Published in final edited form as:

Analyst. 2018 March 26; 143(7): 1599–1608. doi:10.1039/c7an01787a.

Single Gold Nanoparticle Plasmonic Spectroscopy for Study of Chemical-Dependent Efflux Function of Single ABC Transporters of Single Live *Bacillus subtilis* Cells

Lauren M. Browning, Kerry J. Lee, Pavan K. Cherukuri, Tao Huang, Preeyaporn Songkiatisak, Seth Warren, and X. Nancy Xu*

Department of Chemistry and Biochemistry, Old Dominion University, Norfolk, VA 23529, USA

Abstract

ATP-binding cassette (ABC) membrane transporters serve as self-defense transport apparatus in many living organisms and they can selectively extrude a wide variety of substrates, leading to multidrug resistance (MDR). The detailed molecular mechanisms remain elusive. Single nanoparticle plasmonic spectroscopy highly depend upon their sizes, shapes, chemical and surface properties. In our previous studies, we have used size-dependent plasmonic spectra of single silver nanoparticles (Ag NPs) to study real-time efflux kinetics of ABC (BmrA) transporter and MexAB-OprM transporter in single live cells (gram-positive and gram-negative bacterium), respectively. In this study, we prepared and used purified, biocompatible and stable (non-aggregated) gold nanoparticles (Au NPs) (12.4 ± 0.9 nm) to study efflux kinetics of single BmrA membrane transporters of single *Bacillus subtilis* live cells, aiming to probe chemical dependent efflux functions of BmrA transporters and their potential chemical sensing capability. Similar to those observed using Ag NPs, accumulation of the intracellular Au NPs in single live cells (WT and BmrA) highly depends upon the cellular expression of BmrA and NP concentration (0.7 and 1.4 nM). The lower accumulation of intracellular Au NPs in WT (normal expression of BmrA) than BmrA (deletion of *bmrA*) indicates that BmrA extrude the Au NPs out of the WT cells. The accumulation of Au NPs in the cells increases with NP concentration, suggesting that the Au NPs most likely passively diffuse into the cells, similar to antibiotics. The result demonstrates that such small Au NPs can serve as imaging probes to study the efflux function of BmrA membrane transporter in single live cells. Further, the dependence of accumulation rate of intracellular Au NPs in single live cells upon the expression of BmrA and concentration of the NPs is about twice higher than that of the same sized Ag NPs. This interesting finding suggests chemical-dependent efflux kinetics of BmrA and that BmrA could distinguish nearly identical sized Au NPs from Ag NPs and might possess chemical sensing machinery.

TOC image

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*To whom correspondence should be addressed: xhxu@odu.edu; www.odu.edu/sci/xu/xu.htm; Tel/fax: (757) 683-5698.

Conflicts of interest

There are no conflicts of interest to declare.

Keywords

ABC (BmrA) transporter; single cell imaging; multidrug resistance; *Bacillus subtilis*; single nanoparticle plasmonic spectroscopy; gold nanoparticle

Introduction

ATP-binding cassette (ABC) membrane transporters exist in all living organisms and they can selectively transport a wide variety of unrelated substrates across the cellular membranes.^{1, 2} The substrates are often structurally and functionally unrelated, even though the ABC transporters often share a common modular architecture with four core domains: two transmembrane domains (TMD) and two nucleotide-binding domains (NBD).^{3–6} Notably, multidrug ABC membrane transporters can extrude a wide variety of antibiotics out of bacteria or chemotherapeutic agents out of tumor cells, leading to multidrug resistance and ineffective treatment of infection diseases and cancer, respectively.^{7–10} These multidrug ABC membrane transporters are commonly named as efflux pump. Despite extensive studies over decades, it remains not yet fully understood how the structurally similar membrane transporters could selectively extrude a wide variety of structurally unrelated substrates out of the live cells.^{3, 4, 11, 12}

X-ray crystallography and cryo-TEM are two primary means to characterize the structures of membrane transporters at the atomic resolution in the form of purified proteins.^{3–6, 13} However, neither methods can offer temporal and dynamic insights into how the membrane proteins specifically recognize and interact with substrates and assemble membrane transporters to selectively extrude the structurally unrelated substrates out of the cells, and they cannot address whether the membrane transporter processes chemical sensing mechanisms.^{3–6, 13} Furthermore, crystallization and isolation of purified transmembrane proteins are extremely challenging.

Radioisotope and fluorescence assays are common assays to study ensemble accumulation kinetics of substrates in bulk cells.^{14–20} However, individual membrane transporters act distinctively and possess unsynchronized efflux kinetics,¹⁸ which requires real-time probing of efflux kinetics of individual membrane transporters in single live cells in order to depict its efflux function. We have demonstrated that efflux kinetics of single membrane transporters of single live cells can be studied in real-time using thin-layer total-internal reflection fluorescence microscopy and spectroscopy.^{18, 21} However, single fluorophores or

radioisotopes themselves do not possess distinctive size-dependent physicochemical properties that would allow their sizes to be measured *in situ* in real time. Therefore, they cannot serve as various sized pump substrates for the study of size-dependent efflux function of single membrane transporters in single live cells.

Noble metal nanoparticles (e.g., Au and Ag NPs) exhibit high Rayleigh scattering and physicochemical-dependent plasmonic properties. Localized surface plasmon resonance (LSPR) spectra of single NPs highly depend upon their sizes, shapes, dielectric constants, and surrounding environments,^{22–30} which enables us to use size-dependent LSPR spectra of single NPs to quantitatively determine sizes of single NPs in solution, in single live cells and embryos *in situ* in real time.^{26, 30–43}

In our previous studies, we have used size-dependent LSPR spectra of single Ag NPs to study the size and charged dependent efflux kinetics of single BmrA membrane transporters in single live *Bacillus (B.) subtilis* cells (Gram-positive bacterium).^{31, 42, 43} Currently, 78 ABC transporters have been identified in *B. subtilis*,^{44–46} making *B. subtilis* a widely used model organism to study efflux function of ABC membrane transporters. We have also used size-dependent LSPR spectra of single Ag NPs to study transport dynamics of single MexAB-OprM membrane transporters in single live *Pseudomonas aeruginosa* cells (Gram negative bacterium).^{32–34, 47}

In this study, we prepared the purified and stable Au NPs (12.4 ± 0.9 nm) and used LSPR spectra of single Au NPs to study efflux kinetics of BmrA in two strains of *B. subtilis* (WT with normal expression of BmrA and BmrA with deletion of *bmrA*). We have demonstrated that single Au NPs can serve as powerful optical imaging probes to study efflux function of single BmrA membrane transporters in single live *B. subtilis* cells (Figure 1). By comparing with our previous study of efflux kinetics of single BmrA transporters in single live cells using the same sized Ag NPs,³¹ we found the chemical-dependent efflux kinetics of single BmrA transporters in single live cells, suggesting possible chemical sensing capability of BmrA membrane transporter. Furthermore, the study of chemical- and size- dependent transport of single Au NPs in and out of single live cells can offer new insights into the design of more effective drug carriers that could potentially evade multidrug resistance to achieve high efficacy and low side-effects.

Experimental Section

Reagents and Supplies

All chemicals, sodium citrate (99%) and $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (99%), were purchased from Sigma Aldrich, except that LIVE/DEAD *BacLight* viability and counting assay was purchased from Invitrogen. All reagents were used as received. Deionized (DI) water (18 M Ω water, Barnstead) was used to prepare solutions and rinse glassware for synthesis of the Au NPs.

Synthesis and Characterization of Purified and Stable Au NPs (12.4 ± 0.9 nm)

We synthesized Au NPs (12.4 ± 0.9 nm) as described previously.^{30, 48, 49} Briefly, we reduced $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (1 mM) with sodium citrate solution (38.8 mM) in 550 mL of DI water by refluxing, boiling and stirring the solution over 30 min as the solution color

changed from yellow to clear, then to dark purple and finally to burgundy. We stopped heating the solution, cooled it to room temperature, and filtered the solution using a 0.22 μm filter. We thoroughly washed the NPs three times using DI water via centrifugation (7735 relative centrifugal force (rcf), Beckman J-21) to remove byproducts of the synthesis reaction and any excess of chemicals in solution. We then re-suspended the washed NPs in the PBS buffer (0.5 mM phosphate buffer, 1.5 mM NaCl, pH = 7.0) to characterize their stability over hours and to use them for the study of efflux function of BmrA in single live cells.

We determined the concentration, sizes and zeta potentials of Au NPs in solution using UV-vis spectroscopy (Hitachi U-2010) and dynamic light scattering (DLS) (Nicom 380ZLS particle sizing and zeta potential system), respectively. We characterized the LSPR images and spectra, and sizes of single Au NPs using dark-field optical microscopy and spectroscopy (DFOMS) and high-resolution transmission electron microscopy (HRTEM) (JEOL, JEM-2100F), respectively. We have fully described our DFOMS in our previous studies.^{18, 30–34, 36, 38, 40, 41, 47, 50} In this study, a Multispectral Imaging System (Nuance, CRI) was used.^{41, 50}

Cell Lines and Cell Culture

WT (BmrA) was purchased from Bacillus Genome Stock Center (BGSC) and BmrA (previously named as *YvcC* or *bmrA*, devoid of *bmrA*) was provided by J. M. Jault.⁵¹ As described previously,^{31, 43} both WT and BmrA cells were pre-cultured in L-broth (LB) medium (1% tryptone peptone, 0.5% yeast extract, and 0.5% NaCl, pH = 7.2) in a shaker (Lab-line Orbit Envivon-Shaker) (150 rpm, 37°C) for 12 h, and then cultured in the LB medium for another 8 h. We harvested the cultured cells using centrifugation, washed them with the PBS buffer three times, finally re-suspended the cells in the PBS buffer, and adjusted its concentration to $\text{OD}_{600\text{ nm}} = 0.1$ for the study.

Studies have showed that the PBS buffer can retain efflux function of bacterial cells,^{15, 16, 19, 20, 31, 52} and maintain the same number and same growth stage of the bacterial cells over the duration of entire experiment (2 h). In contrast, the cell culture medium would promote the cell growth, create various number and stages of the cells over time, which would make the study of time-dependent efflux function of live cells challenging. Therefore, the efflux function of membrane transporters of bacterial cells has always been studied in the PBS buffer.^{15, 16, 19, 20, 31, 52}

Imaging of Single Au NPs in/on Single Live Cells and Assay of Cellular Viability

We incubated the cell suspension ($\text{OD}_{600\text{ nm}} = 0.1$) with 0.7 or 1.4 nM Au NPs under shaking over time, sampled the mixture (20 μL) into a microchamber every 20 min, and imaged single cells with single Au NPs using DFOMS at 15 cells/min over 2 h, as described previously.^{31, 42, 43} Thus, a large number of single cells (2100 cells) for each sample were imaged for probing of the accumulation kinetics of single NPs in single live cells. The intracellular NPs were determined and plotted versus time to determine accumulation rates of single NPs in single live cells over time (accumulation rate = slope of the plot).

By the end of each experiment, we characterize the viability of the cells with and without Au NPs using LIVE/DEAD *BacLight* bacterial viability and counting assay, as described in the protocol.⁵³ We acquired optical and fluorescence images of single cells using dark-field optical microscopy and epi-fluorescence microscopy, respectively. The live cells emitted the green fluorescence (SYTO9, $\lambda_{\text{max}} = 520$ nm), while the dead cells emitted red fluorescence (propidium iodide, $\lambda_{\text{max}} = 610$ nm), which were determined and counted, respectively.

Data Analysis and Statistics

For study of sizes and LSPR spectra of single Au NPs, a minimal of 300 of single Au NPs were characterized using both TEM and DFOMS with a minimal of 100 Au NPs for each measurement, respectively. Each experiment was repeated three times and histograms of distribution of their sizes and peak wavelength of LSPR spectra were presented. For single cell imaging, ten representative images of each cell suspension incubated with 0.7 or 1.4 nM Au NPs were acquired every 20 min over 2 h using DFOMS to study accumulation rates of intracellular Au NPs in single live cells over time. A minimal of 100 single live cells were imaged every 20 min and 600 single live cells were imaged over 2 h for each measurement, which was repeated three times. Thus, a minimal of 1800 cells were studied for each sample, and accumulative single-cell data for all three measurements at each given time point were presented.

Because efflux kinetics of each individual cell are unsynchronized, imaging of accumulation of intracellular NPs in each individual cell represents a single independent measurement. An average of individual cell measurements with standard deviation would mask the unsynchronized and distinctive efflux kinetics of individual cells. Further, such standard deviations include not only the precision of the three repeated measurements, but also variation functions among single cells. On the other hand, the sum of accumulation of single NPs in single cells from all three measurements at each given time represents the total number of intracellular NPs in 300 individual live cells. Thus, we used cumulative intracellular NPs in 300 individual cells to present the dependence of accumulation kinetics of intracellular NPs upon the NP concentration and BmrA, and used t-test (p-value) to determine the statistically significant difference among individual measurements.

Results and Discussion

Synthesis and Characterization of Stable and Purified Au NPs (12.4 ± 0.9 nm)

We have synthesized and purified the Au NPs as described in Method.^{30, 49} We found that the purified Au NPs were much more stable (non-aggregated) in deionized (DI) water than unwashed Au NPs, similar to those we observed in Ag NPs.^{26, 32, 39} The high-resolution transmission electron microscopy (HRTEM) image in Figure 2A shows the spherical NPs and the histogram of their size distribution in Figure 2B shows an average diameter of (12.4 ± 0.9) nm, ranging from 11 to 16 nm with an aspect ratio of around one. Notably, the crystalline and surface roughness of individual Au NPs differs substantially. The dark-field optical images of single Au NPs and their representative LSPR spectra (Figure 2C and D) show distinctive plasmonic green and orange colors with peak wavelength (full-width-at-half-maximum), λ_{max} (FWHM), of 559 (61) and 618 (95) nm, respectively. The majority of

the NPs, $(88 \pm 4)\%$, exhibit plasmonic green, while a small portion of the NPs, $(12 \pm 4)\%$, show plasmonic orange. Using the approaches as we described previously,^{30, 32} we correlated the size distribution of single NPs measured by TEM with the distribution of their LSPR spectra measured by DFOMS, which show that the sizes of plasmonic green NPs are 11-14 nm, and the sizes of orange NPs are the rare NPs with diameters larger than 14 nm. Therefore, we can use LSPR spectra of single NPs to determine their sizes inside and outside single live cells *in situ* in real time. The larger distribution of LSPR spectra of single Au NPs measured by DFOMS than their size distribution determined by TEM demonstrates that DFOMS is a more effective and higher throughput tool than TEM to effectively characterize rare individual Au NPs in a large population of NPs. Furthermore, DFOMS can effectively characterize the LSPR spectra of individual Au NPs in solution *in situ*, which reflect the sizes, morphologies, roughness and surface properties of individual NPs, while TEM cannot.

To ensure that the concentration and sizes of single Au NPs remain unchanged over the duration of the experiment (2 h), we studied the stability (non-aggregation) of Au NPs (1.4 nM) in the buffer (0.5 mM phosphate buffered saline, 1.5 mM NaCl) over time using both UV-vis absorption spectroscopy and DLS. The absorption spectra of purified Au NPs before and after their incubation with the buffer for 24 h remained unchanged, showing a peak absorbance of 0.393 at 519 nm (Figure 3A). The size distributions of the Au NPs characterized using DLS show that their sizes remain essentially unchanged with an average size at (13.5 ± 3.2) nm and (13.7 ± 1.0) nm (Figure 3B) before and after their incubation with the buffer for 24 h, respectively, which demonstrates that the purified Au NPs are stable in the buffer solution for 24 h (Figure 3). The results show the slightly larger sizes of the Au NPs after their 24 h incubation in the buffer than those measured under vacuum by TEM. The solvation of Au NPs with water is most likely attributed to the larger sizes of NPs observed in water. In this study, we use the sizes of Au NPs (12.4 ± 0.9) nm measured by TEM to describe our findings. Notably, the DLS measures the average sizes of a population of the NPs in the solution. Thus, unlike DFOMS, the DLS cannot detect the sizes of rare individual Au NPs in the population of NPs, further demonstrating the powerful detection capability of DFOMS for characterization of rare individual Au NPs in solution. By the same token, the UV-vis spectra of Au NP solution cannot detect plasmonic absorption of rare individual NPs, but ensemble majority NPs, underscoring the importance of single NP detection.

We measured the zeta potentials of purified Au NPs in the solution as $-(3.1 \pm 0.5)$ mV using Nicomp 380ZLS particle sizing and zeta potential system. Using the same approaches, we also measured the zeta potentials of the nearly identical sized of purified Ag NPs that were used to study the efflux function of BmrA in our previous study³¹ as $-(3.8 \pm 0.2)$ mV. The results indicate that the Au NPs possess similar zeta potentials and charges to those of the Ag NPs.

Probing of Efflux Kinetics of Single BmrA Transporters in Single Live Cells Using Single Au NPs

We imaged and characterized intracellular and extracellular single Au NPs with single live cells using our DFOMS (Figure 4A and 4B). Notably, cellular membranes and vesicles do not possess plasmonic properties and they appear white under dark-field illumination. Thus, the distinctive LSPR spectra (colors) of single Au NPs in Figure 4C enable us to identify single Au NPs over the cellular membrane and vesicles, and track the transport of single Au NPs inside and outside single live cells (WT and BmrA) using DFOMS.

Among single live cells with the NPs, a majority of them (>95%) are one NP per cell as those shown in Figure 4A, while a few cells (<5%) having multiple NPs per cell as those shown in Figure 4B. Three examples of each type of single live cell (WT and BmrA) with either intracellular or extracellular Au NPs acquired by DFOMS in Figure 4A and 4B show cross-sections of single rod-like bacterial cells, and demonstrate that dark-field microscope can be used to image the thin-layer sections of single live cells with single Au NPs. The NPs below and above the focal plane of dark-field illumination are invisible. Notably, the sizes of the Au NPs and thickness of the cellular membrane are both under the optical diffraction limit (~200 nm) and the scattering intensity of single Au NPs is higher than the cellular membrane. Thus, the Au NPs appear to stick outside the cellular membrane (Figure 4A and 4B).

We used scattering intensity of single Au NPs to identify intracellular Au NPs and extracellular Au NPs of single live cells as shown in Figure 4a and 4b, respectively. Note that the dark-field illumination needs to pass through the cellular membrane in order to radiate the intracellular Au NPs. Further, scattering of intracellular Au NPs needs to pass through the cellular membrane in order to reach the detector. The cellular membrane both absorbs and scatters photons, which reduces the intensity of illumination and scattering of the intracellular Au NPs and leads to the dimmer and blurry intracellular Au NPs (Figure 4a). In contrast, the scattering intensity of the extracellular Au NPs on the membrane is the sum of the scattering intensity of the Au NPs and cellular membrane, which enhances the scattering of the extracellular Au NPs and produces the brighter extracellular Au NPs (Figure 4b). Therefore, the intracellular Au NPs show lower scattering intensity (Figure 4a) and they are dimmer than the extracellular Au NPs on the cellular membranes (Figure 4b). In our previous studies, we have used TEM to image individual intracellular and extracellular Ag NPs at the nanometer spatial resolution to validate these approaches.³³ We have used these distinctive features to distinguish individual intracellular and extracellular Ag NPs for study of efflux kinetics of single membrane transporters in single live cells in real time.^{31–34, 42, 43, 47} Notably, the Au NPs are orders of magnitude dimmer than the same sized Ag NPs and they are much harder to be imaged than single Ag NPs.^{30, 38} The results in Figure 4 show that our DFOMS equipped with a highly sensitive multi-spectral imaging detector is sufficiently sensitive to detect and track single intracellular and extracellular Au NPs with single live cells in real time.

The representative LSPR spectra of single Au NPs in Figure 4C show distinctive plasmonic properties of single Au NPs with peak wavelengths (λ_{max}) at 561 (green) and 646 nm (orange). These distinctive LSPR spectra of single Au NPs (Figure 4C) enable us to

effectively identify single Au NPs over any other possible cellular debris and vesicles, to determine the sizes of NPs and to track the stability (non-aggregation) and transport of single Au NPs inside and outside of membrane of single live cells *in situ* in real-time. For instance, should individual Au NPs be aggregated to become the larger NPs, we would have observed the red-shift of LSPR spectra and higher scattering intensity of the NPs. We did not observe such a phenomenon over the duration of each experiment (2 h), which indicates that the Au NPs are stable (non-aggregated) in the PBS buffer and in the cells over time and they can be used to study the efflux function of single membrane transporter (BmrA) in single live cells in real time. Furthermore, we observed the majority of intracellular NPs exhibit plasmonic green, while the extracellular NPs displayed plasmonic orange. As described above, the plasmonic green NPs are smaller NPs (11-14 nm in diameters), while the orange NPs are larger NPs with diameters greater than 14 nm. Note that, $(88 \pm 4)\%$ of NPs are plasmonic green and $(12 \pm 4)\%$ of NPs are plasmonic orange NPs. Therefore, the results suggest that the smaller NPs could be easier to enter the cells than the larger NPs, either due to their higher concentration gradients across the cellular membrane and/or their smaller size.

Study of BmrA-Dependent Accumulation Rates of Single Au NPs in Single Live Cells

Unlike eukaryotes, prokaryotes (bacterial cells, *B. subtilis*) do not possess endocytosis, pinocytosis and exocytosis. Therefore, these mechanisms are not responsible for the transport of Au NPs in and out of the live cells. To determine whether the NPs are extruded out of the cells by BmrA, we investigated the dependence of accumulation of single Au NPs in WT (cells with normal expression of BmrA) and Δ BmrA (cells with deletion of *bmrA*), *in situ* and in real time. The results in Figure 5 show that WT cells accumulate the intracellular Au NPs over time much less rapidly than Δ BmrA, indicating the high dependence of accumulation rates of intracellular Au NPs upon BmrA. Note that WT and Δ BmrA cells share the same membrane permeability and they would have the same accumulation kinetics of intracellular NPs, should the BmrA membrane transporter did not extrude the NPs out of the WT cells. Therefore, the results in Figure 5 indicates that the BmrA extrude the intracellular Au NPs out of the WT cells, leading to less accumulation of intracellular Au NPs in the WT than Δ BmrA cells. The accumulation rates of intracellular Au NPs in WT and Δ BmrA during their incubation with 1.4 nM Au NPs over 50.5 min are 1.1 and 1.8 intracellular NPs/min, respectively (Table 1). After 50.5 min incubation, the accumulation of intracellular Au NPs in Δ BmrA cells continues to increase with time, while the accumulation of intracellular Au NPs in WT cells remain essentially unchanged over time.

The results in Figure 5 are similar to those observed using fluorophore (Hoechst 33342) as we reported previously,^{19, 31} further indicating that single Au NPs, like small fluorescence molecules, are well suited for the study of efflux kinetics of single BmrA membrane transporters in single live cells. Unlike fluorescence probes, LSPR spectra of single plasmonic Au NPs provide size information of the NPs, and enable us to determine the size-dependent efflux kinetics of multidrug membrane transporters. By comparing with our previous study of efflux kinetics of single BmrA membrane transporters in single live cells (WT and Δ BmrA) using the nearly identical sized Ag NPs (Table 1),³¹ we observed twice higher BmrA-dependent accumulation rates of single Au NPs in single live cells than those

of single Ag NPs, suggesting chemical-dependent efflux kinetics of BmrA in single live cells and potential chemical sensing capabilities of BmrA. In this study, we compare the dependence of accumulation kinetics of single Au NPs in single live cells upon Au NP concentration and BmrA, with the dependence of accumulation kinetics of single Ag NPs in single live cells upon Ag NP concentration and BmrA.

Study of Concentration-Dependent Accumulation Rates of Single Au NPs in Single Live Cells

We further studied the dependence of accumulation rates of single Au NPs in single live cells (WT and BmrA) upon the NP concentration, aiming to investigate how the Au NPs enter the cells and whether they passively diffuse into the cells, as those conventional pump substrates (antibiotics). The results in Figure 6 and Table 1 show that the number of intracellular Au NPs in single live cells highly depends upon Au NP concentration for both strains (WT and BmrA), and the number of intracellular NPs increases with the Au NP concentration at any given incubation time. Interestingly, the number of intracellular NPs in WT (Figure 6A) increases less rapidly with the NP concentration than those in BmrA (Figure 6B). For example, as Au NP concentration increases from 0.7 to 1.4 nM, the number of intracellular Au NPs accumulated in single live WT cells increases from 20 to 57 within 50.5 min at the rates of 0.4 and 1.1 NPs/min, respectively. In contrast, the number of intracellular Au NPs accumulated in single live BmrA cells increases from 31 to 89 within 50.5 min at the rates of 0.6 and 1.8 NPs/min, respectively.

These interesting findings suggest that the Au NPs, most likely passively diffuse into the cells and be extruded out of the WT cells by BmrA transporter, similar to antibiotics. The diffusion rates of single Au NPs into the cells highly depend upon the concentration gradients of Au NPs across the cellular membrane of single live cells, and they increase as the concentration gradients of Au NPs increase and vice versa, as those observed in BmrA (absence of BmrA transporter). In contrast, the efflux of pump substrates (e.g., Au NPs) out of the cells against the concentration gradient is an active transport process, and powered by ATP. Therefore, the efflux rates of the Au NPs out of the cells are not proportional to NP concentration. That explains why accumulation of intracellular Au NPs in WT increases much slowly than in BmrA (devoid of BmrA), as NP concentration increases, further demonstrating that the NPs were extruded out of the WT cells by BmrA transporter.

Characterization of Biocompatibility of NPs with Single Live Cells

We characterized the viability of single cells (WT and BmrA) upon their incubation with the Au NPs over time using LIVE/DEAD *BacLight* viability and counting assay,⁵³ to determine whether the Au NPs enter live cells and whether intercellular Au NPs cause the cell death. For this viability assay, SYTO9 enter live bacterial cells and produce green fluorescence ($\lambda_{\text{max}} = 520$ nm), showing the viable cells. Propidium iodide can only enter the dead bacterial cells (but not live cells) as the cellular membranes of the dead cells disintegrate and become more porous, which produces red fluorescence ($\lambda_{\text{max}} = 610$ nm) in the dead cells. Thus, the green fluorescence cells are live cells, while the red fluorescence cells are dead cells.

We acquired optical images and fluorescence images of single cells incubated with Au NPs using DFOMS and epi-fluorescence microscopy, respectively. The DFOMS allow us to image single intracellular and extracellular Au NPs with single cells, while fluorescence microscopy enables to determine the live and dead cells based upon the fluorescence emission of SYTO9 and propidium iodide. The optical images of single cells (WT, BmrA) incubated with Au NPs for 2 h show that the cells with and without Au NPs (Figure 7A) exhibit the green fluorescence (Figure 7B), indicating that the cells are alive. The number of live and dead cells were studied and the percentage of the viable cells were presented. The result shows that 96% of the WT and 99% of BmrA cells are alive and the cells with NPs are all alive (Figure 7C), which indicates that the Au NPs indeed enter into the live cells, and the cells with the intracellular and extracellular Au NPs are alive. These results in Figure 7 further demonstrate that the Au NPs (1.4 nM) are biocompatible to the cells during the entire experimental duration (2 h) and the Au NPs are well suited to serve as optical imaging probes for real-time study of efflux kinetics of single membrane transporters in single live cells.

Distinctive Findings of This Study

In this study, for the first time, we are able to image and track such small single Au NPs (12.4 ± 0.9 nm in diameter) in and out of single live bacterial cells and to use LSPR spectra of single Au NPs to study the efflux function of BmrA transporter in single live cells *in situ* in real time. Note that Au NPs are more biocompatible than Ag NPs,^{30, 54–56} but the scattering intensity of Au NPs are orders of magnitudes weaker than the same sized Ag NPs.^{30, 38, 40} Therefore, this study represents a major advance in detection and characterization of single small Au NPs and offers the possibility of using more biocompatible Au NPs as imaging probes to study efflux functions of membrane transporters, as well as to design more effective drug carriers to treat infectious disease and cancer, and to combat MDR.

To systematically study the size, charge, chemical, and bacterial strain dependent accumulation and efflux kinetics of BmrA membrane transporter, we have to keep our experimental methods and conditions the same among our studies and only alter one parameter at time in each independent study. For example, we studied the accumulation and efflux kinetics of smaller Ag NPs (11.8 ± 2.6 nm in diameter)³¹ and the larger Ag NPs (97 ± 13 nm in diameter)⁴² in single live cells (BmrA and BmrA) to determine size-dependent efflux kinetics of BmrA membrane transporter in single live cells. Furthermore, we functionalized the same sized of Ag NPs (11 nm in diameter) with positively charged and negatively charged peptides and study their accumulation kinetics in single live cells (BmrA and BmrA) to characterize charge-dependent efflux functions of BmrA membrane transporter in single live cells.⁴³

In this study, we used the same methods and same size and dose of Au NPs (12.4 ± 0.9 nm in diameter) as those Ag NPs (12.4 ± 4.3 nm in diameter) used in the previous study,³¹ to study the accumulation kinetics of the Au NPs in single live cells (BmrA and BmrA). These experimental designs enable us to compare the new findings with the previous study that used the nearly identical sized Ag NPs for the study of efflux function of BmrA in single live cells,³¹ and to determine the chemical-dependent efflux functions of BmrA

membrane transporter in single live cells. The results show the dependence of accumulation rates of single Au NPs in single live cells upon BmrA and NP concentration are about twice higher than those of single Ag NPs (Table 1), suggesting chemical-dependent efflux kinetics of BmrA in single live cells and potential chemical sensing selectivity of the BmrA membrane transporters. Notably, we compare the ratio of accumulation rates of intracellular Au NPs in WT and BmrA with the ratio of accumulation rates of intracellular Ag NPs in WT and BmrA. We compare the ratio of accumulation rates of intracellular Au NPs for 1.4 nM and 0.7 nM Au NPs with the ratio of accumulation rates of intracellular Ag NPs in WT and BmrA for 1.4 nM and 0.7 nM Ag NPs. Therefore, any potential variation of measurements for each type of NPs (e.g., detection sensitivity of single Ag NPs vs single Au NPs) would not affect comparison between both studies. The new finding offers the first evidence that multidrug membrane transporter might possess a sensing machinery that provides chemical selectivity, which could lead to the further studies that would address a long-standing important biological question about how multidrug membrane transporter can selectively extrude structurally and functionally unrelated substrates and how MDR occurs.

This study also aim to address the other long standing important question about which physicochemical properties of nanomaterials could affect their interactions with live cells and alter living cellular functions. The general assumptions attribute their unique nanometer sizes to their distinctive interactions with live cells. In this study, we kept sizes, shapes and charges of Au and Ag NPs nearly identical, and only alter chemical properties. We discovered a significant different efflux response of the same live cells to two different types of NPs (Ag and Au NPs), showing that chemical properties are very important.

The findings of this study and our previous studies of the size and charge dependent efflux function of BmrA membrane transporter in single live cells are intertwine and each study is distinctive.^{31, 42, 43} Note that the results of one study cannot be extrapolated from another. Further, if we attempted to alter any experimental conditions and designs, we would not be able to address whether efflux function of BmrA transporter in single live cells depends upon the chemical properties of NPs and whether the BmrA transporter can distinguish the difference between the Au NPs from the Ag NPs. Therefore, these systematic studies are especially important and they are absolutely necessary to address important questions about efflux function of membrane transporters and how MDR occurs in single live cells. Moreover, this study demonstrates the importance of ultrasensitive detection and their roles in advancing our understanding in cell biology and biomaterials.

Summary

In summary, we have synthesized and characterized the purified Au NPs (12.4 ± 0.9 nm). The Au NPs up to 1.4 nM are stable (non-aggregated) in the PBS buffer over 24 h and biocompatible with the cells (WT, BmrA) throughout the 2-h experimental duration. We have demonstrated that we can image such small single Au NPs and use them as photostable optical imaging probes to study efflux kinetics of BmrA membrane transporters in single live cells. We found the lower accumulation rates and lower number of intracellular Au NPs in WT than in BmrA cells, indicating the high dependence of accumulation of single Au NPs in single live cells upon BmrA. The result suggests that BmrA is responsible for efflux

of single Au NPs out of the WT cells, leading to their lower accumulation in WT than

BmrA. Furthermore, the accumulation rates and the number of intracellular Au NPs in WT increase with the NP concentration much less rapidly than those in BmrA, showing high concentration and high BmrA dependence. The result suggests that the Au NPs passively diffused into the cells and were extruded out of the WT cells by BmrA membrane transporters. Such accumulation and efflux mechanisms are similar to those of conventional pump substrates (antibiotics). By comparing with our previous studies of efflux kinetics of BmrA in single live cells using fluorophores and nearly identical sized Ag NPs,^{19, 31} we found that the accumulation kinetics of single Au NPs in single live cells are similar to those observed using the fluorophore (Hoechst 33342), further suggesting that the Au NPs can serve as effective optical imaging probes to study the efflux kinetics of BmrA membrane transporters in single live cells. In contrast, we found about twice higher dependence of accumulation rates of single Au NPs in single live cells upon BmrA and NP concentration than those of single Ag NPs, suggesting chemical-dependent efflux kinetics of BmrA in single live cells and potential chemical sensing selectivity of the BmrA membrane transporters. Further study is needed to completely depict their underlying molecular mechanism and the chemical interactions of NPs with the BmrA membrane transporters, which would offer new insights into rational design of drug nano-carriers that may effectively evade the efflux machinery for more effective therapy.

Acknowledgments

This work is supported in part by NSF (CBET 0507036 & 1450936) and NIH (R01 GM0764401; R21HL127580; R15GM119116). Browning, Lee and Warran appreciate the support of NIH-GRAS, NSF-GRAS, and NSF-REU, respectively. We thank JM. Jault for providing us BmrA (*YvcC* or *bmrA*) cells.

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Figure 1.

Schematic illustration of study of efflux kinetics of single BmrA transporters in single live cells *in situ* using LSPR spectra of single Au NPs. The distinctive LSPR spectra (colors) of single Au NPs enable us to distinguish single Au NPs over cellular membranes and vesicles and to track single NPs in and out of single live cells. As single Au NPs diffuse into the cells and are extruded out of the cells, the scattering intensity of LSPR spectra of single Au NPs decrease and increase, respectively. Further, LSPR spectra of single Au NPs enable us to monitor their stability (non-aggregation) in single live cells in real-time.

Figure 2.

Characterization of sizes, shapes and plasmonic optical properties of single Au NPs. **(A)** TEM image of single Au NPs shows nearly spherical NPs. **(B)** Histogram of the size distribution of single Au NPs measured by HRTEM shows average diameters of 12.4 ± 0.9 nm. **(C)** Optical image of single Au NPs acquired by DFOMS shows a majority of the NPs are plasmonic green and a few of NPs are orange. **(D)** LSPR spectra of single Au NPs acquired by DFOMS show λ_{max} (FWHM) at **(a)** 559 (61) and **(b)** 618 (95) nm. Scale bars are 10 nm in (A) and 2 μm in (C). The scale bar in (C) shows the distances among single NPs, but not their sizes which are under the optical diffraction limit.

Figure 3.

Characterization of stability (non-aggregation) of the Au NPs in the PBS buffer. **(A)** UV-vis absorption spectra ($C_{\text{NPs}} = 1.4 \text{ nM}$) of the Au NPs dispersed in the PBS buffer show peak absorbance of 0.393 at 519 nm (FWHM = 68 nm) at (a) 0 and (b) 24 h. **(B)** Size distributions measured by DLS ($C_{\text{NPs}} = 1.4 \text{ nM}$) of the Au NPs in the PBS buffer show average diameters of (a) $13.5 \pm 3.2 \text{ nm}$ and (b) $13.7 \pm 1.0 \text{ nm}$ at 0 and 24 h, respectively. These results show that the Au NPs are stable (non-aggregated) in the PBS buffer for 24 h.

Figure 4. Imaging of single intracellular and extracellular Au NPs in single live cells using DFOMS. CCD images of individual (A) WT and (B) BmrA cells incubated with the Au NPs (1.4 nM) in the PBS buffer show individual (a) intracellular and (b) extracellular Au NPs. Three examples of single cells with intracellular and extracellular Au NPs were shown in (i-iii). (C) LSPR spectra of single Au NPs show λ_{max} (FWHM) at (a) 561 (87), and (b) 646 (101) nm. The plasmonic colors of the Au NPs in (A) and (B) were characterized using their LSPR spectra similar to those in (C) and their pseudo colors were added into the images based

upon their spectra, showing that the majority of intracellular NPs exhibits plasmonic green color while the extracellular NPs display plasmonic orange color. The scale bars in (A) and (B) are 2 μm .

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Figure 5.

Study of the dependence of accumulation kinetics of single Au NPs in single live cells upon the expression of BmrA. Plots of the number of single intracellular Au NPs in single live cells for (A) WT (normal expression of BmrA) and (B) BmrA (deletion of *bmrA*) cells incubated with the Au NPs (1.4 nM) over time show the high dependence of accumulation kinetics of the Au NPs in single live cells upon the expression of BmrA. Each point represents the sum of three repeated experiments at a given duration and the lines are added to guide the trend. At each point (every 20 min), 300 cells at 15 cells/min are imaged over time. The P value of data in (A) and (B) is 0.043, showing statistically significant difference of intracellular NPs in (A) WT and (B) BmrA with 95% confidence level, and BmrA dependent accumulation kinetics of single Au NPs in single live cells.

Figure 6.

Study of the dependence of accumulation kinetics of single Au NPs in single live cells upon the concentration of Au NPs. Plots of the number of single intracellular Au NPs in (A) WT and (B) BmrA cells incubated with (a) 1.4 and (b) 0.7 nM Au NPs in the PBS buffer over time show the high dependence of accumulation kinetics of the Au NPs in single live cells upon the concentration of Au NPs. Each point represents the sum of three repeated experiments at a given duration and the lines are added to guide the trend. At each point (every 20 min), 300 cells at 15 cells/min are imaged over time. The data in (a) are those in Figure 5. The P value of data of (a) and (b) in (A) is 0.004, showing statistically significant difference of concentration-dependent intracellular NPs in (A) WT for (a) 1.4 nM and (b) 0.7 nM Au NPs, with 95% confidence. The P value of data of (a) and (b) in (B) is 0.013, showing statistically significant difference of concentration-dependent intracellular NPs in (B) BmrA for (a) 1.4 nM and (b) 0.7 nM Au NPs, with 95% confidence level.

Figure 7.

Characterization of the viability of single cells using LIVE/DEAD BacLight viability and counting assay. (A) Dark field optical image and (B) fluorescence image of single (a) WT, and (b) $\Delta BmrA$ cells incubated with 1.4 nM Au NPs over the duration of each experiment for 2 h show that the cells with or without intracellular NPs emit the green fluorescence ($\lambda_{\text{max}} = 520$ nm), indicating viable cells. (C) Plots of percentage of viable cells incubated with Au NPs for 2 h for WT and $\Delta BmrA$ cells show that 96% and 99% of the cells are alive and the cells with NPs are alive. More than 100 of cells are imaged for each experiment. The scale bar in (A-B) is 6 μm .

Study of Dependence of Accumulation Rates and Number of Intracellular Single Au NPs and Single Ag NPs in Single Live Cells upon the Expression of BmrA and NP Concentration

Table 1

Diameter (nm) ^a	C _{NPs} (nM)	Accumulation Rate (NPs min ⁻¹) ^b		Number of Intracellular NPs ^c	
		WT	BmrA	WT	BmrA
Au NPs ^d					
12.4 ± 0.9	0.7	0.4	0.6	20	31
	1.4	1.1	1.8	57	89
Ag NPs ^e					
12.4 ± 4.3	0.7	0.9	1.7	39	79
	1.4	1.3	3.2	60	103

^aDiameters of NPs measured by HRTEM;
^bAccumulation rates (slopes of the plots) at the 50.5 min incubation;
^cNumber of intracellular NPs accumulated in 300 live cells at the 50.5 min incubation.
^dThis study
^ePrevious study³¹