称号及び氏名	博士（工学）　白井　亮洋
学位授与の日付	平成 31 年 3 月 31 日
論　文　名	「Single-Step Immunoassay Microdevices Based on Fluorescence Quenching Function of Graphene toward Point-of-Care Testing（臨床現場即時検査を指向したグラフェンの蛍光消光能に基づく 1 ステップ免疫測定マイクロデバイス）」
論文審査委員	主査　久本　秀明 副査　原田　敦史 副査　松岡　雅也

論文要旨

Point-of-Care Testing (POCT) is medical test and diagnosis conducted nearby patients by medical doctors, and it realizes to reduce testing time and to make patients feel close to the medical tests. Therefore, it contributes proper treatments, nursing, prevention from diseases, and health promotion. Finally, it realizes to improve the quality of life of patients. Recently, POCT has been paid attention due to the diversity of medical diagnosis, and various kinds of compact analyzers and rapid diagnosis kits have been developed to replace large size diagnostic devices in the central laboratories in hospitals. The diagnostic devices generally integrate immunoassay method that is based on specific interaction between antigen and antibody to measure the concentrations of specific disease-related proteins. However, conventional immunoassay has the problems related with the needs of step-by-step procedures, long analysis time, and the expensive reagents. There are two major trends for applying the immunoassay to the field of POCT: immunochromatography and fully automated immunoassay device. The advantages of the former are the visual analysis and mass-production, however it has low quantitativity, and causes false-negative and false-positive results. Therefore, the improvement of quantitativity is indispensable. The latter realizes the high usability and elimination of step-by-step procedures, however the usable place is limited because the connection to power supply is necessary.

The basic strategy for simplification of immunoassay is to eliminate the step-by-step procedures to separate free antibody and immunocomplex, and that for reduction of analysis time and reagent usage is to integrate immunoassay principle into microsystem. Recently, luminescent oxygen channeling immunoassay (LOCI) method was proposed to solve the problems listed above. LOCI method is conductable by mixing sample and reagent solutions in homogeneous system. Therefore,

the problem relating mainly to the step-by-step procedures was solved, however the unstable reagents and expensive equipment are required. Thus, it is not a solution from the viewpoint of application to POCT. As with the LOCI method, using the reagents that realize the homogeneous immunoassay principle is one of the solutions.

In this thesis, the author focused on graphene including its derivatives that is two-dimensional carbon nanomaterial possessing many unique properties. Among these, the author focused on the fluorescence quenching function of graphene and graphene oxide (GO) based on fluorescence resonance energy transfer (FRET). By integrating the homogeneous immunoassay principle using chemically modified and functionalized graphene or GO into microspace of capillary, development of the immunoassay microdevices measurable of the antigen concentrations just by introducing a sample solution by capillary action is possible. This minimal operation is defined herein as “single-step”, and single-step immunoassay microdevices are applicable to the field of POCT.

In the first chapter, the introduction and motivation of my study were summarized referring the previous related studies.

In the second chapter, the experimental results and discussion about integration of the conventional sandwich immunoassay using GO antibody conjugates and fluorescently labeled antibody into a capillary were summarized. It is difficult to apply GO to homogeneous reaction because GO is easily and irreversibly precipitated due to the π–π interaction between GO. Thus, in order to improve the dispersibility of GO, sulfonic acid group was introduced onto GO surface. Then, by activating carboxylic group on GO and covalently immobilizing with amino group of antibody, GO antibody conjugates was prepared. The reagents solution containing poly(ethylene glycol) (PEG) that is water-soluble polymer, GO antibody conjugates, and fluorescently labeled antibody was prepared. By introducing it into glass capillary, and then drying it, the proposed immunoassay microdevice was prepared. The coating containing reagents for immunoassay was uniformly and physically immobilized onto the inner wall of capillary. By introducing the diluted serum containing immunoglobulin G (IgG) as a model antigen into the proposed microdevice, and monitoring the fluorescence intensity, the measurement of IgG concentration in diluted serum was successful. The reaction time was approximately 40 sec and it was much shorter than that of conventional method using optical cell due to the reaction scale. These results indicated that by conducting homogeneous immunoassay in a microscale, the reaction time is expected to be much shorter and contribute the rapid diagnosis with single-step operation.

In the third chapter, the experimental results and discussion about the preparation of GO-containing hydrogel by encapsulating GO with poly(2-hydroxyethyl methacrylate) and application to immunoassay were summarized. The purpose of preparation of GO-containing hydrogel is to give the size separation function to GO. The fluorescence quenching rate in the case of the direct adsorption of fluorescently labeled antibody onto GO surface is expected to be higher than that of the method described in the previous chapter, that is based on the sandwich immunoassay, because the rate depends on the distance between fluorescence dye and graphene surface. By investigating the optimal amount of 2-hydroxyethyl methacrylate monomer to GO, the GO-containing hydrogel that can separate free antibody and immunocomplex based on size difference was successfully prepared. By combining GO-containing hydrogel and poly(dimethylsiloxane) (PDMS) microchannel with a soluble coating containing fluorescently labeled antibody, the proposed single-step immunoassay microdevice was prepared. In the case that the sample solution containing immunoglobulin M (IgM) as a model antigen was introduced into the microdevice, IgM concentration was successfully measured based on the fluorescence intensity change. Though it is difficult to precisely separate free antibody and immunocomplex due to the non-uniform hydrogel structure, and reaction time was long (approximately 20 min) due to the heterogeneous size separation process, the proposed immunoassay principle based on the fluorescence quenching and size separation functions of GO-containing hydrogel was novel, and GO-containing hydrogel is expected to be

inexpensive reagent for immunoassay.

In the fourth chapter, the experimental results and discussion about the development of a single-step competitive bioassay microdevice composed of two PDMS microchannel with soluble coating containing reagents to independently immobilize multiple reactable reagents in a same microchannel were summarized. As a model target molecule, biotin was chosen and GO biotin conjugate was prepared by covalently immobilizing biotin on GO via amino group of poly(ethyleneimine). The reagent solution containing GO biotin conjugates was introduced into PDMS microchannel, and then dried it to form the soluble coating. In the same manner, fluorescently labeled streptavidin was physically immobilized onto inner wall of another PDMS microchannel. By combining these two PDMS microchannels, the proposed competitive bioassay microdevice was prepared. In the case that the sample solution without biotin was introduced into the proposed microdevice, physically immobilized biotin and fluorescently labeled streptavidin were spontaneously diffused and reacted, then the fluorescence was quenched. On the other hand, in the case that the sample solution with biotin was introduced into the proposed microdevice, biotin and GO biotin conjugates competitively reacted with streptavidin and fluorescence intensity depending on biotin concentration was observed. Therefore, by monitoring fluorescence intensity, the biotin concentration was measured with single-step operation.

In the fifth chapter, the experimental results and discussion about the preparation of graphene/PEG hybrids (GPH) and application for immunoassay were summarized. The purpose to propose this nanomaterial is to solve the problem about long analysis time of GO-containing hydrogel sheet-based single-step immunoassay shown in the third chapter. By improving the form of the graphene-containing hydrogel from the sheet-like structure to the particle-like structure, that is dispersible enough to be applicable to a homogeneous immunoassay system, the problem of reaction time is expected to be solved. By encapsulating the graphene surface with PEG based on coacervation method, GPH was successfully prepared. By considering the ratio of graphene and PEG, the optimal condition to separate free antibody and immunocomplex was determined. By physically immobilizing GPH and fluorescently labeled antibody separately onto inner wall of a PDMS microchannel and combining those in the same manner shown in the fourth chapter, the proposed immunoassay microdevice was prepared. Just introduction of the sample solutions containing various concentrations of C-reactive protein (CRP) as a model antigen into the proposed microdevice allows spontaneous fluorescence change based on the size separation and direct adsorption of free fluorescently labeled antibody onto graphene surface. The increase of fluorescence intensity depending on the CRP concentration was observed, and based on these results, potential applicability of the newly prepared GPH to single-step immunoassay was successfully demonstrated.

In the sixth chapter, the results and findings obtained in this study were summarized.

The author worked on the development of single-step immunoassay microdevices from the viewpoint of both chemical modification of graphene or GO, which are applicable to immunoassay, and device preparation based on the modification of the capillary inner wall. Single-step immunoassay microdevice that is measurable of antigen concentrations just by sample introduction by capillary action was developed by physically immobilizing fluorescently labeled antibody and graphene antibody conjugate as a soluble coating onto inner wall of the capillary. Furthermore, by chemically modifying the graphene surface and encapsulating with polymer chain, the function to separate free antibody and immunocomplex based on the size difference was added to graphene, and it was shown as an inexpensive reagent that are applicable to single-step immunoassay. These results contribute to the improvement of the performance of the diagnostic equipment especially utilized in the field of acute care testing, such as disaster medical diagnosis and infectious disease diagnosis, in the field of POCT.

審査結果の要旨

本論文は、グラフェンあるいは酸化グラフェン（GO）の持つ蛍光消光機能とマイクロ流路を活用し、これまで簡便・迅速な分析が困難だった免疫測定法の問題を解決するデバイス開発を行った研究であり、以下の成果を得ている。

（1）免疫測定法の簡便・迅速化のために、毛細管内壁に抗体固定化グラフェンと蛍光標識抗体を物理吸着固定した免疫測定マイクロデバイスを開発した。これは毛細管現象のみの簡便な試料導入操作で、試料中抗原と毛細管内壁にあらかじめ固定した 2 種抗体が均一溶液系で迅速に反応・蛍光消光することを原理としており、このデバイスで簡便・迅速に抗原濃度定量できることを明らかにした。

（2）免疫測定法の簡便化のために GO 含有ハイドロゲルシートを開発した。これは GO の蛍光消光機能に加え、ハイドロゲル網目構造に基づくサイズ分離機能を有する新規材料で、プレポリマー溶液中モノマー量の検討で、未反応抗体と抗原抗体複合体をサイズ分離できることを明らかにした。また流路内壁に蛍光標識抗体を物理吸着固定したポリジメチルシロキサン(PDMS)製マイクロ流路とこれを組合せた免疫測定マイクロデバイスを作製し、免疫グロブリン M を、試料導入のみの簡便な操作で測定できることを明らかにした。

（3）互いに反応性を持つ 2 種の試薬を 2 つの PDMS マイクロ流路内壁に独立固定し、組合せた PDMS 製毛細管の作製と免疫測定応用の基礎検討を行った。蛍光標識ストレプトアビジンとビオチン標識 GO でビオチン検出を試みたところ、還元な蛍光検出に成功し、本デバイスが競合型免疫測定法にも応用できることを明らかにした。

（4）親水性ポリマーであるポリエチレングリコールで被覆したグラフェン粒子を作製し、その蛍光消光機能とサイズ分離機能に基づく簡便な免疫測定応用を達成した。これと蛍光標識抗体を独立固定した PDMS 型毛細管マイクロ流路を用い、炎症反応マーカータンパクである C-reactive protein の迅速(約 2 分)な検出を実現した。

以上の諸成果は、免疫診断の際に課題となっている操作の簡便化・分析時間の短縮を実現するために重要な知見を与えるとともに、分析デバイス開発についても有益な情報を提供したものであり、本分野の学術的・産業的な発展に貢献するところ大である。また、申請者が自立して研究活動を行うのに必要な能力と学識を有することを証したものである。
学位論文審査委員会は、本論文の審査ならびに学力確認試験の結果から、博士（工学）の学位を授与することを適当と認める。