様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）光学的方法を用いた組織間質液イオン環境の非侵襲的測定法開発

研究課題名（英文）Development of new methods for measuring ion concentrations in interstitial fluid using optical spectroscopy

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研究成果の概要（和文）：ラマン分光を用いた新規のpH（酸性度）計測技術を開発し、癌細胞外近傍の微小局所領域約20ナノメートル（１ナノメートルは１メートルの10億分の1の大きさで、20ナノメートルは細胞の大きさの約１千分の1の大きさである）での酸性度合を測定することに成功しました。その結果、癌細胞近傍の超微小局所領域が極度の酸性環境になっていることを明らかにしました。この癌細胞周囲超微小局所環境の極度の酸性環境であることが明らかになったことは、今後癌治療にも応用される可能性も秘めています。本研究成果をもとに、今後は各種癌細胞周囲局所で酸性度合いを検証し、癌転移予防法の新規開発も期待されます。

研究成果の学術的意義や社会的意義
Dysregulated pH is a common characteristic of cancer cells compared with normal cells. The precise measurement of extracellular pH on the cell membrane using Raman spectroscopy can be used in the future to characterize primary and metastatic cancer cell populations and minimal residual disease.

研究成果の概要（英文）：We exploited the remarkable optical properties of gold nanoparticles and their ability to conjugate with different thiol-containing molecular compounds to develop a method for highly localized pH bio-sensing using Raman spectroscopy. The strategy for gold nanoparticles conjugation was specifically designed to efficiently target the cell membrane proteins and to quantify the local pH by collecting the Raman scattering of the 4-MBA monolayer assembled on the gold surface. Experiments on HepG2 human liver cancer cells and MKN28 gastric cancer cells proved the successful anchoring of the gold nanoparticles to the outer cell membrane and showed substantial acidification of the extracellular surface pH. The proposed method of analysis can be used as a simple and viable tool to investigate and unfold the dynamics of proton exchange in cells, even after exposure to different pharmacological treatments or different physiological conditions.

研究分野：Cell physiology

キーワード：Raman spectroscopy　pH　interstitial fluid　gold nanoparticles

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９、ＣＫ－１９（共通）

１．研究開始当初の背景

Regulation of intracellular pH is critically important for many cellular functions. The quantification of proton extrusion in different physiological conditions is pivotal to fully elucidate the mechanism of pH homeostasis in living cells. Gold nanoparticles (AuNP) can be exploited to create a high spatial resolution sensor for measuring extracellular pH in proximity of the membrane proteins of different types of cells. The AuNP surface can be specifically functionalized with thiol-containing molecules that enable to target the outer membrane proteins of the cells and to measure pH using surface enhanced Raman spectroscopy (SERS).

２．研究の目的

The research project was aimed at developing new methods to measure ion concentrations in living cells using non-destructive techniques such as Raman spectroscopy. The main efforts were made on measuring highly localized values of pH (i.e., concentration of H+) on the outer membrane surface of cancer cells. We studied MKN28 gastric cancer cells with and without inhibition of Na+-H+ exchanger (NHE) by ethyl-isopropyl amiloride (EIPA), HepG2 human liver cancer cells, A549 adenocarcinomic human alveolar basal epithelial cells and human epidermal keratinocyte (HEK).

３．研究の方法

Cell surface labelling with pH-sensitive gold nanoparticles

The detailed explanation of the method is reported in our recently published paper “Bioconjugation strategy for cell surface labelling with gold nanostructures designed for highly localized pH measurement” Nature Communications, 9, 5278 (2018). In Fig. 1 are reported an explanatory sketch of the pH nanosensor and an example of transmission electron microscope (TEM) image confirming the anchoring of AuNP to the outer cell surface.

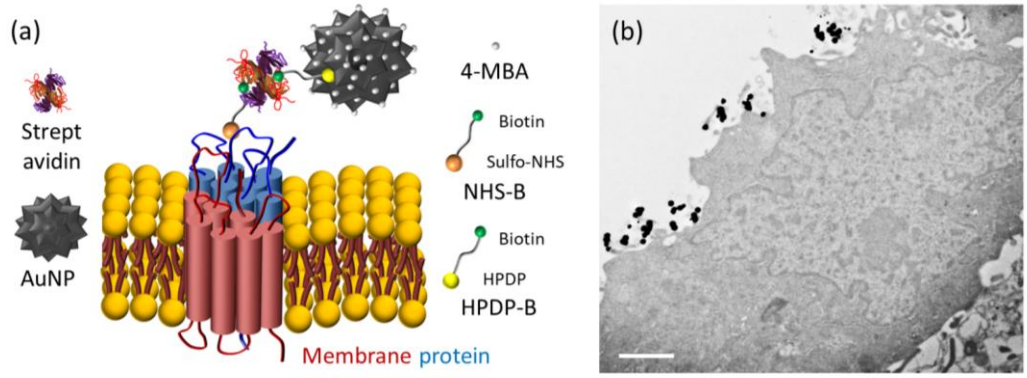

Figure 1: (a) Explanation of the bioconjugation strategy for cell surface labelling using AuNP. (b) TEM image collected from MKN28 gastric cancer cells after attachment of AuNP (scale bar 1 μm). The cells were fixed with glutaraldehyde/paraformaldehyde in 0.1 M PBS and sliced using an ultra-microtome 1 hour after the completion of AuNP treatment, in order to confirm also the lack of endocytosis during the typical time of Raman experiments.

The pH-sensitive AuNP were prepared adding 4-MBA and a pyridyldithiol-biotin compound (HPDP-B) to colloidal solutions of 90 nm gold nano-urchins (Sigma Aldrich, St. Louis, Missouri, USA), which conjugated via thiol-gold interaction. Cells were seeded on glass bottom dishes and treated for 30 min at 4℃ with EZ-link Sulfo-NHS-SS-biotin (NHS-B), which is a surface protein biotinylation reagent. After rinsing with Ca2+ containing ice-cold PBS (PBS-2Ca), we treated the cells with streptavidin in PBS-2Ca at pH 7.4 for 30 min at 4℃. Following rinsing in PBS-2Ca, the cells were incubated with conjugated AuNP solution for 15 min at 4℃ followed by 15 min at 21℃. After robust washing by PBS-2Ca, Raman analyses were carried out in buffer solution containing 115 mM NaCl, 15 mM NaNO3, 5 mM KCl, 1 mM Ca(NO3)2, 1 mM Mg(NO3)2, 10 mM HEPES, 10 mM glucose, 10 mM sucrose and adjusted to pH 7.4. Further validation of the protocol was provided by MTT cell viability assay, which was performed 3 hours after AuNP anchoring. The results are shown in Fig. 2 (a) for gastric MKN28 and liver HepG2 cancer cell lines, respectively. As compared to their controls, MKN28 and HepG2 labelled with AuNP did not show statistically significant difference in cell viability (two-tailed unpaired t-test, n = 3, p = 0.803 and 0.533, respectively). In addition, staining by fluorescent propidium iodide (PI) red dye, which is only permeant to dead cells, provided further evidence regarding the lack of cell

apoptosis induced by the treatment with AuNP, as shown in Fig. 2 (b) and (c).

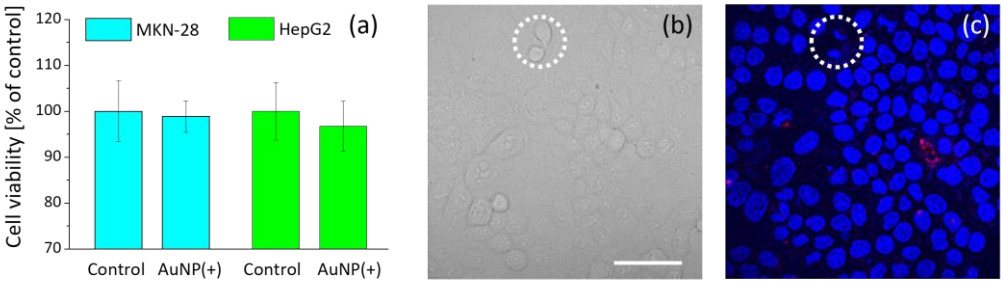

Figure 2: (a) MTT assay results reported as percentage of living cells with respect to the average of the control samples. (b) Bright-field and (c) Hoechst nuclei dye (blue) – PI dye (red) merged fluorescence images collected from the same location of an AuNP treated MKN28 sample, respectively. In addition, the region inside the dotted white circles are shown to emphasize the occurrence of nuclei fragmentation without detection of red PI fluorescence, which indicates cell mitosis and it represents an additional proof of normal physiological activity of the cells (scale bar 50 μm).

Measurement of pH using 4-MBA conjugated AuNP and Raman spectroscopy

In the Raman spectrum of 4-MBA, the intensity of -COO- symmetric stretching at 1395-1414 cm-1 is pH-dependent, as shown in Fig. 3 (a)-(b). Conventional Raman scattering is very weak and undectabled at sub-micromolar concentrations, which are the object of investigation in physiology. Nonetheless, using AuNP we can exponentially enhance the Raman scattering of the 4-MBA molecules attached to the gold surface, since AuNP can localize plasmon polaritons in their close vicinities once illuminated by the laser beam. Using the signal enhancement given by AuNP, the pH probe size is in the range of few nm. In fact, only the nanometer thick self-assembled monolayer of 4-MBA on the gold surface contributes to the collected Raman intensity, as shown in Fig. 3 (d). This advanced technique is called surface enhanced Raman spectroscopy (SERS). We exploited SERS to measure pH after anchoring 4-MBA conjugated AuNP on the outer cell membrane.

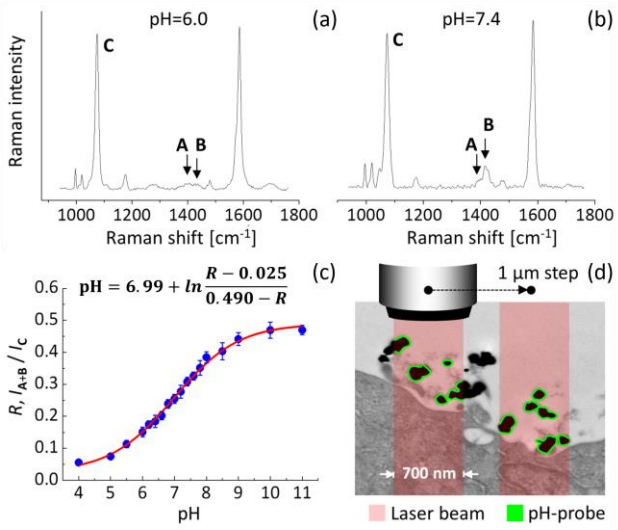

Figure 3: In (a) and (b) are reported two typical 4-MBA spectra collected from AuNP in buffer solutions at pH 6.0 and 7.4. The intensity of bands A+B increases at alkaline pH and it can be normalized with the intensity of band C. (c) Experimental curve of the intensity ratio R = IA+B / IC as a function of pH. The SERS measurements were collected from AuNP suspended in cell buffer solutions at different pH. The best-fitting equation in the inset can be used to calculate pH using SERS. (d) The pH-sensitive 4-MBA monolayer of each AuNP anchored to the cell can be probed when illuminated by the laser beam (SERS active layer depicted in green). The lines does not represent the real thickness of the layer, which can be assumed as 0.78 nm thick.

4. 研究成果

Examples of SERS hyperspectral maps of pH collected from different types of cells after AuNP anchoring are shown in Figs. 4 (a)-(e). In Fig. 4 (f) we compared the mean values of pH calculated from 3 cells for each of the 5 investigated cases. According to our findings, the mean surface interstitial pH in MKN28 cells was 6.2 ± 0.2 (n = 3 cells); the most acidic and alkaline values were estimated to 5.0 and 7.4, respectively. The mean standard deviation of pH in a single cell was 0.4, which denotes highly localized variations of proton concentration on the outer cell surface. The increase of [H+] detected in cancer cells was presumably correlated to upregulation of V-type H+-ATPase, monocarboxylate transporters (MCTs), NHE and carbonic anhydrases (CAs), which is a peculiarity of most cancer histotypes. In order to confirm this hypothesis and to test the sensitivity of this method, we also carried out experiments on MKN28 cells treated with EIPA, an inhibitor of NHE. The mean surface interstitial pH on EIPA-treated MKN28 increased to 6.7 ± 0.04 (n = 3 cells). In some locations, we still detected pH lower

than 6.5, which may be correlated to the higher activity of V-type H+-ATPase and MCTs. In the case of HepG2, A549 and HKC cells, the average pH was 6.7 ± 0.1, 6.6 ± 0.06 and 7.2 ± 0.04, respectively (n = 3). All the experiments were carried out in buffer solutions at pH 7.4, namely also in normal cells, such as HKC, we detected acidification of the extracellular milieu at nanometer distance from the outer cell surface.

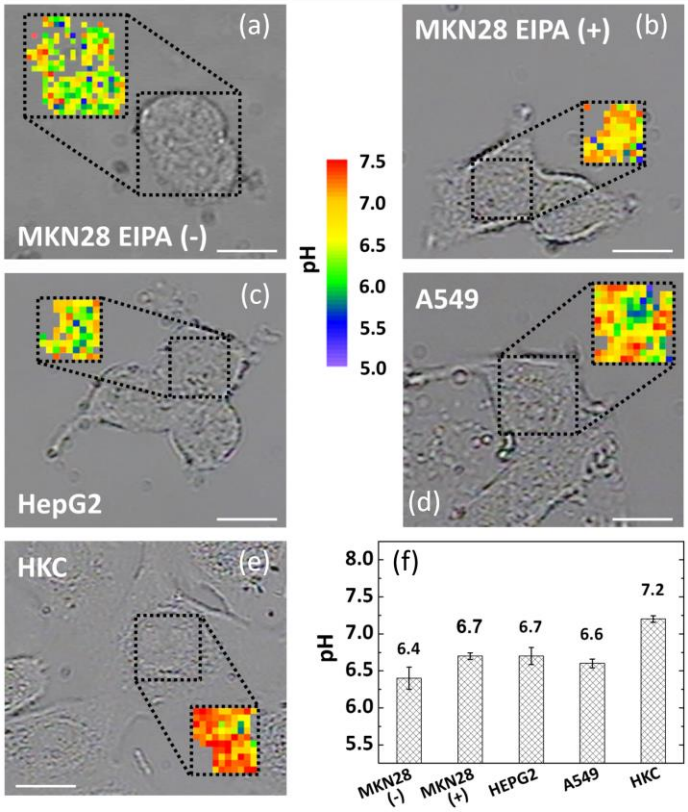

Figure 4: Examples of SERS pH mapping on the surface of MKN28 (a), MKN28 30 minutes after the application of EIPA (b), HepG2 (c), A549 (d) and HKC (e) cells (scale bar 10 µm). (f) Mean values of surface pH in the five investigated cases (n = 3 independent experiments for each case).

The nanometer size of the AuNP sensor attached to the outer cell membrane surface and the use of SERS enabled us to visualize highly localized variation of extracellular pH induced by H+ transport, which was particularly upregulated in cancer cells. The proposed method of analysis can be used to investigate and unfold the dynamics of proton exchange in cells, even after exposure to different pharmacological treatments or different physiological conditions. In addition, this protocol for unspecific labelling of outer membrane proteins can be adapted to the future use of different types of gold nanosensor, which can vary in size, morphology and choice of SERS active compound.

５． 主な発表論文等
〔雑誌論文〕（計２件）

1. Bioconjugation strategy for cell surface labelling with gold nanostructures designed for highly localized pH measurement. Puppulin L, Hosogi S, Sun H, Matsuo K, Inui T, Kumamoto Y, Suzaki T, Tanaka H and Marunaka Y. Nat Commun, 9, 5278 (2018). IF: 12.353.
2. Raman micro-spectroscopy as a viable tool to monitor and estimate the ionic transport in epithelial cells. Puppulin L, Pezzotti G, Sun H, Hosogi S, Nakahari T, Inui T, Kumamoto Y, Tanaka H, Marunaka Y. Sci Rep 7; 3395 (2017). IF: 4.122.

〔学会発表〕（計０件）

〔図書〕（計０件）

〔産業財産権〕
○出願状況（計０件）

名称：
発明者：
権利者：
種類：
番号：
出願年：
国内外の別：

○取得状況（計 0 件）

名称：
発明者：
権利者：
種類：
番号：
取得年：
国内外の別：

〔その他〕
ホームページ等

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