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Investigation of surface enhanced Raman spectroscopy for hemozoin detection in malaria diagnosis

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ABSTRACT

We report two methods of surface enhanced Raman spectroscopy (SERS) for hemozoin detection in malaria infected human blood. In the first method, silver nanoparticles were synthesized separately and then mixed with lysed blood; while in the second method, silver nanoparticles were synthesized directly inside the parasites of *Plasmodium falciparum*.

Keywords: Raman Spectroscopy, surface enhanced Raman scattering, hemozoin, malaria, silver nanoparticles.

1. INTRODUCTION

Malaria is a global disease which causes 584,000 deaths per year in the world.[1] Malaria parasites in infected erythrocytes metabolize hemoglobin resulting in the production of hemozoin which can serve as a unique biomarker. Currently microscopic examination of Giemsa-stained blood smears is regarded as the "gold standard" in malaria diagnosis. However, the process is time consuming and requires trained operators to ensure parasite detection especially at low parasitemia levels.[2]

Raman spectroscopy has been used to characterize the electronic structure of β-hematin (a biocrystal with close Raman characteristics resemblance to hemozoin) or hemozoin[3] and its change with drug treatment or disease progression.[4] However, the amount of hemozoin produced in the early ring stages normally seen in peripheral blood is very small. Therefore, the enhancement of Raman signals is crucial for the detection of hemozoin in early ring stages so as to provide the sensitivity needed for diagnosis.

Surface enhanced Raman spectroscopy (SERS) has been reported to enhance the Raman signal of hemozoin or β-hematin by several orders of magnitude.[5] The lowest detection limit of SERS in the current literature is about 0.0005%, which was achieved using a gold coated substrate based on butterfly wings[6]. However, this method requires the development of a cost-effective and reproducible method to fabricate complex butterfly wing nanostructures. Moreover, it is time consuming at a low parasitemia level to locate the hot spots. We have previously used magnetic field enriched SERS to augment the interaction between β-hematin and silver nanoparticles[7, 8] enabling a detection limit as low as 5-nM β-hematin. However, the requirement of an external magnetic field is inconvenient. Here, we report two enhanced SERS methods for the detection of hemozoin from *Plasmodium falciparum* infected human blood. In Method 1, silver nanoparticles are synthesized separately and then mixed with the processed sample. While in Method 2, silver nanoparticles are synthesized directly inside parasites to achieve closer contact with hemozoin, which is a new technique enabling ultrasensitive detection of hemozoin.

2. MATERIALS AND METHODS

2.1 Parasite culture

Plasmodium falciparum parasites (3D7) were cultured in fresh red blood cells (RBCs) at 5% hematocrit with media constituted of bicarbonate buffered RPMI 1640 (RPMI 1640 Medium, Life Technologies, Grand Island, USA)

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supplemented with 5% albumax (AlbuMAX® I Lipid-Rich BSA, Life Technologies, Grand Island, USA), 200 μM hypoxanthine and 20 μg/ml gentamycin as described previously.[9] The cells were maintained at 37°C and a gas mixture of 5% CO_2, 1% O_2 and 94% N_2. After 5 cycles of parasite growth, the mixed culture was synchronized using 5% D-sorbitol (D-Sorbitol, Sigma, St. Louis, USA) treatment[10] and allowed to grow one more cycle then were washed to get rid of floating hemozoin. Giemsa stained microscopy was used to confirm the stages of parasites and parasitemia level. It turned out that nearly 98% of parasites were in the ring stage and 2% in the early trophozoite stage in this study.

2.2 Sample preparation in Method 1 (synthesizing nanoparticles separately)

In Method 1, silver nanoparticles were synthesized separately and then mixed with isolated hemozoin. Silver nanoparticles were synthesized using the reduction method.[11] A total of 33-μl Triton X-100 (Triton X-100 Detergent, Bio-Rad Laboratories, Hercules, USA) was mixed with 5-ml hydroxylamine hydrochloride (0.03 mM, MP Biomedicals, Santa Ana, USA) and NaOH (0.15 mM). The mixture was added drop-wise over a period of 8 min to 45 ml aqueous $AgNO_3$ (Silver nitrate, Merck, Kenilworth, USA) with a concentration of 1.11 mM. The resulting solution was then sonicated (Elma E30H, Elma, Wetzikon, Switzerland) for 30 min. Finally, the generated silver nanoparticles were centrifuged and resuspended in 5 ml solution. The suspension was sonicated for 5 min for later use.

Hemozoin crystals were extracted by simple cell lysis and centrifuging. A total of 10 μl infected blood sample were dispersed in 50 ml deionized water mixed with 100 μl Triton X-100 and then sonicated for 5 min. Released hemozoin crystals were collected by centrifuging at 5000 rpm for 5 min (Sartorius 2-16, Sigma Laborzentrifugen, Ostrode, Germany) and resuspended in 5 ml NaOH solution (0.05 mM).

Then silver nanoparticles suspension and hemozoin suspension were mixed together (1:1 v/v). The mixture was sonicated for another 2 min. During Raman measurements, the sample was smeared on a slide covered by aluminum foil as the substrate. On each slide, five random locations were selected for Raman measurements while the sample was still wet.

2.3 Sample preparation in Method 2 (synthesizing nanoparticles inside parasites)

In Method 2, silver nanoparticles were synthesized inside parasites directly to achieve closer contact with hemozoin than Method 1. A total of 10-μl lysed blood was dispersed in 50-ml deionized water and then sonicated for 5 min to achieve blood cell lysis. The mixture was pushed through a filter with a pore size of 0.2 μm (Supor Syringe filters, Pall Life Science, Washington, USA) in a syringe to remove hemoglobin in the lysed blood sample. Then, the residue in the filter was flushed out and suspended in 45-ml $AgNO_3$ (Silver nitrate, Merck, Kenilworth, USA) solution with a concentration of 1.11 mM. Then 33-μl Triton X-100 (Triton X-100 Detergent, Bio-Rad Laboratories, Hercules, USA) was mixed with 5-ml solution of hydroxylamine hydrochloride (0.03 mM, MP Biomedicals, Santa Ana, USA) and NaOH (0.15 mM), which was then added drop-wise over a period of 8 min to aqueous $AgNO_3$ to reduce silver. The resulting solution was then sonicated (Elma E30H, Elma, Wetzikon, Switzerland) for 30 min. Finally, the sonicated solution was pushed through a 0.2-μm filter (Supor Syringe filters, 25mm, 0.2 μm, Pall Life Science, Washington, USA) in a syringe to filter away small excessive nanoparticles and chemicals and leave analyte on the filter paper. The filter paper was taken out and deposited on a slide covered by aluminum foil for Raman measurements. For each filter paper, five random locations were selected for Raman measurements.

2.4 Raman measurements and data processing

A micro-Raman spectrometer system (inVia, Renishaw, Aberdeen, UK) coupled with a microscope (Alpha 300, WITec, Ulm, Germany) in a backscattering geometry was used to measure all Raman spectra. The exposure time was 10 seconds and the excitation power was 2.5 mW for all SERS measurements. To obtain the contribution of hemozoin alone in the measurement of an infected blood sample for convenient visualization in Method 1, the clean Raman spectrum of an infected blood sample was modeled as the summation of the contribution from the normal blood sample and that from hemozoin. Then the contribution of each component to the infected blood spectrum was estimated using a least square regression method[12] implemented by the lsqcurvefit function in MATLAB with an option of trust-region-reflective algorithm.

To determine the detection limit for Method 1, a *t*-test was conducted to evaluate the difference in hemozoin contribution between every infected blood sample with a progressively decreasing parasitemia level and the normal blood sample. When there was no difference between the two samples, the parasitemia level in the previous infected blood sample that still demonstrated a significant difference in hemozoin contribution compared to the normal blood sample was

determined to be the smallest detectable parasitemia level. The lowest detectable parasitemia level for Method 2 was determined in the same manner by conducting a series of t-tests except that the Raman peak intensity at 1623 cm^{-1} instead of hemozoin contribution was compared.

3. RESULTS AND DISCUSSIONS

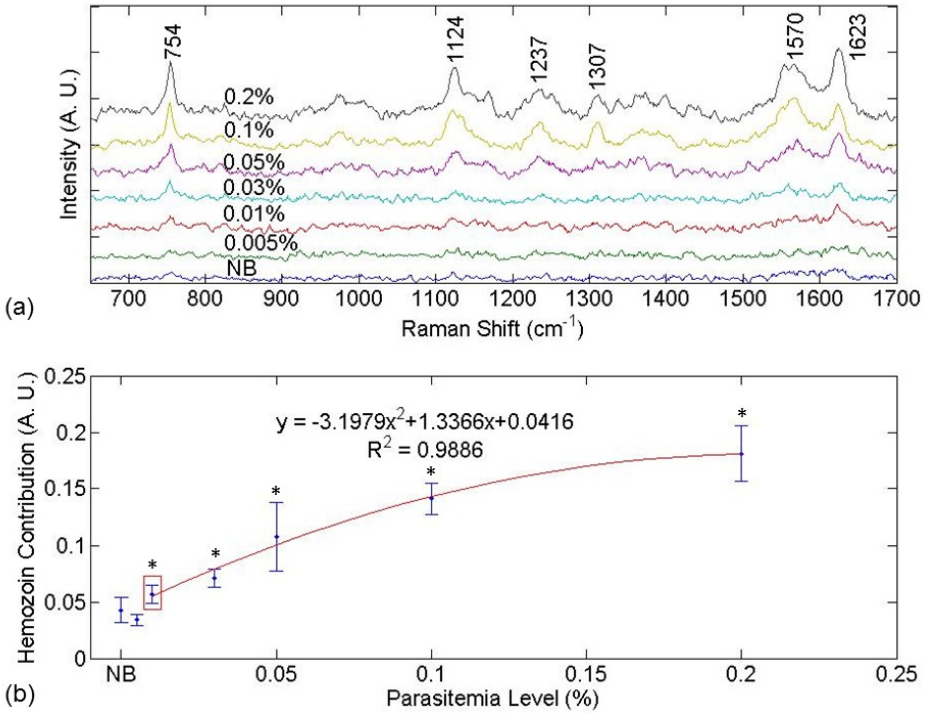

Figure 1. SERS spectra and hemozoin contribution in Method 1. (a) SERS spectra contributed by hemozoin in infected blood treated by Method 1. (b) Hemozoin contribution as a function of parasitemia level. In (a), the data point corresponding to a parasitemia level of 0.01% is marked by a red box. In (b), the resulting curve of the second-order polynomial fitting for the data points corresponding to parasitemia levels in the range of 0.01% to 0.2% is shown in red. The data corresponding to normal blood samples, labeled as "NB", is added manually to facilitate comparison.

Figure 1 shows (a) the SERS spectra and (b) hemozoin contribution as a function of parasitemia level for infected and normal blood samples obtained using Method 1. Hemozoin contribution refers to the weight of the basic component spectrum corresponding to hemozoin resulting from the least square regression as described in data processing section. The lowest detectable parasitemia level was determined to be 0.01% after conducting a series of *t*-tests to compare the hemozoin contribution in infected blood samples with that in the normal blood sample ($p<0.05$). There appears to be a strong correlation between hemozoin contribution and the parasitemia level in Fig. 1(b).

Figure 2 shows the SERS spectra and ν_{10} peak intensity as a function of parasitemia level obtained using Method 2, in which silver nanoparticles were synthesized inside parasites. Compared with the spectra in Fig. 1, the Raman spectra in Fig. 2 are much stronger. During silver nanoparticles synthesis, the use of detergent Triton X-100 and hydroxylamine hydrochloride likely converted the residue hemoglobin to heme and then hematin[13] which contributed to the Raman peaks[14]. Note that hematin and hemozoin share most peaks including 1623 cm^{-1}. The detection limit for Method 2 is determined to be 0.00005% parasitemia level in the ring stage, after comparing the Raman peak intensity at 1623 cm^{-1} measured from infected blood samples with that from the normal blood sample with *t*-test ($p<0.001$). The detection limit is exceptionally low compared to most relevant publications we can find.

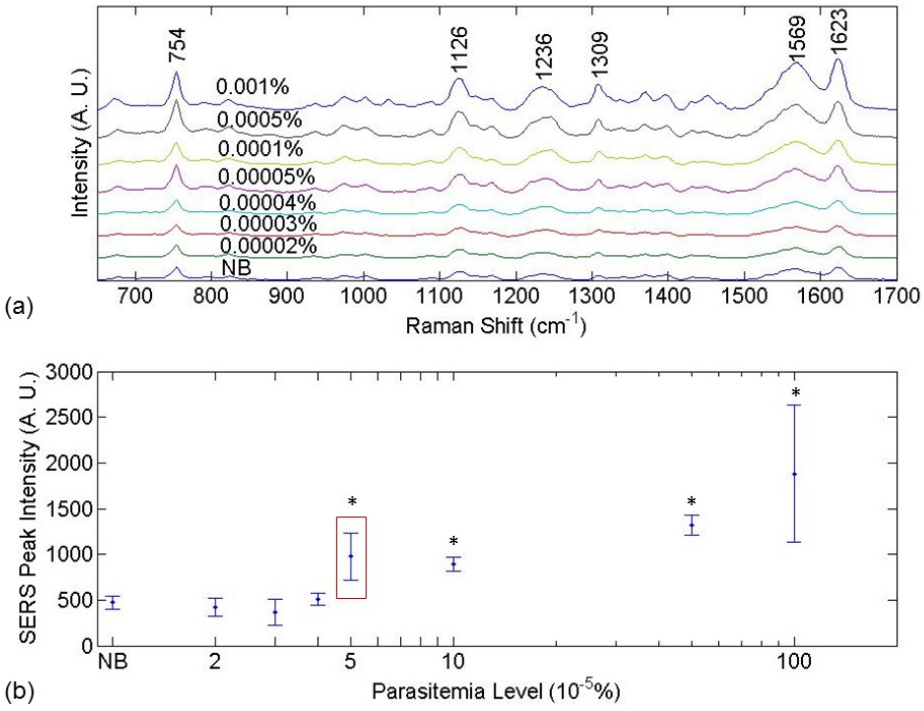

Figure 2. SERS spectra and hemozoin Raman peak distribution in Method 2. (a) SERS spectra of normal blood and infected blood sample treated by Method 2. (b) SERS peak intensity at 1623 cm^{-1} as a function of parasitemia level. The data corresponding to normal blood samples, labeled as "NB", is added manually to facilitate comparison.

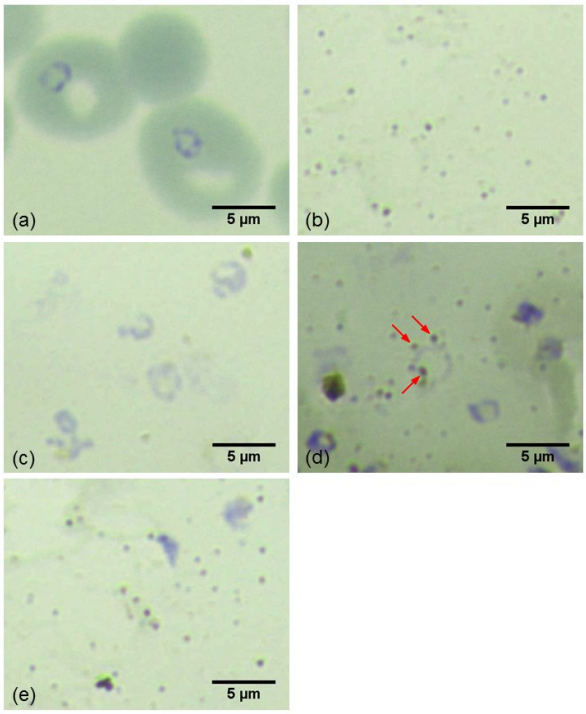

Figure 3. Giemsa stained images of blood samples treated by Method 1 and Method 2. (a) a blood sample with parasites in the ring stage prior to cell lysis, (b) silver nanoparticles alone, (c) a blood sample after blood cell membrane lysis but prior to nanoparticle synthesis treated by Method 2, (d) the blood sample after nanoparticle synthesis treated by Method 2 and (e) a blood sample treated by Method 1 and then mixed with nanoparticles synthesized separately.

To illustrate the difference in the separation of parasites and nanoparticles between Methods 1 and 2, Fig. 3 shows the Giemsa stained images of a typical infected blood sample, silver nanoparticles and a blood sample treated by Method 2 before and after nanoparticle synthesis as well as a blood sample treated by Method 1 for comparison. Figure 3(a) illustrates the purple rings superimposed on top of red blood cells, which are the stained DNAs of malaria parasites in the ring stage. Figure 3(b) displays the Giemsa stained image of silver nanoparticles serving as a reference to facilitate search for nanoparticles in subsequent images, in which aggregated nanoparticles show up as sparsely distributed brown or black dots. In Fig. 3(c), red blood cells have been lysed as treated by Method 2 so the blood cells do not exist in background, which is visibly different from Fig. 3(a). However, parasites were not lysed because the concentration of the lysing agent was purposefully reduced. For this reason, the stained DNA rings are clearly seen. In Fig 3(d), nanoparticles have been synthesized in the lysed blood sample as treated by Method 2. It can be clearly seen that some nanoparticles are in the close proximity to the DNA rings of parasites as indicated by red arrows. This phenomenon can be observed in roughly one out of every ten rings in samples treated by Method 2. This suggests that silver nanoparticles were more likely to be close to hemozoin inside the same parasites. Because hemozoin was not released to the outside of parasites, the original local hemozoin concentrations in the parasites were maintained, which created higher SERS signals than otherwise. For the purpose of comparison, Fig. 3(e) illustrates the Giemsa stained image of a blood sample treated by Method 1, in which lysed blood samples and parasites were mixed with nanoparticles separately synthesized. Because parasites have been lysed, no DNA rings were observed and hemozoin inside parasites were thus released into the entire sample. Hemozoin concentration in the sample was thus much lower than that in the original parasites. The SERS signal from hemozoin was thus much lower as expected.

4. CONCLUSIONS

In conclusion, the simplicity and speed of our SERS based techniques indicate their great promise in malaria diagnosis. In the case of P. falciparum infections in which parasites observed in peripheral blood are predominantly in the ring stage, Method 1 would allow the relatively accurate determination of the parasitemia level. While infections containing asynchronous stages would be more difficult to accurately quantify as the hemozoin level varies between the different stages unless an extra synchronization step is employed.

5. ETHICS STATEMENT

The use of human blood strictly followed protocols and guidelines that were approved by the domain-specific review board of Nanyang Technological University (IRB number: NTU-IRB 11/12/2011). Blood component collection service was provided by Blood Transfusion Service and Blood Donation Centre of National University Hospital. All individuals gave informed consent.

6. ACKNOWLEDGEMENTS

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