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科学研究費助成事業　　研究成果報告書

令和　４ 年　 ６ 月　 ９ 日現在

機関番号：１３３０１
研究種目：基盤研究(C)（一般）
研究期間：2019～2021
課題番号：１９Ｋ０５２２８

研究課題名（和文）Nanometer scale resolution imaging of pH and reactive oxygen species on the outer membrane surface for fast and effective cell discrimination in cancer tissues.

研究課題名（英文）Nanometer scale resolution imaging of pH and reactive oxygen species on the outer membrane surface for fast and effective cell discrimination in cancer tissues.

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：細胞表面の活性酸素種であるH2O2を定量化するためのナノセンサーを開発に成功した。ナノセンサーとして、バイオコンジュゲート金ナノ粒子を用い、表面増強ラマン分光法（SERS）による細胞表面でのH2O2の定量化を可能とした。肺癌細胞株を用いてH2O2の定量に成功し、さらに、以前開発した細胞表面のpHイメージングによる測定をより広域での測定を可能とするため、レーザーライン励起モードにて、細胞表面の広領域から400本のSERSスペクトルを同時に解析可能とした。正常細胞株（ヒトおよびマウス由来線維芽細胞株、）および、がん細胞株（肺がん３株、膵癌３株）を網羅的に解析し、測定可能であることを実証した。

研究成果の学術的意義や社会的意義
Quantification of pH and hydrogen peroxide on the cell surface is critically important to study many cellular functions and can be exploited to discriminate cancer cells from normal cells in tissues. The nanosensors developed in our research project showed great potential to accomplish these goals.

研究成果の概要（英文）：We developed and tested a nano-sensor for quantification of hydrogen peroxide (H2O2) on the cell surface. The nanosensor is based on bioconjugated gold nanoparticles that can be anchored to the extracellular cell surface and can quantify H2O2 using surface enhanced Raman spectroscopy (SERS). We successfully tested the new nanosensor on adenocarcinomic human alveolar basal epithelial cells (A59 cell line) for highly localized quantitative measurements of H2O2. In addition, we optimized the use of our previously developed pH nanosensor for cell surface pH imaging. Using a laser line illumination mode, we simultaneously acquired 400 SERS spectra from a wide area of the cell sample. We analyzed different cell lines: pancreatic cancer cell BxPC; pancreatic cancer cells AsPC-1; murine fibroblast cells NIH/3T3; human fibroblast WI-38; lung cancer A549.

研究分野：Biophysics

キーワード：Raman spectroscopy　Nanosensors　pH　Hydrogen peroxide　cell surface

１．研究開発当初の背景 (Introduction)

The subtle mutation of the biological environment in close proximity to the cell surface play critical roles in the physiological function of cells. In particular, variations of pH and hydrogen peroxide (H_2O_2) are pivotal for the modulation of various cellular activities including proliferation, signal transduction, and apoptosis. At present, experimental techniques for reliable and accurate quantification of endogenous extracellular pH and H_2O_2 are still a work in progress. Raman spectroscopy is a non-destructive technique that is suitable to unravel the presence of biomolecules in liquid environment. Nonetheless, the use of conventional Raman spectroscopy is not feasible in physiology to quantify meaningful molecular concentrations in the micro/nanomolar range, since the Raman scattering is weak and undetectable at those low levels of concentration. Nanoparticles made of noble metals such as gold (AuNP) can localize plasmon polaritons on their surface once illuminated with a laser beam of a suitable wavelength in resonance with surface plasmons. The highly localized exponential increase of the electromagnetic field can be exploited to detect the Raman scattering of molecules located in proximity of the metal surface even at concentrations as low as picomoles. This experimental approach to collect Raman spectra is known as surface enhanced Raman spectroscopy (SERS) and it can be exploited to develop highly-sensitive nanosensors for biological applications.

２．研究の目的 (Aim of research)

This research project was aimed at developing new methods to measure pH and H_2O_2 on the surface of cell samples using SERS. We aimed at creating new nanosensors based on bioconjugated gold nanoparticles that can be anchored to the extracellular surface of any type of cell. The surface of the nanostructures is also functionalized with specific molecules that are sensitive to variations of proton concentration (i.e., pH) or H_2O_2 concentration. The concentration of the sensing molecule can be detected and measured by SERS. As far as H_2O_2 is concerned, although it can act as a 'secondary messenger', relaying or amplifying certain signals between cells, it is generally toxic because of its oxidant character. The latter means that it converts (oxidizes) biochemical molecules like proteins and DNA. The oxidizing property of H_2O_2 is of potential therapeutic relevance for cancer, though, deliberately causing tumor cells to increase their H_2O_2 concentration would be a way to destroy them. In light of this, but also for monitoring pathologies associated with H_2O_2 overproduction, we aimed at reliably quantify concentrations of endogenous hydrogen peroxide in the extracellular environment for the first time. In addition, we aimed at improving the method for surface pH imaging in a wide area of the cell sample, which can be instrumental to discriminate normal cells from cancer cells in cell tissues. In fact, the acidity of the cell surface is different from normal to malignant cells.

３．研究の方法 (Experimental methods)

Taking advantage of the ability of gold to readily conjugate with different thiol-containing molecular compounds, we have the opportunity to design various gold nanosensors capable of targeting specific locations of the cell and measuring the concentration of sensing molecules that are selectively correlated to the concentration of the target analyte. We achieved AuNP anchoring to the plasma membrane using two molecular compounds containing biotin: a pyridyldithiol-biotin compound (EZ-link-HPDP-biotin), which conjugates to AuNP via thiol-gold interaction, and a sulfo-NHS-ester-biotin compound (EZ-link-NHS-biotin) that reacts with the primary amines of lysine and the amino-termini of membrane proteins.

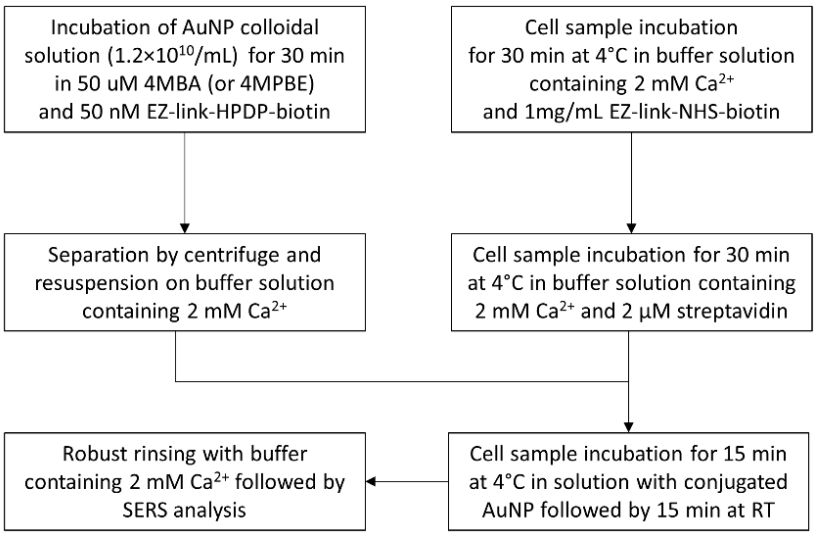

Figure 1. Flowchart describing the experimental protocol.

The addition of streptavidin provides the strong and selective bond between biotinylated AuNP and membrane proteins. The quantification of pH or H_2O_2 is achieved by the SERS analysis of molecular compounds that are also assembled on the gold surface and are selectively modified by the target analyte. These compounds should possess a large Raman scattering cross-section and include a thiol group to form the self-assembled monolayer (SAM) on AuNP surface. Figure 1 summarizes the details of the experimental protocol with the preliminary preparation of the conjugated AuNP and the three consecutive steps of incubation of the cell sample for AuNP anchoring. 4-mercaptobenzoic acid (4MBA) or 4-mercaptophenylborinic acid pinacol ester (4MPBE) are used as sensing compounds for measurement of pH or H_2O_2 on the cell surface, respectively. In our SERs experiments, a 671 nm laser excitation source was used to illuminate la cell surface and acquire maps of SERS spectra by a Raman microscope (Raman-11, Nanophoton, Osaka, Japan). Typical laser power and acquisition time for each spectrum were set to 2 mW and 5 s, respectively.

4．研究成果 (Results)

Measurement of cell surface pH using 4MBA conjugated AuNP and Raman spectroscopy

In Fig. 2 are reported representative results of SERS experiments for assessment of cell surface pH using the newly developed nanosensor. Upon acidification of the liquid environment, the SERS spectrum of the sensing compound (i.e., 4MBA) shows clear decrease of the band intensities at 1390 and 1415 cm^{-1}, which are assigned to COO- stretching (see Fig. 2 (a)). As shown in Fig. 2 (b), the sum of these two bands can be normalized by the intensity of the v12 ring vibration at 1068 cm^{-1} and the ratio can be used to calibrate the nanosensor response as a function of pH. SERS hyperspectral maps of pH collected from A549 lung cancer cells and human keratinocytes (HKC) are shown in Fig. 2 (c). Figure 2 (d) compares the mean pH values of the two investigated cases (n=3 cells). All the experiments were carried out in buffer solutions at pH 7.4. According to our findings, the mean extracellular surface pH in A549 cells is 6.6 ± 0.06. These results denote highly localized variations of proton concentration on the outer surface of A549 cells. The increase of [H+] detected in cancer cells is correlated to upregulations of V-type H+-ATPase, monocarboxylate transporters, Na^+/H^+ exchanger and carbonic anhydrases, which are a peculiarity of most cancer histotypes. Interestingly, also in normal cells like HKC we detected some acidification of the extracellular milieu at nanometer distance from the outer surface (mean pH = 7.2 ± 0.04).

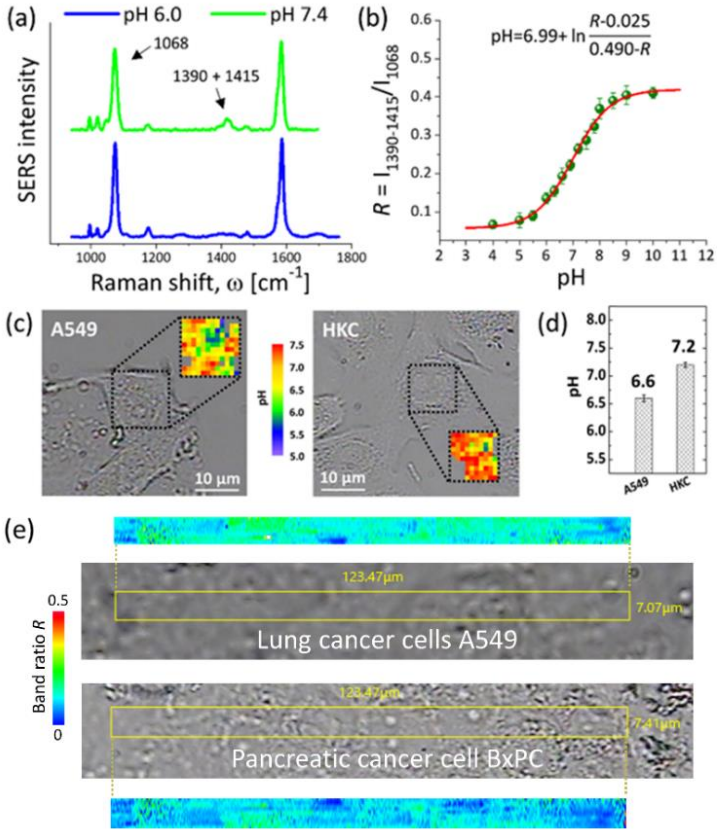

Figure 2. (a) 4MBA SERS spectra at different pH. (b) Calibration curve of SERS band ratio R as a function of pH (adapted from [4]). (c) SERS hyperspectral maps of surface pH collected from A549 and HKC cells. (d) Mean surface pH measured from the 2 different cell lines. (e) Maps of band ratio *R* collected from full confluence samples of lung and pancreatic cells using line illumination.

Measurement of H_2O_2 using 4MPBE conjugated AuNP and Raman spectroscopy

We created a high spatial resolution nanosensor for measuring concentrations of extracellular and intracellular H_2O_2 in proximity of the cell membrane using surface enhanced Raman spectroscopy (SERS). The method was tested in human lung adenocarcinoma A549 cells. The use of H_2O_2-responsive gold nanoparticles aimed at measuring cell

surface H_2O_2 is unprecedented. In fact, previous studies reported only measurements in the intracellular space after non-specific endocytosis of nanoparticles. The second peculiarity of this study is the analytical approach to estimate also the correlated concentrations of endogenous intracellular H_2O_2. This newly designed method combines the experimental outcomes of the SERS analysis with those of advanced methods of redox biology for calculation of H_2O_2 gradients across cell membranes. Although several calculated values of endogenous extracellular H_2O_2 are in the submicromolar range, the proposed method of analysis clearly reveals localized spots on the cell surface at higher concentration, up to 12 µM. The mean concentration of intracellular H_2O_2 is estimated as 0.7 ± 0.2 nM, with peaks of 5.1 ± 1 nM. In Fig. 3 are shown representative results of H_2O_2 quantification on the cell surface. The H_2O_2–sensitive Raman band at 998 cm-1 normalized by the ring stretching at 1074 cm-1 can be used to calibrate the SERS response of the nanosensor to different concentration of H_2O_2 (see Figs. 3 (a) and (b)). In Fig. 3 (c) is reported an example of SERS hyperspectral map of surface H_2O_2 collected from an A549 cell. Upregulation of H_2O_2 production in some locations of the surface are correlated to the activity of NADPH oxidases and superoxide dismutase, which stimulate cell proliferation and preservation of the transformed state in cancer cells. Overall, these results demonstrate that the nanosensor anchored to the outer cell membrane can be used as a simple and viable tool to investigate and unfold dynamics of H_2O_2 production in living cells. In addition, we showed that the quantification of extracellular H_2O_2 ($[H_2O_2]_e$) by SERS enables us to estimate also the correlated concentration of intracellular hydrogen peroxide ($[H_2O_2]_i$) using the typical transmembrane gradient obtained with advanced methods of redox biology. As illustrated in the schematic of Figure 3 (a), the activity of basal H_2O_2 in the proximity of the plasma membrane can be estimated using a kinetic model including the absorption of endogenously produced extracellular H_2O_2 and the reaction of scavenging by the predominant cytoplasmic antioxidant (i.e., peroxiredoxin in our case). At steady-state conditions, the rate of adsorption equals the rate of consumption by peroxiredoxin. When the cells are exposed to extracellular H_2O_2 in the micromolar range, the $[H_2O_2]_i$ is much lower than $[H_2O_2]_e$ and the most of the antioxidant pool is in the reduced form. Under these assumptions, both reactions of the model can be approximated to two pseudo-first order reactions (Huang and Sikes, Redox biology 2, 955-962, 2014) and the gradient of H_2O_2 across the plasma membrane, $R = [H_2O_2]_e/[H_2O_2]_i$, can be expressed as the ratio between the two rate constants K_{prx} and K_{abs}. We determined the two kinetic rate constants by in-vitro experiments on A549 cell samples according to protocols reported in (Antunes and Cadenas Anticancer research 32(7), 2599-2624, 2000; Huang and Sikes, Redox biology 2, 955-962, 2014). Figure 4 (b) shows the trend of adorption of exogenous H_2O_2 added to intact A549 cells (i.e., initial concentration of 100 µM in buffer solution). The time-dependent concentration of extracellular H_2O_2 was measured monitoring the absorbance of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS). The slope of the natural logarithm of H_2O_2 versus time represents K_{abs} and it is estimated equal to 4.5 × 10-12 s^{-1} $cell^{-1}$ L. In Figure 4 (c) is reported a typical outcome of experiments designed for estimating the kinetic reaction between H2O2 and the intracellular peroxiredoxins of A549 cells. Cell

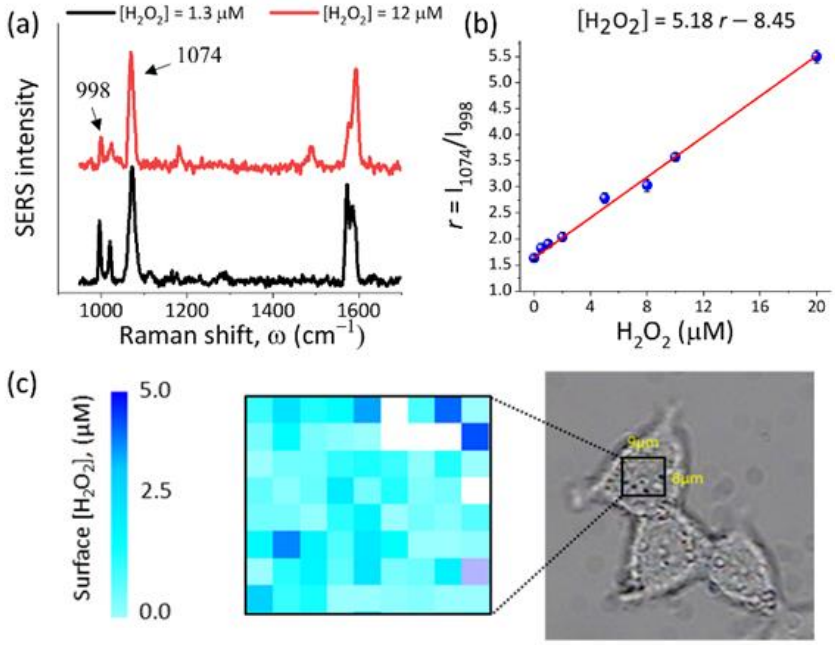

Figure 3. (a) 4MPBE SERS spectra at two different concentrations of H_2O_2. (b) Calibration curve of SERS band ratio r as a function of H_2O_2 concentration. (c) Example of SERS hyperspectral map of concentration of extracellular surface H_2O_2 collected from an A549 cell.

lysates treated with excess of thioredoxin, thioredoxin reductase and NADPH were analyzed after addition of a bolus of H_2O_2 producing an initial concentration of 20 µM. The consumption of H_2O_2 by peroxiredoxins is equivalent to the depletion of NADPH, which can be monitored by measuring the variation of absorbance at 340 nm. The pseudo first-order kinetic rate constant K_{prx} represents the average concentration of the peroxiredoxins pool in the A549 cell lysate multiplied by the typical second-order rate constant of the reaction between the antioxidant and H_2O_2. It was determined by fitting the experimental data with a computational routine adapted from the method introduced in (Huang and Sikes, Redox biology 2, 955-962, 2014). We found a K_{prx} of 1.1 ± 0.06 × 10-8 s-1 cell^{-1} L. Based on these results, we quantified R = 2444. The $[H_2O_2]_e$ obtained by SERS analysis divided by R estimates the respective $[H_2O_2]_i$ beyond the plasma membrane. According to our analysis of n=3 A549 cells, the mean $[H_2O_2]_i$ is 0.7 ± 0.2 nM and the peak of $[H_2O_2]_i$ is 5.1 ± 1 nM. Results are summarized in Fig. 4 (d).

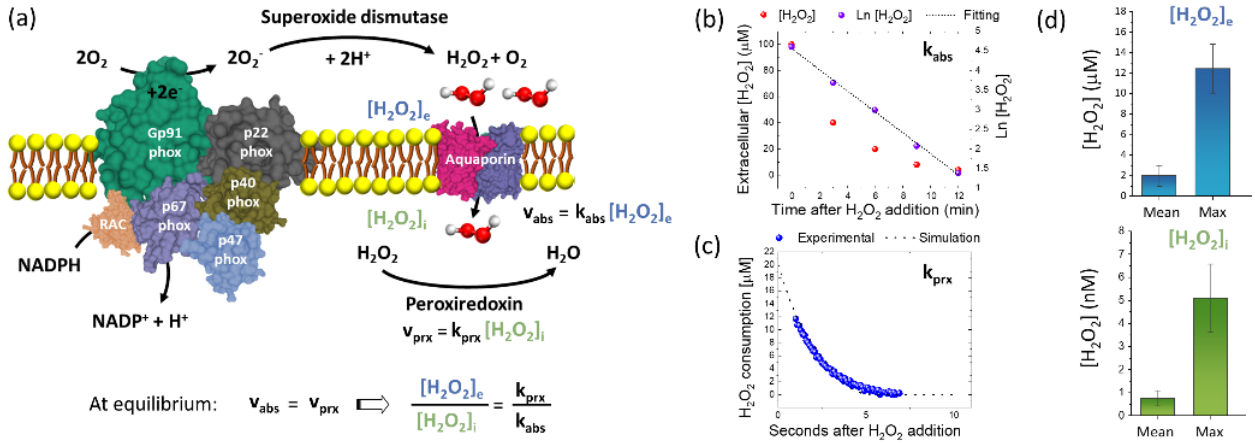

Figure 4. a) Explanatory schematic of the extracellular production of H_2O_2 by NOX complex, the absorption through Aquaporin channels and the reaction with intracellular peroxiredoxin. At steady-state condition, the ratio between extracellular and intracellular concentrations of H_2O_2 can be estimated by the ratio between the rate constants k_{prx} and k_{abs}. b) Absorption of exogenous H_2O_2 added to intact A549 cells as measured by ABTS based assay. c) Typical results of experiments on A549 lysates to measure k_{prx}.. The fitting line was calculated with a numerical simulation based on the system of differential equations reported in (Huang and Sikes, Redox biology 2, 955-962, 2014). d) Results of extracellular and intracellular concentrations of H_2O_2 quantified using the newly proposed analytical method for A549 cells.

5．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　0件／うち国際共著　0件／うちオープンアクセス　0件）

1．著者名 Hosogi Shigekuni、Marunaka Yoshinori、Ashihara Eishi、Yamada Tadaaki、Sumino Ayumi、Tanaka Hideo、Puppulin Leonardo	4．巻 179
2．論文標題 Plasma membrane anchored nanosensor for quantifying endogenous production of H2O2 in living cells	5．発行年 2021年
3．雑誌名 Biosensors and Bioelectronics	6．最初と最後の頁 113077～113077
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.bios.2021.113077	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計1件（うち招待講演　1件／うち国際学会　0件）

1．発表者名 Puppulin Leonardo
2．発表標題 Surface enhanced Raman spectroscopy probe for nanometer-scale measurement of pH and hydrogen peroxides on the outer membrane of cells
3．学会等名 The 97th Annual Meeting of the Physiological Society of Japan（招待講演）
4．発表年 2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

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7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関