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Tracking Bismuth Antiulcer Drug Uptake in Single *Helicobacter pylori* Cells

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Supporting Information

ABSTRACT: Bismuth-based drugs have long been used for the treatment of *Helicobacter pylori* infection. In this work, the metal content in *H. pylori* was monitored at the single-cell level by time-resolved inductively coupled plasma mass spectrometry, and ~2.9 × 10^7 Mg atoms/cell was determined for the wild-type. Bacteria treated with a Bi antiulcer drug deposited nearly 1.0 × 10^6 Bi atoms/cell, whereas the uptake process took ~3 h to reach the half-maximum. Interference of ferric ions on bismuth uptake was demonstrated, suggesting that the metallodrug can utilize certain iron-transport pathways in the pathogen. The approach provides a general strategy for monitoring metals in single cells, facilitating exploration of metal-relevant bioprocesses.

It has been well-known that at least 25 elements are essential for life, among which most are metals or metalloids.[1] Metals are commonly involved in (patho)physiological processes. Malfunction of metal regulatory proteins causes diseases such as Menkes disease and Wilson's disease, which are due to copper deficiency and overload, respectively.[2] Metal complexes are also used as therapeutic and diagnosis agents.[3] The success of the platinum-based anticancer drug cisplatin has stimulated the use of metal-containing compounds in medicine.[4] Bismuth complexes, such as colloidal bismuth subcitrate (CBS) and ranitidine bismuth citrate (RBC), together with antibiotics, have been used for decades for the treatment of *Helicobacter pylori* infections and peptic ulcers.[3a,5] New techniques and strategies such as metallomics and metalloproteomics have been developed for the systematic study of biometals and metallodrugs.[6] Inductively coupled plasma mass spectrometry (ICP-MS) is one of the useful methods because of its high sensitivity. For example, ICP-MS was used to determine the zinc content in *Escherichia coli* to understand the regulation of intracellular Zn.[7] It has also been used in genome-wide metallomic surveys to search genes and gene networks that are associated with metal hemostasis in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*.[8]

ICP-MS measurements on a biological sample typically require acid digestion of the sample, which is tedious and prone to contamination and sample loss. Single-cell analysis using time-resolved ICP-MS requires minimal sample preparation and provides immediate information on the metal content of individual cells and the related variance in a population of cells[9] (see the Supporting Information). Intact cells are introduced directly into the ICP. ICP-MS spikes, corresponding to individual cells, are produced. The sensitivity of single-cell analysis using time-resolved ICP-MS is high: 10^6 atoms/cell can be readily detected. The technique is also relatively robust with respect to the distribution of spike intensities. The spike intensity is proportional to the quantity of the analyte ions in the cell. The peak maximum of the spike intensity distribution can be used for the determination of the average metal content of the cells, as the peak maximum is relatively insensitive to the presence of sporadic large spikes in the distribution.[10] Importantly, the average metal content in the cells can be quantitatively determined from the peak maximum using polydisperse metal nanoparticles as a calibration standard.[9]

In view of the medical value of Bi-based drugs toward the gastric-disease-causative agent *H. pylori*, their pharmaco-metallomic profiles were studied in this work using time-resolved ICP-MS. The application of such a methodology to track metallodrug uptake was demonstrated, and the initiative paves the way for building up a macroscopic view of the drug uptake process on a single-cell basis.

The feasibility of monitoring individual cells was demonstrated by tracking Mg^{II}, the most abundant divalent cation in living cells. For example, the number of Mg atoms in *E. coli* was previously measured to be on the order of 10^8 per cell.[11] Figure 1A shows the ICP-MS temporal profile of ^{24}Mg (abundance 78.99%)[12] for a suspension of wild-type *H. pylori* having a cell number density of 10^5 cells/mL. Mg spikes, corresponding to individual *H. pylori* cells, were observed. The average frequency of the spikes was ~1 spike/s, or 0.01 spikes every 10 ms (the integration window of the ICP-MS instrument). Assuming a Poisson distribution of the appearance of spikes, the chance of two spikes overlapping in the ICP within the integration window was 0.005%. Overlap of ICP-MS intensity spikes within the ICP-MS integration window was thus insignificant. Each Mg spike should correspond to one *H. pylori* cell only. As shown in Figure 1E, the distribution of ^{24}Mg spike intensity of the *H. pylori* suspension was approximately log-normal with peak maximum of 2.04, which corresponds to 2.85 × 10^7 Mg atoms/cell using MgO nanoparticles as calibration standard as we did previously.[9] To validate the methodology, the ^{24}Mg content was also determined using a batch-type method and found to be 2.52 × 10^7 Mg atoms/cell, in agreement with the single-cell measurement. In addition, the distribution showed a full width at half-maximum (fwhm) of 0.42, indicative of biological variations in a population of cells. ICP-MS intensity spikes of ^{24}Mg can therefore serve as a marker for the presence of intact *H. pylori* cells.

The uptake of metallodrug was exemplified using CBS, which is the mainstay for the eradication of *H. pylori*. The inhibition of *H. pylori* by the metallodrug has been associated with the disruption of the functions of key proteins.[13] No ^{209}Bi (abundance 100%)[12] ICP-MS

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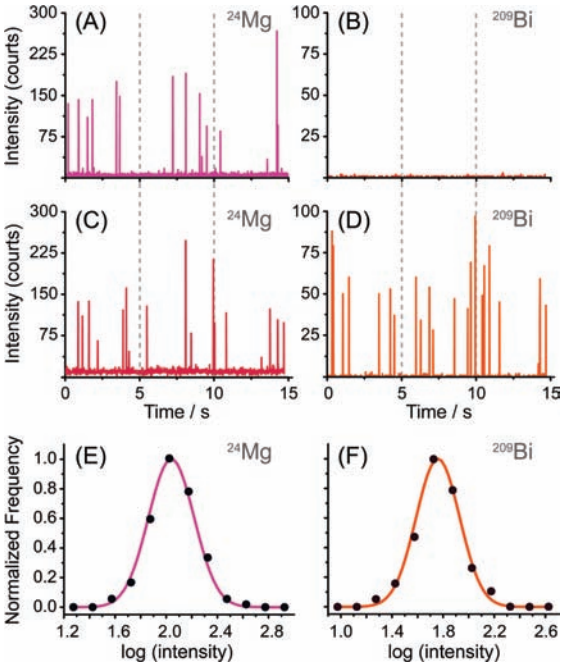

Figure 1. Feasibility of using time-resolved ICP-MS for tracking the presence of individual cells and metallodrug uptake. (top) ICP-MS temporal profile of an *H. pylori* suspension. ^{24}Mg and ^{209}Bi were measured from *H. pylori* grown in Brucella broth with (A, B) 0 and (C, D) 20 μg/mL of CBS, respectively. (bottom) Corresponding distributions of ICP-MS spike intensities for ^{24}Mg and ^{209}Bi.

intensity spikes were observed for the *H. pylori* suspension prior to the incubation of the metallodrug (Figure 1B), suggesting that no Bi is present in the pathogen under normal conditions. In contrast, ^{209}Bi spikes corresponding to Bi in individual *H. pylori* cells were readily detected from *H. pylori* treated with 20 μg/mL CBS (~30 μM Bi) for 16 h (Figure 1D). No ^{209}Bi spikes were detected for a solution containing only 20 μg/mL CBS. ^{24}Mg spikes for the treated *H. pylori* were also measured separately. For the *H. pylori* suspension having a cell density of 10^5 cells/mL, the frequency of the Bi spikes was ~1 spike/s, the same as the Mg spike frequency for untreated *H. pylori*. The distribution of ^{209}Bi spike intensity for the *H. pylori* suspension was also approximately log-normal with a peak maximum of 1.74 and fwhm of 0.40 (Figure 1F). Interestingly, the Mg spike frequency was reduced to ~0.7 spike/s after CBS treatment (Figure 1C). The reduction was probably due to the loss of live and/or intact *H. pylori* cells. The Bi spikes in Figure 1D correspond to Bi in individual *H. pylori* cells. The fwhm is comparable to that of the Mg distribution.

To investigate the potential effect of ferric ions on the uptake of the bismuth drug as previously proposed,[14] a competition study involving ferric citrate and CBS was performed, and the potential interference by citrate was also evaluated. *H. pylori* cells treated with 20 μg/mL CBS in the presence of 0, 25, 50, and 100 μM Fe^{III} (as ammonium ferric citrate) for 16 h were cultured and washed prior to the ICP-MS measurement. The peak maximum of the ^{209}Bi spike intensity distribution (corresponding to the average Bi content in the cells) was found to decrease monotonically with increasing concentration of Fe^{III}, whereas the growth of *H. pylori* (as a percentage), measured in terms of the optical density at 600 nm (OD_{600}) of the *H. pylori* suspension, increased slightly (~10%) under the same conditions (Figure 2A). In contrast, almost no changes in the peak maximum

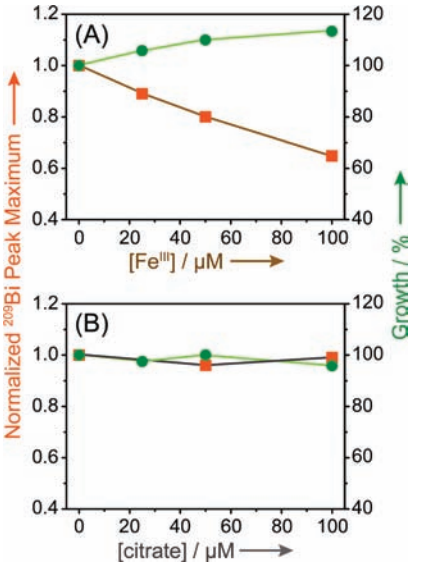

Figure 2. Protective effect of ferric citrate against CBS accumulation in *H. pylori*, as demonstrated by plots of the normalized peak maximum of the ^{209}Bi spike intensity distribution (■) and the percentage growth (●) of *H. pylori* as functions of the concentrations of (A) Fe^{III} (as ammonium ferric citrate) and (B) citrate (as sodium citrate).

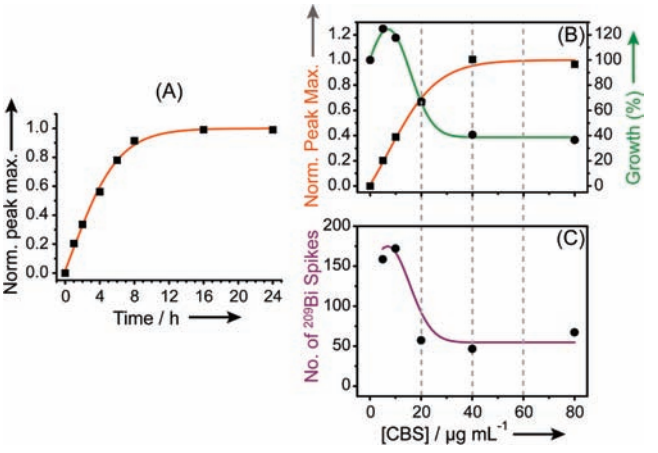

Figure 3. Kinetics of CBS uptake in *H. pylori* and the relationship between number of spikes and cell viability. (A) Normalized peak maximum of the ^{209}Bi spike intensity distribution vs incubation time for *H. pylori* treated with 20 μg/mL CBS. (B) Normalized peak maximum of ^{209}Bi spike intensity distribution (■) and percentage growth (●) and (C) the number of ^{209}Bi spikes measured in 60 s as functions of CBS concentration.

and percentage growth were observed for *H. pylori* suspensions treated with 20 μg/mL CBS in the presence of different amounts of sodium citrate (Figure 2B), indicating that citrate was responsible for neither the growth nor the interference with the metallodrug uptake. Indeed, Fe^{III} protects *H. pylori* from Bi stress in a concentration-dependent fashion. Such protection may be attributed to competition in the transport of the metals into the cells through, for example, the ferric citrate outer membrane receptor Fec A, which is responsible for ferric citrate translocation.[15]

The time- and dose-related bismuth uptake upon CBS treatment in *H. pylori* was monitored to investigate the correlation between bismuth intake and cell viability. To study the kinetics of the metallodrug

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uptake, *H. pylori* cultured in the presence of 20 μg/mL CBS was prepared, harvested, and washed after 0, 1, 2, 4, 6, 8, 16, and 24 h, respectively. The suspensions were subjected to time-resolved ICP-MS measurements of ^{209}Bi immediately after washing. As shown in Figure 3A, the peak maximum of the ^{209}Bi spike intensity increased gradually within the first 8 h of incubation and leveled off after 8 h. The uptake of Bi was relatively slow, with a half-life of ~3 h.

The dependence of CBS concentration on Bi uptake was subsequently examined in *H. pylori* suspensions after treatment with 0, 5, 10, 20, 40, and 80 μg/mL CBS for 16 h. As shown in Figure 3B, the accumulation of Bi was linear for [CBS] < 20 μg/mL and then gradually reached a maximum. The peak maximum of the ^{209}Bi spike intensity distribution increased with [CBS] from 0−40 μg/mL and became saturated for [CBS] > 40 μg/mL. Since no appropriate bismuth nanoparticle was available for quantification, a batch-type method was used to determine the metal content. A digested sample of *H. pylori* treated with 20 μg/mL CBS showed an accumulation of 9.77×10^5 Bi atoms/cell, of which a significant fraction of Bi entered the periplasm and cytoplasm (see the Supporting Information).

Interestingly, the growth of *H. pylori* (as indicated by OD_{600}) was enhanced in the presence of low concentrations of CBS (<10 μg/mL) and started to drop below 100% at higher drug concentrations. No further inhibition was observed for [CBS] > 40 μg/mL. The same concentration range also revealed a constant peak maximum of the ^{209}Bi spike intensity. Our results show that uptake of a small amount of Bi (i.e., <50% of Bi uptake limit) stimulates the growth of *H. pylori*, and the growth is inhibited afterward. The change in the number of ^{209}Bi spikes as a function of the CBS concentration followed a similar profile as the percentage growth of *H. pylori* (Figure 3B,C). The number of Bi spikes reflects the population of *H. pylori* upon CBS treatment, which is consistent with the OD measurement since both methods monitor the total biomass in the samples.

In summary, we have demonstrated that the changes in bismuth content in bacteria with volumes in femtoliter range can be rapidly monitored and that single-cell analysis offers valuable biologically relevant insights into the uptake of bismuth-based drugs. The changes in the peak maximum of the spike intensity distribution with time provided a snapshot of the bismuth uptake in *H. pylori*. The large variation observed for both the ^{24}Mg and ^{209}Bi spike intensities showed that cells were growing at different stages in life cycle. Importantly, the competition between ferric ion and bismuth was demonstrated, indicative of a competitive transport pathway involving the two metals in *H. pylori*. In addition, the study has launched new directions in using time-resolved ICP-MS for extensive single-cell-based applications, including in vivo simultaneous multielemental monitoring,[16] rapid diagnosis of metal-relevant diseases, investigation of cellular heterogeneity,[17] and identification of metal-related genes.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and bismuth content determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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