the first one is that we used No-Touch palm measurement instead of hand-held method, which ensure the measurement result cannot be affected by pressure fluctuation when different subjects are tested by different operators. Another one lies on that No-Touch palm measurement can test directly without considering color difference of palm from different population.

For optical measuring device, the stability of the light source is critical to the accuracy of the measurement. The simulation experiment result show that light intensity can reach stable state within

4 S, with a constant current source design, the light intensity variation is within 1% after reaching the stable state. Stable light source intensity will ensure the accuracy of skin cholesterol measurement.

The result of this study suggested that our device can potentially distinguish high risk atherosclerosis, atherosclerosis patients and healthy individuals, as shown in Fig. 7., atherosclerosis patients group and high risk atherosclerosis group exhibited higher skin cholesterol content than normal group. It is consistent with the previous study reported in Korea population[15], and the similar result that increased skin cholesterol can identify individuals at increased cardiovascular risk was also reported in 565 asymptomatic subjects from 6 sites in North America[16]. Our results first offered the relationship between skin cholesterol and atherosclerotic disease in Chinese population, which may be of great significance in the following research worldwide.

To verify the accuracy of the device, porcine skin containing gradient concentration of cholesterol was obtained using the method described in previous research[17], As shown in Fig. 5, we detected a gradual decreased levels of cholesterol in porcine skin with the extension of extraction time. Furthermore, we compared the measurement value performed with non-invasive detection system and the gas chromatography measurement results, Fig. 6 indicated that there is a strong significant correlation with each other, which further prove the accuracy of the detection accuracy. Both in vivo and in vitro results imply that our device is reliable and capable of identifying different amounts of cholesterol in the skin.

This research demonstrated the feasibility of a new system that creates a link between skin cholesterol and atherosclerosis disease. While additional experimental results and clinical data are needed to establish the reliability of this technology, the potential for atherosclerosis disease assessment and non-invasive pre-clinical atherosclerosis screening is demonstrated. However, a study from Medical University of Vienna revealed that skin tissue cholesterol concentration determined by the PREVU POC Skin sterol Test are not related to the presence of cerebrovascular disease(CVD) and peripheral arterial disease(PAD) or to an elevated cardiovascular risk, they also point out previous reported higher concentrations of skin cholesterol which indicated a relation between the presence of coronary heart disease(CHD) differed significantly between the studies and suffer from high standard deviations and high interquartile ranges[18]. These differences may resulted from pressure fluctuation and color difference of palm from different individuals conducted by different operators. Therefore, judging whether skin cholesterol can be used as a reliable indicator of atherosclerosis require a more stable and precise approach. Furthermore, multi-center clinical studies and prospective controlled trial evaluating cardiovascular disease from different races around the world is needed to clarify the role of skin cholesterol in cardiovascular risk assessment.

Conclusions

In this paper, we proposed a No-Touch palm measurement device for non-invasive skin cholesterol detection that comprises a constant current source, a light source, a sample platform, and a spectrum detection module. Promising results from the experiments have shown that the cholesterol value

measured by our device has a significant strong correlation with the results of gas chromatography, which verifies the accuracy and reliability of this technology. Meanwhile, clinical data suggest that healthy individuals and atherosclerosis patients, as well as high risk atherosclerosis population can also be recognized by our device. This is the first time that the non-invasive method has been used in distinguishing high risk atherosclerosis population or atherosclerosis patients from healthy individuals in Chinese population. Therefore, No-Touch palm measurement device is a promising approach for atherosclerosis risk assessment.

Methods

Detection steps

The detailed detection steps are as follows: First, Attach the Teflon gasket with a detection well in the middle to the small thenar part of the palm; Then, Add 100 μl of detection reagent to the detection well, after a 1-min incubation, the reagents are removed by blotting and the 30 μl of TMB is added to the well, after an additional 2-min incubation, 30 μl of the reaction solution is transferred to the sample pool with pipettor on the sample platform. Finally, the value of cholesterol content is acquired according to the absorption spectrum of the reaction solution.

Skin cholesterol detection reagent

The detection reagent is digitonin–copolymer–horseradish peroxidase (HRP) conjugate, which has beed proposed as the specific reagent for skin cholesterol detection[9], in simple terms, digitonin have strong affinity with cholesterol, and it can combine with cholesterol to form cholesterol-digtonin complex[19-21], the copolymer ensures a tight bond between digtonin and horseradish peroxidase, and the horseradish peroxidase can catalyze the color change of 3, 3’, 5, 5.-tetramethylbenzidine (TMB) substrate, which is dependent on the amount of cholesterol bound by the detection reagent. The reagent was synthesized following the steps described in the patent (authorization number: US05489510), TMB chromogenic reagent was purchased from Sigma-Aldrich (Shanghai, China). The detection reagent was diluted to 5 μg/ml , 2.5 μg/ml, 1.25 μg/ml, 0.625 μg/ml, 0.3125 μg/ml and 0.15625 μg/ml with deionized water to catalyze with TMB, the amount of colored products produced by the reaction were measured with our system.

Gradient Cholesterol extraction from porcine skin

Abdominal skin samples were obtained from six Tibetan pigs weighing 30-35 kg, and were provided by the animal center of Anhui medical university. Normal saline (shanghai, sigma) was used to soak the skin after subcutaneous tissue was removed so as to exclude hemoglobin and other pollution. The skin was then dried in the shade of the nature after cutting into 1.5*7.5 cm rectangular cubes. Skin cholesterol was extracted afterward in the mixture of ethanol and ethyl ether with a proportion of 3:1 for different time course (0 min, 1 min, 2 min, 3 min and 4 min) to obtain skin containing gradient cholesterol.

Participants

26 patients (45-80 years of age) diagnosed with atherosclerosis according to clinical information and coronary arteriography or carotid artery ultrasonography analysis were enrolled in The First Affiliated Hospital of University of Science and Technology of China from February to May 2019. Extent of disease was defined as the number of vessels with ≥50% stenosis and patients were further classified as having angiographic disease if the extent of disease was at least “one”. 79 high risk population(>10) and 58 low risk individuals(≤10) assessed by Framingham scoring from Health Management Center of Renmin Hospital of WuHan University were enrolled as control groups. Meanwhile, another 72 volunteers were recruited to participate in the accuracy verification, briefly, everyone involved in this experiment would measure skin cholesterol with non-invasive detection system, then the detection site will extracted with 400 ul of absolute ethanol for 2 minutes, cholesterol in extractive liquid were determined with gas chromatography immediately. Exclusion criteria included current use of cholesterol lowering medications, hepatitis, pregnancy, or skin disease on either hand.

Cholesterol measurement with gas chromatography

Cholesterol standard (Sigma-Aldrich) is dissolved in absolute ethanol to the concentration of 1 ug/ml, 2 ug/ml, 5 ug/ml, 10 ug/ml, 25 ug/ml and 50 ug/ml. Gas chromatography is used for detection of cholesterol content followed the previously reported method[22], briefly, detection condition is as follows: Column, DB-5 elastic quartz capillary column. Carrier gas, high purity nitrogen, purity ≥ 99.999 %; Constant flow rate, 2.4 mL/min; Column temperature (programming temperature): initial temperature is 200 ° C, hold for 1 min, increase to 280 ° C at 30 ° C / min, for 10 min; Inlet temperature, 280 °C; Detector temperature, 290 °C; Injection volume: 1ul; Injection method: no split injection, open the valve after 1 minute of injection; Air flow: 350 mL / min; Hydrogen flow rate: 30 mL/min. The cholesterol standard solution was separately injected into the gas chromatograph, and the peak area of the standard solution was measured under the above chromatographic conditions, and the standard curve was prepared by taking the concentration as the abscissa and the peak area as the ordinate. Then the extract was injected into the gas chromatograph to measure the peak area, and the concentration of cholesterol in the sample solution was obtained from a standard curve.

Statistic analysis

One-way ANOVAs (Graphpad, Prism 5) were utilized for multiple-group comparisons. All analysis was presented as means ± SD, A P value < 0.05 was considered statistically significant.

Abbreviations

SC: skin cholesterol; CIMT : carotid intima-media thickness; HRP: digitonin–copolymer–horseradish peroxidase; TMB: 3,3’, 5,5'-tetramethylbenzidine; LED: Light emitting diode; CCD: Charge-coupled device

Declarations

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Authors' contributions

Jingshu Ni: Conceptualization, Validation, Resources, Validation, Investigation, Formal analysis, Writing - original draft, Visualization, Writing - review & editing. Haiou Hong: Methodology, Investigation, Formal analysis, Validation. Yang Zhang: Investigation, Validation. Shiqi Tang: Methodology, Validation. Yongsheng Han: Methodology, Validation, Formal analysis. Zhaohui Fang: Resources, Investigation. Yuanzhi Zhang: Software, Formal analysis, Investigation. Nan Zhou: Project administration, Supervision. Quanfu Wang: Supervision. Yong Liu: Supervision, Project administration, Resources. Zhongsheng Li: Validation, Investigation. YiKun Wang: Supervision, Project administration, Writing – review, Funding acquisition. Meili Dong: Supervision, Writing – review, Funding acquisition.

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Availability of data and materials

All data used and analyzed during the study are available from the leading corresponding authors Meili Dong and Yikun Wang(wyk@aiofm.ac.cn; dongmeili@aiofm.ac.cn) on reasonable request.

Ethics approval and consent to participate

All procedures performed in studies involving human participates were in accordance with the 1964 Helsinkli declaration and its later amendments, and were approved by the ethics committee of the The First Affiliated Hospital of University of Science and Technology of China and Renmin Hospital of WuHan University. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflict of interest.

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Figures

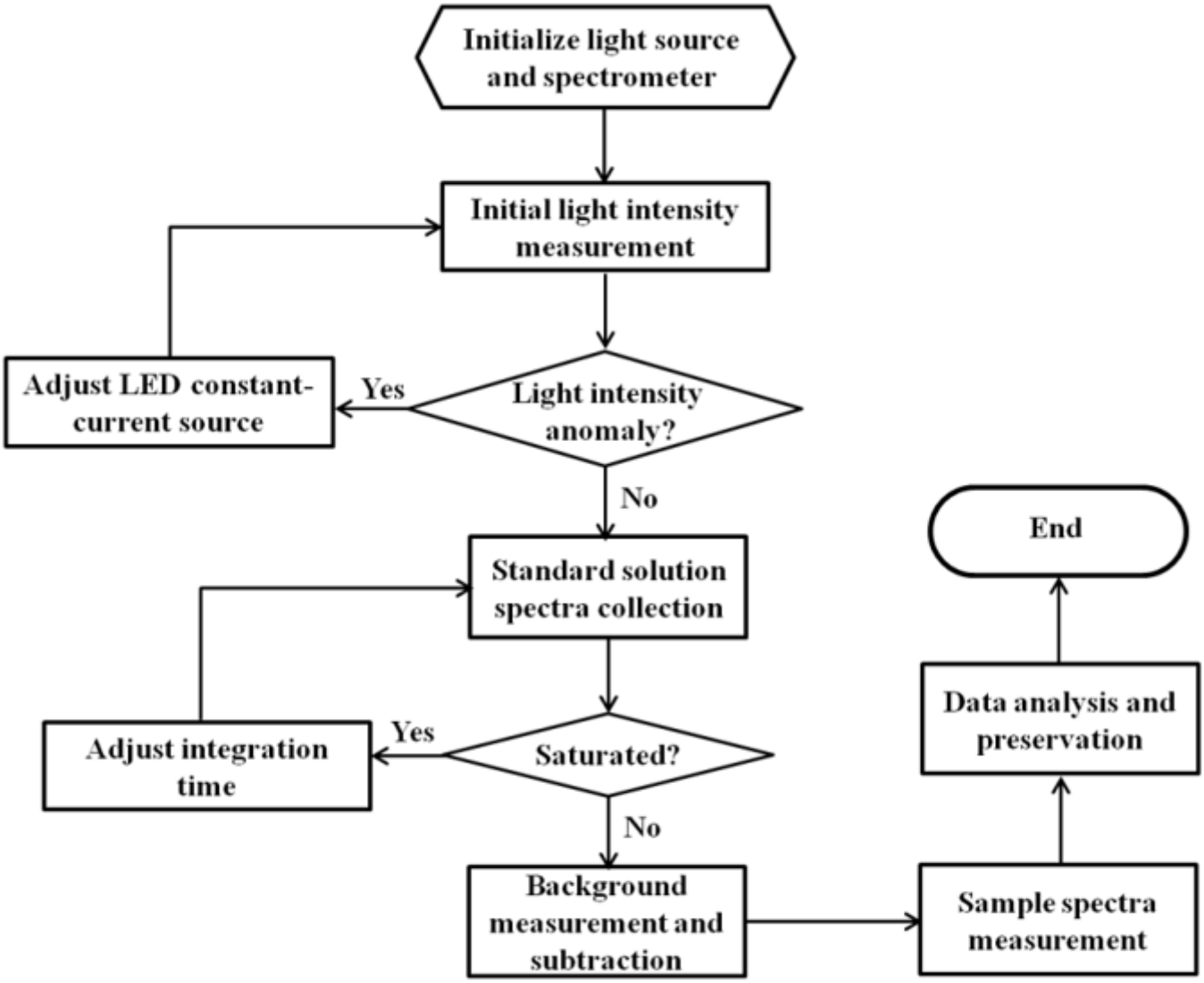

Figure 1

Schematic of optical system（A）and the structure of sample platform（B）

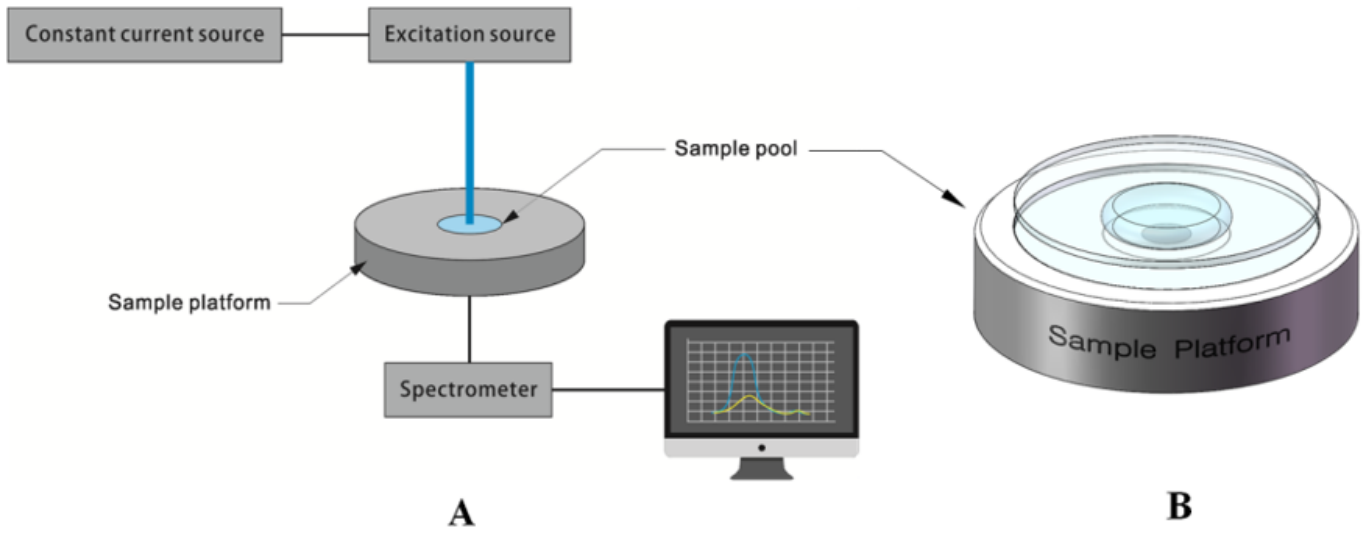

Figure 2

Flow chart of light source intensity control and spectra collection

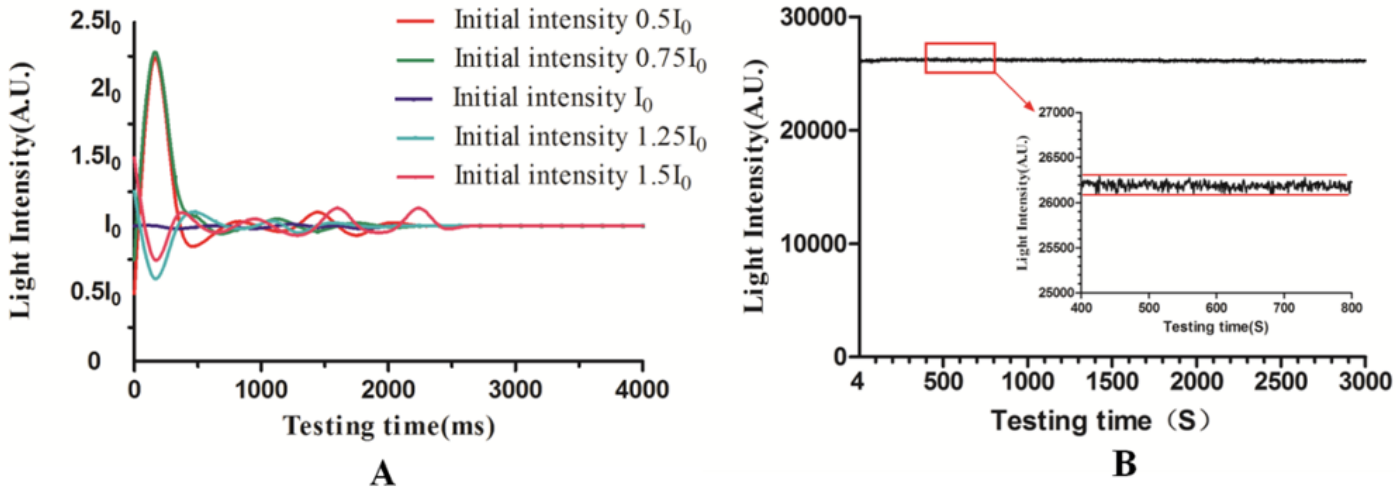

Figure 3

The process of LED dynamic state response(A) and LED steady state response(B)

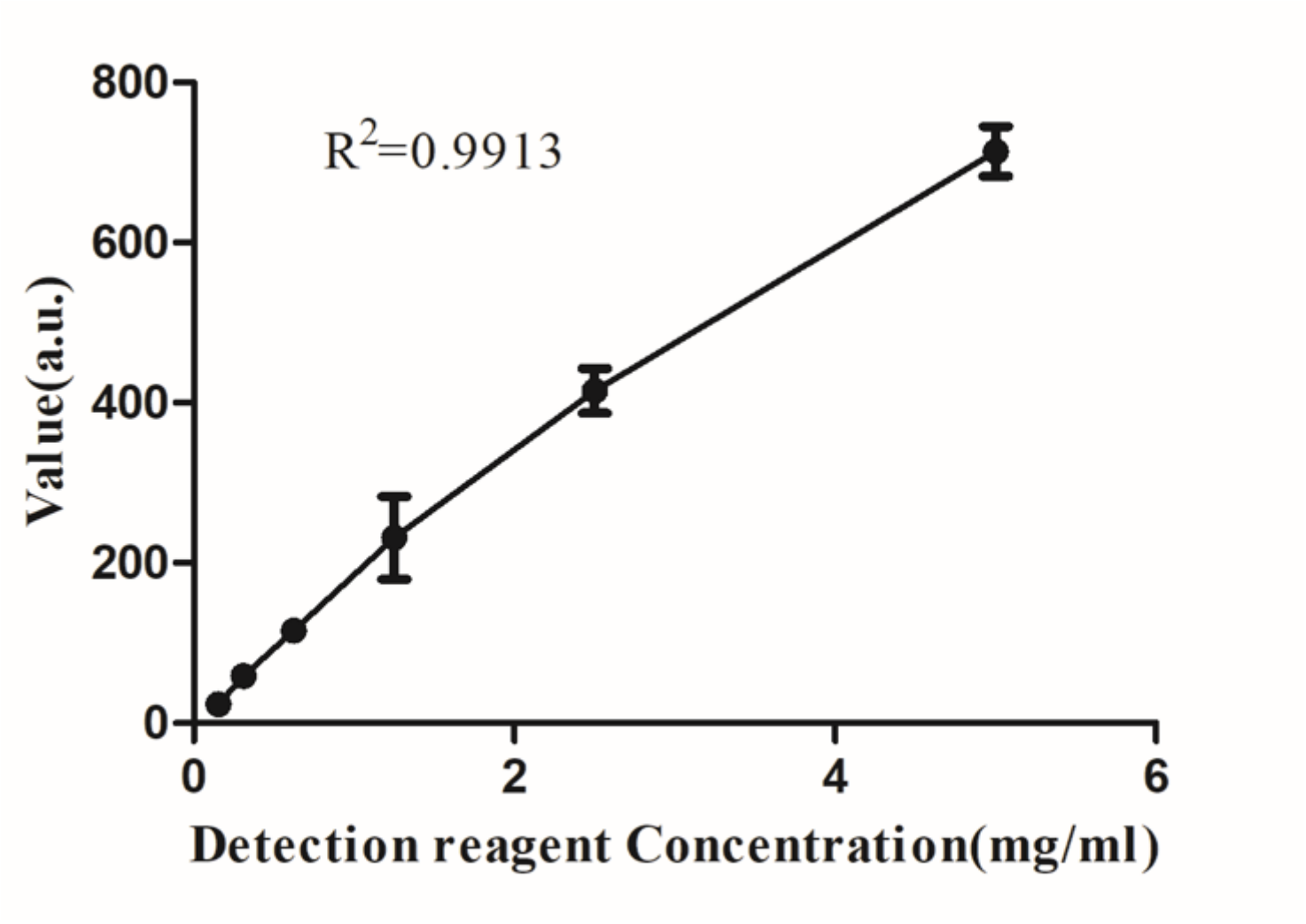

Figure 4

The fitted curve of different gradation of color induced by TMB and detection reagent.

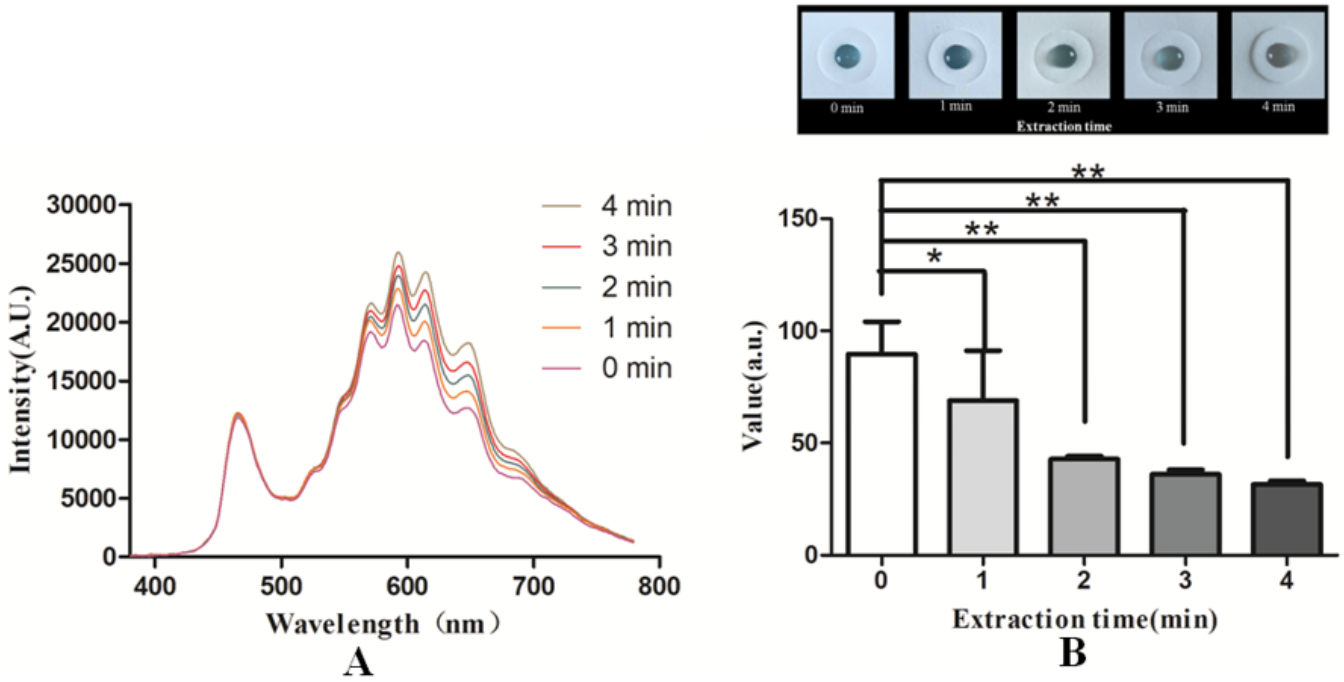

Figure 5

No-Touch palm measurement device can distinguish gradient skin cholesterol in pig skin extracted with the mixture of ethanol and ethyl ether with a proportion of 3:1 for different time course. A) The absorption spectroscopy under different extraction time; B)The variation of values with the increased extracting time.

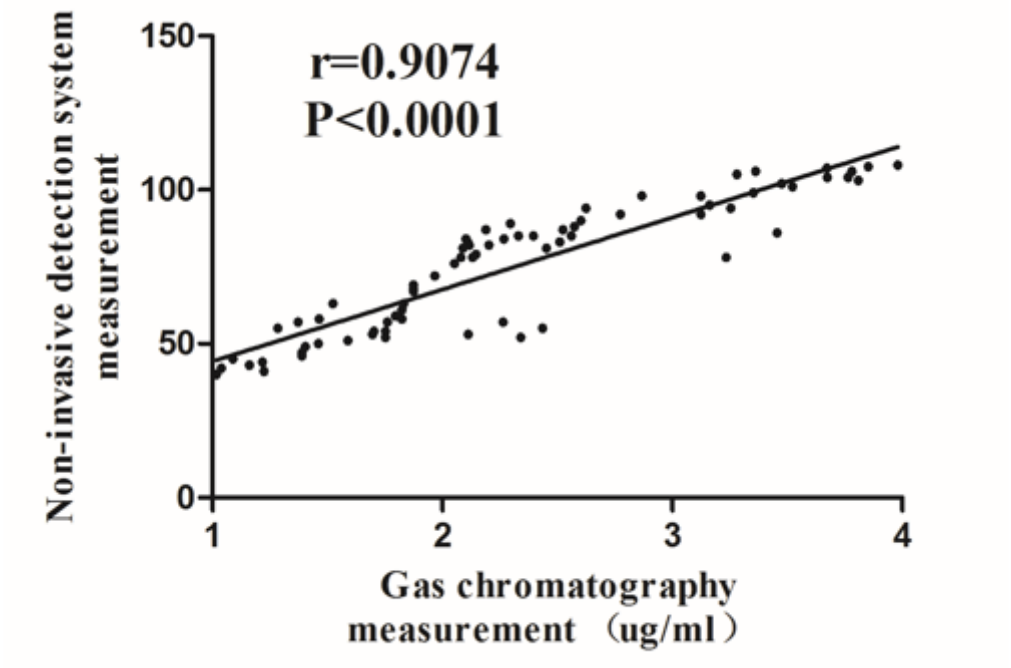

Figure 6

The correlation between skin cholesterol content measured by gas chromatography and non-invasive detection method.

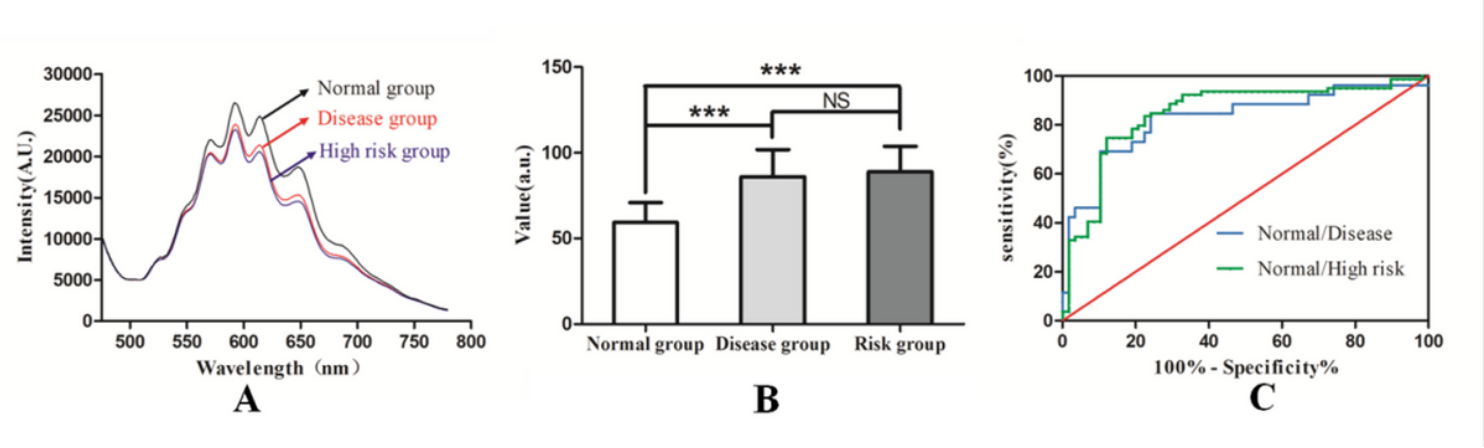

Figure 7

No-Touch palm measurement device can distinguish subclinical atherosclerosis, atherosclerosis patients and healthy individuals. A) The absorption spectroscopy of normal group, disease group and high risk group. B) Skin cholesterol value of normal group, disease group and high risk group detected by non-invasive measurement system. C) Receiver-operating characteristic(ROC) curves for distinguishing Normal/Disease group and Normal/High risk group.