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A rapid readout for many single plasmonic nanoparticles using dark-field microscopy and digital color analysis

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ABSTRACT

The integration of plasmonic nanoparticles into biosensors has the potential to increase the sensitivity and dynamic range of detection, through the use of single nanoparticle assays. The analysis of the localized surface plasmon resonance (LSPR) of plasmonic nanoparticles has allowed the limit of detection of biosensors to move towards single molecules. However, due to complex equipment or slow analysis times, these technologies have not been implemented for point-of-care detection. Herein, we demonstrate an advancement in LSPR analysis by presenting a technique, which utilizes an inexpensive CMOS-equipped digital camera and a dark-field microscope, that can analyse the λ_{max} of over several thousand gold nanospheres in less than a second, without the use of a spectrometer. This improves the throughput of single particle spectral analysis by enabling more nanoparticles to be probed and in a much shorter time. This technique has been demonstrated through the detection of interleukin-6 through a core-satellite binding assay. We anticipate that this technique will aid in the development of high-throughput, multiplexed and point-of-care single nanoparticle biosensors.

1. Introduction

The trend in the development of next-generation biosensors is towards the detection of smaller and smaller amounts of materials, with the goal being the detection of a single molecule (Gooding and Gaus, 2016). The detection of single molecules has the potential to provide new information about biological systems by: i) uncovering the heterogeneity within samples, and ii) the potential to identify rare species or subtle changes in analyte concentration (Holzmeister et al., 2014). However, with the detection of single molecules comes the challenge of having collected sufficient single molecule events to be able to obtain quantitative information (Gooding and Gaus, 2016).

Owing to the characteristic of plasmonic nanoparticles (e.g. gold or silver nanoparticles), known as localized surface plasmon resonance (LSPR), the intense scattering of light by these nanoparticles can be directly imaged in the wide field of view of a dark-field microscope (Anker et al., 2008; Sriram et al., 2015). This widefield imaging of nanoparticles offers a unique opportunity to build a biosensor for single nanoparticle assays, capable of detecting and counting single molecules. Beuwer et al. (Beuwer et al., 2015) used a modified dark-field microscope to monitor the stochastic interactions of hundreds of single proteins on individual gold nanorods. Other approaches involve the use of single particle spectroscopy/spectrometry or laser imaging to obtain high resolution spectra from single nanoparticles, that monitor and analyse single nanoparticles in high spectroscopic detail (Chen et al., 2013; Taylor and Zijlstra, 2017). These methods are of great use in characterizing single nanoparticle interactions. However, for biosensing applications, such a high level of spectral detail is not as important as acquiring specific and relevant information from many single nanoparticles. Hence, an approach that is capable of rapid and high-throughput single nanoparticle analysis would be advantageous.

Recently, a new strategy to analyse and monitor the plasmonic

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properties of a nanoparticle known as colorimetric analysis has been developed. This approach analyses the color of scattered light from plasmonic nanoparticles images using dark-field microscopy. The wavelength of the scattered light is directly related to the LSPR of the nanoparticle and its size, shape and local environment. By analyzing the RGB (red, green and blue) of the light scattered by the nanoparticles, this technique has been used to identify the sizes of gold nanoparticles (Jing et al., 2012) and also detect single antibody-antigen binding events (Ungureanu et al., 2010; Verdoold et al., 2011). The technique was further modified to streamline the colorimetric detection, by measuring hue (a single color variable) to detect the formation of a DNA monolayer on gold nanoparticles (Zhou et al., 2016).

The recent development of single nanoparticle analysis using CCD detectors costing thousands of dollars for colorimetric analysis, has opened the possibility for high-throughput single nanoparticle assays to be conducted (Xie et al., 2017). However, in order to move towards achieving a portable device for point-of-care detection, it is important for the tools used in these approaches to be readily accessible and cost effective. Although consumer-grade CMOS cameras do not provide the same level of image quality as CCD detectors (Lustica, 2011), the technology has reached a point where image quality is sufficient to perform image processing tasks at the single nanoparticle level. The other main advantages that these CMOS cameras provide are significantly lower costs of production, the capability for fast read-out speeds and their wide availability in commercially available cameras and smartphones (Waltham, 2013). The last point is especially significant as this would allow for smartphones to be employed in biosensing, a step towards a point-of-care device.

In this study, we demonstrate the ability of a commercially available consumer-grade CMOS camera, when coupled with a dark-field microscope, to analyse the LSPR of single plasmonic nanoparticles. Dark-field images are analysed using an in-house developed MATLAB algorithm that can process thousands of nanoparticles within seconds, a signficant improvement on the longer times of previous spectroscopic techniques. Furthermore, through the calibration of the CMOS camera, we demonstrate that the λ_{max} of up to 5000 nanoparticles can be obtained within seconds, without the use of a spectrometer. In order to demonstrate the ability of this technique to be employed in the detection of an analyte, interleukin-6 (IL-6) was chosen as the model analyte. IL-6 is known to be associated in the inflammatory and auto-immune processes of many diseases, such as Alzheimer's disease (Wang et al., 2015). Using a core-satellite binding assay, we demonstrate the potential of this technique to be employed in biosensing.

2. Material and methods

2.1. Chemicals and materials

Sodium citrate tribasic dihydrate, gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), (3-aminopropyl)triethoxysilane (APTES), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and streptavidin were purchased from Sigma-Aldrich (Sydney, Australia). Small glass coverslips were purchased from Thermo Fisher Scientific (Australia) (22 × 22 mm, 0.16 – 0.19 mm thickness). Large coverslips for use in the flow cell were purchased from Thermo Scientific (USA) (Gold Seal™, 35 × 50 mm, 0.13 – 0.17 mm thickness). Sulfuric acid (H_2SO_4), dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Merck (Australia). Hydrogen peroxide (H_2O_2), 96% ethanol, 2-propanol and potassium carbonate (K_2CO_3) were purchased from Chem-Supply (Australia). Monoclonal mouse anti-IL-6 antibodies, biotinylated polyclonal rabbit anti-IL-6 antibodies and human IL-6 proteins were purchased from Abcam (Australia). 2-(2-((11-mercaptoundecyl)oxy)ethoxy)acetic acid ($HS\text{-}C_{11}\text{-}EG_6\text{-}OCH_2\text{-}COOH$) was supplied by ProChimia Surfaces (Poland). 10 nm and 80 nm citrate-capped gold nanoparticles were purchased from NanoComposix (USA). All water used was Milli-Q grade (18 MΩ cm) (Millipore Australia) unless otherwise stated.

2.2. Gold nanoparticle synthesis

Gold nanoparticles (67 nm) were synthesized as reported in a previous protocol (Bastús et al., 2011). The protocol for the synthesis of the AuNPs is as follows. Sodium citrate solution (2.2 mM, 150 mL) was brought to boil and, under vigorous stirring, $HAuCl_4$ (25 mM, 1 mL) was added. The solution immediately changed from yellow to grey and then to red. The temperature of this solution was then dropped to 90 °C and then $HAuCl_4$ (25 mM, 1 mL) was injected and the solution allowed to stir for 30 min. This process was repeated twice, then the solution was diluted by extracting the sample solution (55 mL), followed by the addition of water (53 mL) and sodium citrate solution (60 mM, 2 mL). This process was repeated 8 times to yield 67 nm AuNPs (2.2×10^{11} NPs/mL, 367 pM). Mean particle diameter was measured with TEM to be 67.2 nm, $s = 7.8$ nm, $n = 310$ (see Fig. S1).

2.3. Surface fabrication

Small and large glass coverslips were both cleaned by immersing them in freshly prepared piranha solution (3:1 mixture of H_2SO_4:H_2O_2) for 1 h, followed by thorough rinsing with water and ethanol. The coverslips were then immediately immersed in a 10% v/v solution of APTES in ethanol for 1 h. Freshly modified coverslips were then rinsed thoroughly with ethanol, sonicated twice in ethanol, once in water (for 30 s) and then baked for 1 h at 120 °C. Slides were stored in ethanol at 4 °C, for a maximum of 1 week, until used.

67 nm AuNPs were diluted 1:200 in water (1.8 pM, 1 mL) and then drop cast onto the surface for 15 min. The colloid solution was removed and then the surfaces rinsed thoroughly with ethanol and water, then dried under a stream of nitrogen. Modified coverslips were stored in ethanol at 4 °C for later use.

2.4. LSPR imaging and analysis

Fig. S2 shows the dark-field microscope (Olympus BX51) setup used for imaging and analyzing the nanoparticles. A 100 W halogen lamp was used as the excitation source and was focused through a dark-field condenser (NA > 0.8). The scattered light was collected by a 40x dark-field objective (NA = 0.65) and then directly imaged using a commercially available complementary metal-oxide-semiconductor (CMOS) camera (Canon 100D, 22.3 mm x 14.9 mm sensor size, 4.3 µm x 4.3 µm pixel size). The images were saved and processed as Canon RAW (.CR2) 14-bit color files.

Analysis was performed using an in-house written MATLAB algorithm (see SI for link to download). Briefly, the images are read into MATLAB and then are converted from the RGB color space to the HSV color space, to separate the intensity and color variables. The image is then processed using a bandpass filter to remove noisy pixels and then single nanoparticles are identified based on the morphology and intensity of the point-spread-functions. Once the nanoparticles are located, they are labelled and color data (hue) is extracted using intensity-weighted averaging, with a mask (3 pixel radius) around the pixel with the highest intensity.

2.5. Hue to wavelength calibration of camera

A Canon 100D was mounted on an optical table and adjusted such that light emitted from the TILL Photonics Polychrome V monochromator would be directed directly onto the imaging sensor, without any lens attachments. The light output from the Polychrome V was initially set to 400 nm and images were captured. Wavelength was increased at increments of 5 nm, with images taken at each increment, until 690 nm.

RAW (.CR2) images were then read into MATLAB and converted