Real-time Monitoring of Colloidal Nanoparticles using Light Sheet Dark-field Microscopy Combined with Microfluidic Concentration Gradient Generator (μFCGG-LSDFM)

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For real-time monitoring of colloidal nanoparticles (NPs) in aqueous media, a light sheet type dark-field microscopy system combined with a microfluidic concentration gradient generator (μFCGG-LSDFM) was developed. Various concentrations of colloidal Au NPs were simultaneously generated with the μFCGG and characterized with the LSDFM setup. The number concentrations and hydrodynamic size distributions were measured via particle counting and tracking analysis (PCA and PTA, respectively) approaches. For the 30 nm Au NPs used in this study, the lower detection limit of the LSDFM setup was 3.6 ng/mL, which is about 400 times better than that of optical density measurements under the same μFCGG system. Additionally, the hydrodynamic diameter distribution of Au NPs was estimated as 39.7 ± 12.2 nm with the PTA approach, which agrees well with DLS measurement as well as the manufacturer’s specification. We propose this μFCGG-LSDFM setup with features of automatic generation of NP concentration gradient and real-time monitoring of their physico-chemical characteristics (e.g., number concentration, and hydrodynamic size distribution) as an important component of future high-throughput screening or high-content analysis platforms of nanotoxicity.

Key Words: Light sheet dark-field microscopy, Microfluidic concentration gradient device, Particle tracking analysis, Real-time monitoring, Colloidal nanoparticles

Introduction

During the last few decades, colloidal nanoparticles (NPs) become increasingly important in various application areas. For instance, they are used as contrast agents for various bioimaging techniques and drug carriers to target cells of living organisms. Widespread use of NPs has also induced increasing concerns about their potential harmful effects on environment and human health. However, due to the ever-increasing numbers of NPs and related consumer products, toxicity assessment of all these novel NPs using currently available testing methods seems impractical. Therefore, high-throughput screening (HTS) and high-content analysis (HCA) approach is considered as promising alternative for the efficient toxicity assessment of NPs and screening NPs with higher potential toxicities for further in vitro and in vivo testing. Currently, high-density multi well plates are typically used as a platform for high-throughput screening. However, as the number of wells per plate has increased, many technical issues have arisen, such as practical limitations in handling sub microliter liquids in an open well system and further increasing well-densities per plate. Among the many alternatives, microfluidic technology is considered as one of the most promising approaches, which can provide miniaturized, integrated and automated HTS platform to enhance the efficiency and reduce the assay cost, labor, chemical reagents and toxic wastes. For instance, we recently reported microfluidic image cytometry (μFIC) to assess toxicities of Cd2+, Paclitaxel, and Ag NPs. However, in addition to these μFIC approach to quantify cellular responses based on their morphology, absorbance, and fluorescence signals, it is urgently needed to develop systems to monitor physicochemical properties (e.g., concentrations, size distribution, agglomeration, sedimentation, etc.) of NPs for further improvement of microfluidic based platform for high-throughput nanotoxicity screening.

Generally, transmission electron microscopy (TEM) has been employed to characterize core size, shape, and aggregation of NPs and inductively coupled plasma optical emission spectrosocpy or mass spectrometry (ICP-OES or ICP-MS) have been also used to determine the composition of colloidal NPs and their distributions in biological tissues. However, they are basically ex situ techniques with limitations of time-consuming sample preparations approach. Therefore, real-time monitoring of NPs’ physicochemical properties (e.g., number concentrations, hydrodynamic diameter, agglomeration and sedimentation) in relevant physiological conditions and miniaturized HTS system should have great importance in developing further applications in biological, medical, and toxicological studies. Dynamic light scattering (DLS) has been widely used for the characterization of the hydrodynamic size distribution of NPs. However, DLS is also known to have limitation in determining hydrodynamic sizes of the NPs suspensions with multimodal size distributions, which may cause problems in testing typical NPs with high degrees of aggregation/agglomeration. On
the other hand, particle counting and tracking analysis (PCA & PTA) technique\textsuperscript{15-18} has been suggested as a promising technique for real-time monitoring of colloidal NPs.\textsuperscript{19-23} PCA can be used to obtain number concentrations of NPs via image acquisition and analysis procedure, while hydrodynamic diameter of NPs can be estimated via PTA technique from the average displacement within a fixed time frame due to Brownian motion. In contrast to DLS, PTA approach has advantages in characterizing NPs with multimodal size distributions, as it measures individual particle properties rather than the averaged properties.\textsuperscript{14}

In this study, for the development of a microfluidics-based nanotoxicity assessment platform, a light sheet type dark-field microscopy system was combined with a microfluidic concentration gradient generator (\textmu FCGG-LSDFM) and real-time monitoring of colloidal Au NPs in aqueous media was performed using particle counting and tracking analysis (PCA & PTA) approaches. The \textmu FCGG-LSDFM system proposed in this study will be an important component of future HTS or HCA platforms for nanotoxicity assessment.

**Experimental**

**Configuration of the \textmu FCGG-LSDFM System.** The \textmu FCGG-LSDFM system was constructed to detect scattered light from NPs in a dark-field illumination mode. The schematic diagram of the \textmu FCGG-LSDFM system is presented in Figure 1(a), which consists of 1) a blue laser (MGL-III-473, CNI Inc., Changchun, China), 2) an optical system for light sheet (LS) formation, 3) a microfluidic device as sample cells, and 4) an inverted-type microscope (IX71, Olympus, Tokyo, Japan).

The optical system has three mirrors (Cat. No. 21004, Chroma, VT, USA), a cylindrical lens (LH1695RM, Thorlabs, NJ, USA) and an illumination objective lens (UPlanFLN, Olympus, Tokyo, Japan). In this optical system, the blue laser light is guided into a cylindrical lens by three mirrors, M1, M2 and M3, and the cylindrical lens transforms this circularly-symmetrical Gaussian beam into an elliptically shaped beam. The illumination objective lens then focuses this elliptical shape beam into a light sheet within the microfluidic channels, where the focal plane of the detection objective lens is located. The microfluidic device is composed of two inlets, the microfluidic concentration gradient generator (\textmu FCGG), and eight outlet channels with different concentrations of Au NPs (Figure 1(b)). The scattered light from NPs is then collected using the inverted-type microscope equipped with a detection objective lens (LUCPlanFLN, Olympus, Tokyo, Japan), x-, y-, z- adjustable sample stage (Thorlabs, NJ, USA) and a CCD camera (Fast 1394, QImaging, BC, Canada) (Figure 1(c) and (d)).

**Fabrication of the Microfluidic Device.** The microfluidic device used in this study was made of polydimethylsiloxane (PDMS) using a typical, soft lithography technique. SU-8 50 photore sist (Microchem, MA, USA) was spin-coated and photolithographically patterned on a silicon substrate. After
patterning, Sylgard 184 PDMS prepolymer and curing agent (Dow corning, MI, USA) were mixed in a 10:1 weight ratio and these mixtures were poured on a patterned substrate in Petri dish. Then, they were degassed in a vacuum chamber for 10 min before being incubated at a 60 °C oven for 2 h. Holes for the inlets were punched out of the PDMS layer using flat-tip needles and outlet channels with volumes of 20 μL (2 mm × 5 mm × 2 mm) were made by casting with Teflon mold during the curing process. The PDMS was bonded to a glass coverslip (24 mm × 60 mm) via oxygen plasma treatment (CUTE-B, FemtoScience, South Korea).

Dark Field Image Acquisition and Analysis. All time-lapse dark-field images were processed with ImageJ (ver. 1.41a, NIH, USA) and the Interactive Data Language (IDL) software package (ver. 6.3, Exelis Visual Information Solutions, CO, USA). The image acquisition rate and exposure time were controlled by using the Micro-Manager software package (ver. 1.3, UCSF, CA, USA). Typically, the exposure time was set at 10 ms and the time between images was maintained at 10 ms. The field of view for particle counting and tracking analysis were approximately 250 × 250 pixels, which typically contain 50-100 particles per image. ImageJ macro was modified for counting number of particles in hundreds images, automatically. Extracting x- and y- positions of each NP, and PTA were performed by using the IDL particle tracking code developed by Crocker, and Weeks.

Optical Density Based Concentration Measurement. The concentration of colloidal particles can be calculated from the optical density (OD) measurements according to the Beer-Lambert law. Although the OD values are generally measured using spectrophotometer, conventional bright field microscopy was used in this study. The sample was injected in a microfluidic chamber with 1 mm height. The image was captured with exposure time of 16 ms. The average transmission values of the selected regions was obtained by ImageJ and then OD value was obtained in triplicates.

Results and Discussion

Light Sheet Characterization. Since the scattered light intensity by colloidal NPs rapidly decreases as the particle size reduces, detection of the scattering signal from a small nanoparticle requires a very good signal-to-noise ratio. To achieve a good signal-to-noise ratio in LSDFM, a thin-focused light sheet, illuminating the sample perpendicular to the detection pathway, is required, such that only a narrow region near the observation plane is illuminated, while samples outside this observation plane are not illuminated and do not generate background signals. Furthermore, samples outside the observation plane are not damaged by the laser radiation, which is one of the important benefits of LSDFM in contrast to the bright-field illumination or epi-illumination microscopes. Thus, light sheet microscopy with a very thin illumination volume is required for achieving small nanoparticle counting and tracking in an aqueous solution. In literature, the thickness of the light sheet was reported as 1-10 μm (z-axis).

In this study, the light sheet was obtained by directing an elliptical beam formed by a cylindrical lens to the illumination objective lens. As previously mentioned, since the dimension of light sheet defines the field of view and the optical sectioning thickness of this LSDFM system, the thickness and width of the light sheet was carefully measured using 1 μM fluorescein solution. As shown in Figure 2, the light sheet used in this study has a horizontal width of 251.7 μm and vertical thickness of 4.05 μm, which are similar with those reported in literature and within an appropriate range for our purpose.

Visualization of Au NPs in Aqueous Solution. By using the above mentioned LSDFM setup, stacks of dark-field images were collected for suspensions of 30 nm Au NPs with various concentrations. In Figure 3, a representative dark-field image and time-dependent changes in the signal-
to-noise ratio (SNR) are presented. The SNR of each image, which is calculated based on the following formula, was $13.10 \pm 2.46$.

$$\text{SNR} = \frac{\langle I_{\text{particle}} \rangle - \langle I_{\text{background}} \rangle}{\sigma_{\text{background}}} \quad (1)$$

where $I_{\text{particle}}$ is the intensity of the particle image, $I_{\text{background}}$ is the intensity of the background, angle brackets refer to the statistical average of the contained quantity and $\sigma_{\text{background}}$ is the standard deviation of the background. Each SNR value plotted in Figure 3(b) was obtained from approximately 1000 particles, and they were maintained nearly constant over the observation period, confirming that the LSDFM setup provide reproducible and reliable data for colloidal NPs in an aqueous solution.

Concentration and Size Distribution Measurement. One of the easiest ways to measure metallic NP concentrations in microfluidic device are the optical density (OD) based measurements of NP concentrations. For comparison purposes, the OD measurement approach using conventional bright field microscopy was used to determine concentrations of Au NPs. However, as shown in Figure 4(a), the OD measurement showed decent linearity down to the level as low as ug/mL level with a lower detection limit of 1.5 $\mu$g/mL. In contrast, when using the LSDFM setup combined with the PCA approach, the LSDFM setup is capable of detecting Au NPs at the ppb level with a lower detection limit of 3.6 ng/mL. This sensitivity enhancement (~400 times) confirmed us that the LSDFM is better than the conventional OD measurement methods for the detection of NPs at lower concentrations.

In addition to this PCA approach, image stacks were further analyzed with PTA approach. With suitable image adjustment settings, NPs in each image were identified and located on a frame-by-frame basis and their root mean squared displacements were determined while trajectories of individual NPs were tracked. Among the trajectories of NPs, those with a sufficiently long lifetime were selected to ensure accurate PTA results. The diffusion coefficient (D) and hydrodynamic diameter (d) of Au NPs could be determined by the following Stokes-Einstein equation:

$$D = \frac{k_B T}{3 \pi \eta d} \quad (2)$$

where $k_B$ is Boltzmann’s constant, $T$ is temperature and $\eta$ is the viscosity of water. Thus, from the PTA of the image stack, the particle size distributions can be estimated. As presented in Figure 4(b), particle size distributions of the 30 nm colloidal Au NPs suspension was estimated as $39.7 \pm$...
12.2 nm, which agrees reasonably well with the manufacturer’s specification.

**Application in the Microfluidic Chip.** By using μFCGG-LSDFM setup, the number concentrations and hydrodynamic diameter distributions of colloidal Au NPs were monitored. As shown in Figure 1(b), the device is consisted of two inlets, a CGG, eight channels for NP monitoring with LSDFM setup. Au NPs with concentration, 93.8 ng/mL and deionized (DI) water were injected through each inlet, respectively and then concentration gradient of Au NPs was generated via laminar flow and diffusive mixing of media and NP streams in a CGG, which was composed of six successive steps of mixing to ensure complete mixing of NPs. Then, stacks of Au NPs with concentration, 93.8 ng/mL and deionized water were injected through each inlet, respectively and then concentration gradient of Au NPs was generated via laminar flow and diffusive mixing of media and NP streams in a CGG, which was composed of six successive steps of mixing to ensure complete mixing of NPs.

As shown in Figure 1(b), the device is consisted of two inlets, a CGG, eight channels for NP monitoring with LSDFM setup, the number concentrations and hydrodynamic diameter distributions of colloidal Au NPs were monitored.

Because of these capabilities to form linear concentration gradients via tree-shaped microfluidic network design, the needs for labor-intensive manual operations or high cost robotic-based HTS systems for the nanotoxicity assessment will be significantly reduced. Various microfluidics-based approaches for quantifying cellular responses (e.g., morphology-, absorbance-, and fluorescence-based μFIC) can be combined with this μFCGG-LSDFM setup, in which quantitative dose-response relationships of NPs can be investigated via measuring physicochemical properties and biological responses of NPs. We believe that this development of μFCGG-LSDFM system with features of automatic generation and real-time monitoring of colloidal NP concentration gradient can be used as an invaluable component of future high-throughput screening or high-content analysis platforms of nanotoxicity assessment.

**Conclusion**

In this study, a μFCGG-LSDFM setup was constructed for a real-time monitoring and sensitive detection of colloidal NPs. The μFCGG-LSDFM system was used to continuously generate concentration gradients of colloidal NPs and monitor their concentrations and hydrodynamic diameter distributions using PCA and PTA approaches. Particularly, we have demonstrated that this system is able to continuously generate and monitor number concentrations and hydrodynamic diameter distributions of 30 nm Au NPs with a lower detection limit of 3.6 ng/mL, which is about 400 times better than that of OD based measurements under the same μFCGG system. Furthermore, this system has greater potential as a key component of nanotoxicity assessments platform. By combining with the recently reported μFIC approach for quantifying cellular responses (e.g., absorbance-, and fluorescence-based μFIC) can be combined with this μFCGG-LSDFM setup, in which quantitative dose-response relationships of NPs can be investigated via measuring physicochemical properties and biological responses of NPs. We believe that this development of μFCGG-LSDFM system with features of automatic generation and real-time monitoring of colloidal NP concentration gradient can be used as an invaluable component of future high-throughput screening or high-content analysis platforms of nanotoxicity assessment.

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