氏　　名	Md. Abu Sayed
学位（専攻分野）	博士（理学）
学位記番号	総研大甲第1216号
学位授与の日付	平成２１年３月２４日
学位授与の要件	物理科学研究科　構造分子科学専攻 学位規則第６条第１項該当
学位論文題目	Construction of New Infrared Reflection Absorption Spectroscopy (IRRAS) System for Solid-Solution Interface Biomaterials
論文審査委員	主　査　教授　大島　康裕 教授　宇理須　恆雄 教授　藥師　久彌 准教授　藤井　浩 准教授　平野　愛弓（東北大学）

論文内容の要旨

The study of protein–surface interactions represents one of the most important topics in the field of biomaterials. The immobilization of proteins on solid surfaces is an important step in biosensor fabrication as well as medical devices. Infrared spectroscopy is a powerful technique for the determination of conformation and orientation of lipids and proteins including membrane proteins, and of antibody-antigen reactions on solid surfaces. Infrared reflection absorption spectroscopy (IRRAS) is one of the FT-IR techniques for determination of biomaterials on IR reflective metals, and very few IRRAS systems are found to determine the adsorbate at solid-solution interfaces.

For his doctoral research work, he has constructed a new narrow gap infrared reflection absorption spectroscopy (NG-IRRAS) system with a prism/narrow solution layer/substrate arrangement, to which substrates for biosensors and biochips can be directly attached. Advantages of this new NG-IRRAS system over other conventional-IR systems are i) different IR reflective materials can be used as a substrate ii) having a sufficient large gap (~8 μm) to flow reagent solution, and iii) a solution injection system is included to introduce reagent solution onto the sample substrate from outside. Another advantage of this IRRAS system is that this system can easily be rearranged to vacuum IRRAS system. There were two problems to be solved for the standardization of this NG-IRRAS system, i) the baseline was fluctuated due to the change of solution layer thickness, and ii) sample biomaterials was adsorbed on prism surface.

In the first stage of his PhD work, he constructed the new NG-IRRAS system and investigated the conditions for the flat and stable baseline. Firstly, he found that baseline was fluctuated due to the change of solution layer thickness. He developed a new sample holder for controlling the solution layer thickness between the prism and substrate surfaces (Fig. 1). Thermal effects, adsorbed water on the entrance of optical components, bubbles in solution, and injection flow rate were considered as factors which distorted the baseline. He has investigated the experimental conditions and found that the following procedures are crucial for the stability of the baseline: i) evacuation of the sample chamber at least for 6 h was necessary to minimize the adsorbed water vapor effect, ii) injection flow rate was kept less then 2 mL/h, iii) room temperature fluctuation was controlled within 1°C, and iv) Ni spacer thickness was 8 μm.

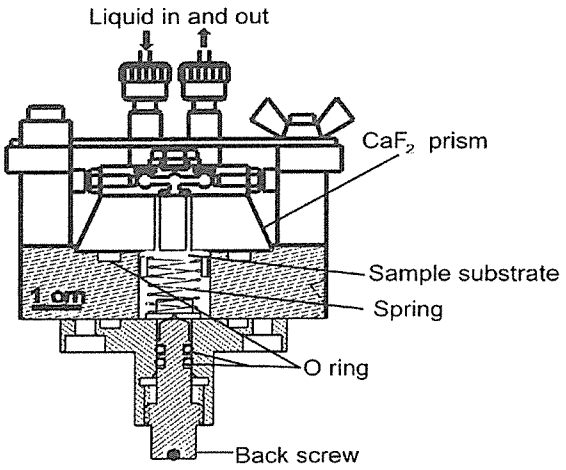

Fig. 1 Schematic diagram of the sample holder for solid-solution interface IRRAS measurement.

After the standardization of the NG-IRRAS, he started the IRRAS measurement of solid surfaces in three different conditions. i) The observation of CaF_2 prism surface in total internal reflection (TIR) mode to investigate the adsorbed biomaterials on prism surface. ii) The observation of biomaterials adsorbed at the interface between the sample substrate and solutions. Two IR-reflective substrates, Si wafer with buried metal layer (BML) and gold were used. iii) The observation of adsorbed biomaterials on the solid surface in vacuum.

Fibronectin (FN) and immunoglobulin G (IgG) were selected for observation at solid-solution interface. But, during the experiments, IR absorbance from the adsorbed biomaterials on the prism surface overlapped the IRRAS spectra of the biomaterials on the sample substrate. Thus, he controlled the adsorption of biomaterials on the prism surface by regulating the effects of salt and pH of the solution and by coating the CaF_2 prism with 2-methoxy-(polyethylene) oxypropyltrimethoxysilane (PEG). Interestingly, the adsorption tendency on the prism surface was completely opposite with salt effects between these two biomaterials (Fig. 2).

FN is easily adsorbed on SiO_2 surface and often used as an extra cellular matrix in the cell culture on SiO_2 substrates. Therefore, FN was chosen in this work to detect at BML-solution interface. He has investigated the condition, in which FN adsorbed only on the BML surface, but not on the PEG-coated prism surface. He found that FN adsorbed on the prism surface in D_2O based phosphate buffered saline (PBS) solution, but ignorable adsorbed FN was found on the PEG-coated prism surface when pure D_2O was used in the experiment (Fig. 2a).

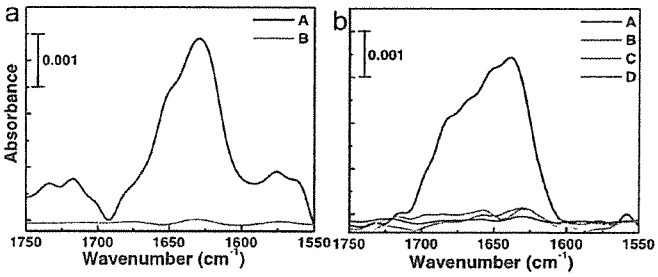

Fig. 2 The amide I bands of adsorbed proteins on a PEG-coated prism surface measured with the total internal reflection (TIR) arrangement.
a) Spectra of FN using (A) D_2O-based PBS solution and (B) pure D_2O as solvents,
b) Spectra of IgG using (A) pure D_2O, (B) NaCl (140 mM)

The IRRAS of adsorbed FN was observed at BML-D_2O interface using this condition. The protein amide I band appear in the range of 1600 – 1700 cm^{-1} assigned to the C=O stretching. The fine structure in the amide I supplies the information of the protein secondary structure, because the peak shift of υ (C=O) due to the hydrogen bonding characteristics of the secondary structure, e.g. υ (C=O) of β-sheet at 1613 -1638 cm^{-1}, α-helix at 1645 - 1657 cm^{-1} , and β-turn at 1662 -1683 cm^{-1}. The amide I band of FN was observed at approximately 1637 cm^{-1} (β sheet) with shoulders around 1671 and 1683 cm^{-1} (β turn) (Fig. 3a). The ignorable conformational change of FN was found due to the adsorption on BML-D_2O interface.

Immobilization of IgG on gold is an active research field for designing immunosensor for medical diagnostic purpose. The adsorption state of the IgG at the gold-solution interface was investigated by the NG-IRRAS. IgG was adsorbed on gold surfaces i.e. coated by the 16-mercaptohexadecanoic acid (MHA)-SAM and by the MHA-SAM activated by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). It was also found that IgG was easily adsorbed on the PEG-coated prism surface in pure D_2O, while IgG adsorption on the PEG-coated CaF_2 surface was suppressed sufficiently in NaCl/D_2O (140 mM) and D_2O based PBS solutions (Fig. 2b). He chose D_2O based PBS solution containing 140 mM NaCl for IRRAS measurement of IgG at the gold-solution interface.

The IR spectra showed that IgG easily adsorbed on the MHA-SAM coated and activated-MHA-SAM coated gold surfaces. The conformation change of the IgG adsorbed on the MHA-SAM coated gold surface was ignorable when the IRRAS spectrum was compared with the FT-IR spectra of IgG dissolved in solution. Absorption spectra at the amide I band region of the IgG in the solution phase and at the solid-solution

interface were measured with good reproducibility. The amide I band of the IgG molecule covalently bonded to the MHA-SAM (Fig. 3b) was quite similar to that of the IgG on the MHA-SAM coated gold surface. The covalent bond is formed between the COOH-terminated MHA-SAM and the lysine residue of the IgG. Because of lysine residue distributes almost homogeneously on the IgG surface, the covalently bonded IgG had random orientation similar to the physisorbed IgG on MHA-SAM coated gold surface.

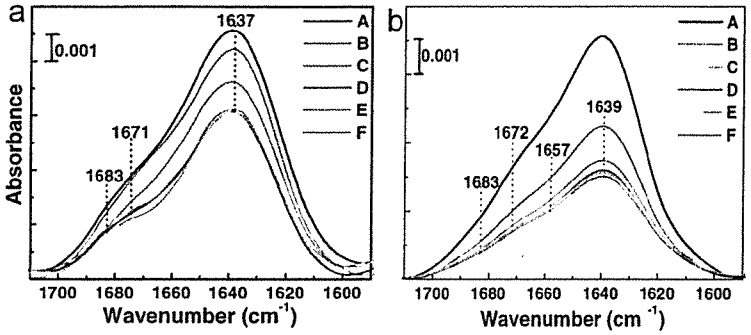

Fig. 3 The amide I bands of adsorbed proteins at solid-solution interface observed by the new NG-IRRAS.

a) Spectra of FN using pure D_2O as the solvent.

b) Spectra of IgG using NaCl (140 mM) added D_2O based PBS solution.

A) Spectra for protein injection and waiting 3 h (FN) and 30 min (IgG) for adsorption on solid surfaces,

Spectra were taken every after 0.5 ml D_2O solutions flushes; B) 0.50 ml C) 1.0 ml D) 1.5ml E) 2.0 ml, and F) 2.5 ml.

After the solid-solution interface IRRAS experiments, the same substrates were examined in the vacuum IRRAS. The amide I band shape was significantly different from that in the solution for both FN and IgG. This change of amide band is due to the denaturation of the proteins during the removal of water from the substrate surface.

As a whole, he has succeeded for the first time in constructing a new NG-IRRAS system having 8 μm gap to flow reagent solution for monitoring the chemical reaction. A specially designed sample holder is used to keep the gap constant during the injection of the reagent solution within a certain injection speed. Adsorption of proteins on the prism surface, which interferes with precise measurement, is suppressed using PEG-coating of the prism surface and controlling the solution pH and the effects of the salt. The amide I bands of the IgG molecules dissolved in the solution and covalently bonded to the COOH-terminated SAM surface at solid-solution interface have been clearly recorded for the first time. This new IRRAS instrument can be easily applicable in the characterization of different antibody-antigen reactions on IR-reflective metal surfaces in physiological condition.

論文の審査結果の要旨

生体材料の赤外分光では、その材料のもつ本来の機能を失わないために、水中の環境で計測することがしばしば必要となる。このような場合、これまで、ATR(Attenuated Total Reflection)法によって測定がなされて来たが、この方法は赤外光に不透明な材料表面に吸着した生体材料に適用することができない。本研究では、このような赤外光に不透明な基板表面に吸着した生体材料の水中での赤外反射吸収スペクトルを測定できる装置を、いくつかの困難な課題を克服して、はじめて製作に成功している。論文は第一章：序論、第二章：固体—液体界面の赤外反射吸収分光の原理、第三章：装置の構造と製作、第四章：材料および表面処理などの各種手法、第五章：ベースライン歪みの問題解決、第六章：タンパク質計測への応用、第七章：まとめ、から構成されている。

ATR 法では、たとえば、表面プラズモン共鳴(SPR)や水晶振動子共鳴(QCR)などの重要なバイオセンシング法について、その基板表面の生体材料の水中赤外吸収スペクトルをそのまま測定することはできない。これらのバイオチップの分野では、いずれも非特異吸着等の問題があり、吸着種の分子構造情報を簡便に得られる水中赤外吸収スペクトル測定に対するきわめて強い要求がある。本研究ではこのニーズに答えるため、CaF_2のプリズムとこれらのバイオチップの基板との間に 8μm のギャップをもうけ、ここに溶液をみたし、基板表面上の生体材料の反射赤外吸収を測定する新しい NG-IRRAS 装置の開発に初めて成功した。従来のこのタイプの装置はギャップが 1～2 μm と狭くギャップ内の溶液交換ができないため、バイオチップの性能評価に必要な表面での化学反応を見ることができないという致命的な問題があった。本研究ではこの技術的問題の解決に成功した。

NG-IRRAS 法の最大の技術課題はギャップの厚みの制御である。Si 基板表面の吸着水素の赤外吸収実測データを例にとると、単分子膜の吸光度は約 5×10^{-4}程度である。他方タンパク質等巨大分子では、この数値は 5×10^{-3}程度になる。すなわち、バイオチップの評価に利用する観点からは、$(0.5\sim1)\times10^{-3}$程度の感度が要求されるが、これはギャップが水で満たされている場合に測定中のギャップ厚みの変動をほぼ 4mm.以内におさえる必要があることを意味する。この要求の厳しさが、これまでこの装置が実用化されなかった原因である。Abu 氏は、試料ホルダーの構造に工夫をこらし、かつ丁寧に溶液注入速度依存性を測定し、ベースラインひずみを 1×10^{-3}以下に抑えることに成功した。さらに本装置をフィブロネクチンや IgG 抗体タンパク質の計測に応用し、水中での良好なスペクトルが測定できることなどを示し、また、表面を親水性にしておくと IgG 吸着時に構造変化を起こさないこと等も明らかにした。

以上いずれも独創性の高いアイデアと粘り強い実験により、技術的に困難とされていた固体—液体界面の生体材料についての赤外反射吸収スペクトル測定装置の開発に成功し、さらに、実際の固体界面に吸着した生体分子の赤外スペクトルの測定に応用しており、非常に優れた研究成果である。よって、審査委員会は本申請論文が博士（理学）の学位論文に十分値すると判断した。