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研究課題名（和文）疾患早期発見を目指す金ナノ粒子－表面増強ラマン散乱法による細胞内炎症反応の検出

研究課題名（英文）Early detection of disease using surface enhance Raman scattering (SERS)-dynamic gold nanoparticle to detect inflammatory response in cells

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研究成果の概要（和文）：疾患の初期段階の炎症反応を迅速に検出するために、金ナノ粒子による表面増強ラマン散乱（SERS）効果を用いた。ラマンプローブである4-メルカプト安息香酸を結合させた金ナノロッド（GNR）に、細胞間接着分子-1抗体（抗ICAM-1抗体）を結合させた。このナノ粒子により、リポ多糖を１時間処理したマクロファージにおいてICAM-1を検出することができ、ELISAや蛍光検出法と比べて短い処理時間での検出が可能であった。

研究成果の概要（英文）：The investigation of the use of surface enhanced Raman scattering (SERS) gold nanoparticles for rapid monitoring of inflammatory responses at the early stage of diseases. Gold nanorods (GNRs) combined with Raman probes (4-mercaptobenzoic acid) are conjugated with intercellular adhesion molecule-1 antibody (anti-ICAM-1). The prepared particles could be used as a SERS probe to detect the expression of ICAM-1 in macrophages treated lipopolysaccharide for 1 h which is faster than using ELISA or fluorescent labeling techniques.

研究分野：　総合領域

科研費の分科・細目：　人間医工学・医用生体工学・生体材料学

キーワード：　金ナノ粒子　SERS　炎症反応　ICAM-1

１．研究開始当初の背景

Excessive inflammation can cause many diseases such as atherosclerosis, rheumatoid arthritis, cancer, and Alzheimer`s diseases[1,2]. Especially in atherosclerosis, the inflammation is the key factor in causing this disease [3,4]. To prevent the occurrence of disease, early disease detection is required to find health problems before the symptoms of disease appear. In recent years, it has appeared that biomarkers play an important role for diagnosis of diseases. Inflammatory biomarkers in inflamed cells are also a key factor used as an effective signal to managing the risk of disease. In this proposal, the early symptom of atherosclerosis will be used as a study model. The inflammatory response in atherosclerosis is indicated by the high expression of inter cellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α)[5]. The increase of basal levels of these biomolecules leads to a risk of cardiovascular disease. Therefore, the ability to detect these inflammatory response molecules at different levels of concentration will show different bioactivities that will provide useful information for predicting cardiovascular events. Many approaches such as ultrasound, magnetic resonance imaging, electron-beam computed tomography, and fluorescence, have been used to detect inflammatory responses. However, it seems that these techniques are still not distinctively to detect the change of biomolecules/biochemical during atherogenesis and some techniques cannot detect the inflammatory response in the early stage due to a low concentration of inflammation biomarkers[6,7]. SERS spectroscopy provides a lot of advantages for many applications. Recently, SERS is becoming of high interest for biological applications for the following reasons: 1) providing a significant amount of information from biological samples such as cells, biological fluids, and living tissues, 2) relative ease in sample preparation compared to other non-invasive methods, and 3) offering high sensitivity for detection of changes in concentration, type, or structure of molecules. This high sensitivity of the Raman signal is enhanced by 13 to 15 orders after the targeted/probed molecules attach to metallic nanoparticles. In general, gold, silver, and copper are likely to be typical nanomaterials for SERS. However, in the term of biological/biomedical applications it seems that gold nanoparticle provides more benefits than other nanomaterials due to properties such as its strong optical extinction peak, easy attachment of organic molecules on its surface, and low cytotoxicity[8]. Many recent reports showed that gold nanoparticles have been extensively used as a substrate for SERS to study the interaction of drugs with proteins and diffusion[9,10], cancer detection in vitro[11]. However, these methods use Raman reporters such as fluorescent dyes. These chemicals may cause of cytotoxicity to living cells and may effect on biological activities of cells. The SERS-dynamic gold nanoparticle proposed here is an approach to detect inflammatory response in inflamed cells without using dyes or reporters. However, after trying this technique, it was not sensitive enough to distinguish different periods of cells stimulated by lipopolysaccharide. To solve this problem,

another technique was replaced. This technique used gold nanorods bound with 4-mercaptobenzoic acid (4MBA), wrapped with polymer, and then attached with antibody specified to interested inflamed molecules (ICAM-1). This technique will help detect disease in the early stage and then lead to effective treatment.

References

(1). Fernando, V. V. Inflammation in the molecular pathogenesis of cancer and atherosclerosis. *Reumatología Clínica* **5, Supplement 1**, 40-43, (2009).

(2). Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K. & Sethi, G.. Inflammation and cancer: How hot is the link? *Biochemical Pharmacology* **72**, 1605-1621, (2006).

(3). Hansson, G. K. Inflammation, atherosclerosis, and coronary artery disease. *New England ournal of Medicine* **352**, 1685-1695, (2005).

(4). Ross, R. Atherosclerosis - An inflammatory disease. *New England Journal of Medicine* **340**, 115-126, (1999).

(5). Collins, R. G.., Velji, R., Guevara, N. V., Hicks, M. J., Chan L., Beaudet, A. L. P-selectin or iIntercellular adhesion molecule (ICAM-1) deficiency substantially protects against atherosclerosis in apolipoprotein E–deficient mice. *The Journal of Experimental Medicine* **191**, 189-194, (2000).

(6). Fayad, Z. A., Fuster, V. Clinical Imaging of the high-risk orvulnerable atherosclerotic plaque. C*irculation Research* **89**, 305-316, (2001).

(7). Motz, J. T, Fitzmaurice M., Miller A., Gandhi S. J, Haka, A. S., Galindo, L. H., Dasari, R. R., Kramer, J. R., Feld, M. S. In vivo Raman spectral pathology of human atherosclerosis and vulnerable plaque. *Journal of Biomedical Optics* **11,** 021003, (2006).

(8). Pissuwan, D. Valenzuela, S. M., Corie, M. B. Prospects for gold nanorod particles in diagnostic and therapeutic applications. *Biotechnology and Genetic Engineering Reviews* **25**, 93-112 (2008).

(9). Farquharson, S., Shende, C., Sengupta, A., Huang, H., Inscore, F. Rapid detection and dentification of overdose drugs in saliva by surface-enhanced raman scattering using fused gold colloids. *Pharmaceutics* **3**, 425-439 (2011).

(10). Bálint, S., Rao S, Sánchez M. M, Huntosová V, Miskovský P, Petrov D. Diffusion and cellular uptake of drugs in live cells studied with surface-enhanced Raman scattering probes. *Journal of Biomedical Optics* **15,** 027005 (2009).

(11). Lee, S., Chon, H., Lee, M., Choo, J., Shin, S. Y., Lee, Y. H., Rhyu, I. J., Son, S. W., Oh, C. H. Surface-enhanced Raman scattering imaging of HER2 cancer markers overexpressed in single MCF7 cells using antibody conjugated hollow gold nanospheres. *Biosensors and Bioelectronics* **24**, 2260-2263 (2009).

２．研究の目的

The research proposed here was designed to use SERS gold nanorod measurement of the inflammatory markers by building a set of proposed approach to detect inflammatory response in inflamed cells. This technique will help detect disease in the early stage and then leading to effective treatment.

３．研究の方法

The well-known inflammatory agent, lipopolysaccharide (LPS), was used to activate the immune response in the Raw 264.7 cells because recent reports found that LPS can promote atherogenesis by activating leukocytes[1]. The intercellular adhesion molecule-1 (ICAM-1) was selected as a

targeted inflammatory response molecule because it is one of the specific proteins located on the surface of leukocytes and epithelial cells. The expression of ICAM-1 molecules is highly expressed after inflammation and ICAM-1 plays a major role in adhesion of inflamed/stimulated cells. This adhesion can lead to atherosclerosis plaque that can later cause of cardiac death. Therefore, the idea here is that the stimulated Raw 264.7 cells were used as an inflamed model cell. The anti ICAM-1 which is specific to ICAM-1 was used as a model probe for detection the expression of ICAM-1 on the surface of inflamed Raw 264.7 cells by using SERS-gold nanorods. The plan and methods to achieve the goal of this research are explained below:

Step 1. Preparation of gold nanorod probe and anti-ICAM-1 conjugates

The first plan was to use spherical shape gold nanoparticles to detect inflammatory response. However, after trying, it was not sensitive enough to distinguish the change of biomolecules (ICAM-1) on the cell surface after stimulation macrophages with LPS at different hours of stimulating period. Therefore, the preparation of gold nanorod (GNR) probe was used to complete the experiment. In this case, GNRs were mixed with 4-mercaptobenzoic acid (4MBA) and then coated with poly(allylamine hydrochloride)(PAH). Following this, the GNR probe was conjugated with anti-ICAM-1 antibody. The final complex was called as GNR/4MBA@anti-ICAM-1.

Step 2. Cell preparation and study of cell inflammation

Murine macrophage cells (Raw 264.7) were cultured. Lipopolysaccharides (LPS595 from Salmonella Minnesota R595) at various concentrations were used to treat the cells at different incubation times (0, 1, 3, and 5 h). Following this, cells were fixed with methanol before treating with GNR/4MBA@anti-ICAM-1.

Step 3. ICAM-1 analysis

The ICAM-1 expression of Raw 264.7 cells was detetced using mouse soluble ICAM-1 (CD54) ELISA kit and fluorescent dyes to compare with proposed SERS technique.

Step 4. Investigation of gold nanorod probe-anti-ICAM-1 reacted with ICAM-1

Before starting SERS investigation in inflamed cells, the SERS spectra of GNR probe-anti-ICAM-1 reacted with ICAM1 was investigated. The laser excitation at the wavelength of 785 nm was used.

Step 5. Cellular immune response SERS investigation

Fix Raw 264.7 cells, with and without treatment with LPS, was incubated with GNR probe-anti-ICAM-1 conjugates. The cells were washed after incubation. The SERS signal was detected using Raman spectroscopy.

Step 6. Extra experiments

Since GNRs were used as a tool to detect inflamed cells, for the next step of the future work by using GNRs in live cells or in vivo, the investigation of the effect of GNRs on inflammatory response is important. Therefore, the effect of GNRs on inflammatory response in macrophage cells was investigated. Another experiment of the investigation of intracellular SERS signals of different surface modified GNRs

was also performed.

Step 7. Consolidation

Findings of this proposed research will be published in ISI journals.

Reference

(1). Takeshi, T., Keisuke, N., Takaaki, I., Makoto, Y., Tatsuji, N. Involvement of adhesion molecule in in vitro plaque-like formation of macrophages stimulated with Aggregatibacter actinomycetemcomitans lipopolysaccharide. *Journal of Periodontal Research* **45**, 550-556 (2010).

４．研究成果

GNR probe conjugated with anti-ICAM-1 was successfully used as a tool to detect the inflamed cells at the early stage under Raman spectroscopy. The detected SERS signals showed that the spectrum (at peak position of ~1075 cm^{-1}) of SERS probe detected in RAW 264.7 cells treated with LPS for 1 h was significantly enhanced comparing with the SERS intensity of cells treated with LPS for 3 and 5 h (Tukey-Kramer`s test with significant set at $P < 0.05$.). This means that the detection of ICAM-1 molecules could be detected after macrophages cells were stimulated only for 1 h. The comparison of this SERS GNR probe technique with fluorescent dye and ELISA technique shows that the expression of ICAM-1 molecules could be detected after cells were treated with LPS for 5 h by using ELISA and fluorescent dye techniques. These results show that the sensitivity of ICAM-1 detection by using fluorescent dye and ELISA is lower than using SERS GNR probe. Therefore, the prepared SERS probe GNRs can be used as a tool for detecting the biomolecular change in cells. This probe could be developed further to diagnose diseases at the early stage, which will help improve the treatment of diseases.

The investigation of the effect of GNRs coated with different surfaces also shows that the concentration of three different surfaced modified GNRs (coating with polyethylene glycol, polystyrene sulfonate, and poly(diallyldimethylammonium chloride)) at 5 ug mL^{-1} has no effect on induction of inflammatory cytokines. The results of using GNRs for intracellular SERS signals show that GNRs coated with poly(diallydimethylammonium chloride) (PDAC-GNRs) can distinct groups of surface-enhanced Raman scattering (SERS) spectra in different regions of the cells. Spectra with unique features could be clustered into sets of molecules in Raw 264.7 cells. This technique is useful for studying the analytes in interested live cells. The results of these two studies were published in ISI journals.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 2 件）

1.Pissuwan, D., Hobro, A., Pavillon, N., Smith, N. Distribution of label free cationic polymer-coated gold nanorods in live macrophage cells reveals formation of groups of intracellular SERS signals of probe nanoparticles, *RSC Advances*, 2014, Vol.4, pp.5536-5541 *corresponding author*

2. Pissuwan, D., Kumagai, Y., Smith, N. Effect of surface-modified gold nanorods on the inflammatory cytokine response in macrophage cells, *Particle and Particle*

System Characterization, 2013, Vol. 30, pp. 427-433 *corresponding author*

〔学会発表〕（計　3　件）

(1). Pissuwan, D., Hobro, A., Pavillon, N., Smith, N. The uptake and intracellular signal monitoring in macrophage cells of different surface modified gold nanorods using surface enhanced Raman scattering spectroscopy, 7th International Conference on Advanced Vibrational Spectroscopy (ICAVS), August 25-30, 2013, Kobe, Japan

(2). Pissuwan, D., Smith, N., Gold nanorods: Tool to detect early stage inflammation and their surface effects on inflammatory response in macrophage cells, The 4th International Nanomedicine Conference, July 1-3, 2013, Sydney, Australia

(3). Pissuwan, D., Cortie, M., Niidome, T., Smith, N. Intersection of light and gold nanoparticles for biomedical applications, Photonics Global Conference, December 13-16, 2012, Singapore (Invited speaker)

〔その他〕
ホームページ等
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