PROCEEDINGS OF THE INTERNATIONAL CONFERENCE NANOMATERIALS: APPLICATIONS AND PROPERTIES
Vol. **1** No 3, 03PCSI04(3pp) (2012)

Characterization of Nanomaterials in Liquid Phase for Nanotoxicological Assessment

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(Received 4 June 2012; published online 18 August 2012)

Nanosized particles are currently of interest due to their attractive physical, photochemical, and catalytic properties. However, such small size particles could possess not only unique and advantageous properties, but could also have potentially new types of toxicity to humans. To estimate the true nano toxicity, it is therefore improtant to perform carful characterization of nano colloidal suspension.

To elucidate important parameters for nano toxicity assessment of nano colloidal materials, experiments were carried out using pulsed field gradient nuclear magnetic resonance (PFG-NMR), asymmetrical flow field-flow fractionation (AFFFF), and dynamic light scattering (DLS) methods.

For the assessment of the in nano toxicity, the amounts of total and bulk bovine serum albumin (BSA) molecules in nano colloidal suspensions were determined using the PFG-NMR and AFFFF methods. Because the amount of bulk BSA molecules in the cell culture medium is a significant factor in inducing cell growth and because BSA can strongly adsorb onto the nano sized particles, this value is an important parameter for in vitro toxicological assessment. In addition, structural analysis of the colloidal nanomaterials were successfully performed using AFFFF-multi angle light scattering (MALS) and DLS methods.

Keywords: PFG-NMR, DLS, FFFF, Nanotoxicity, Liquid phase.

PACS number: 81.15.Lm

1. INTRODUCTION

In this decade, a numerical number of experimental, theoretical attempts on nano sized materials have been widely performed [1-7]. Nano sized materials are currently a subject of interest because of their attractive physical, photochemical, and catalytic properties. Their potentials of small sized materials are increased with decreasing of the sizes of the materials, therefore, the number of studies on the reduction of the nano sized materials are produced acceleratory. For example, a good photocatalyst needs a large catalytic surface area since the size of the primary size of the catalyst nanoparticle defines the surface area available for adsorption and decomposition of the organic pollutants [3].

However, such a small size induced not only a novel superior properties but also entirely new risk on human health [8]. The unexpected adverse effects of nano materials on human health are received a lot of attentions, therefore, many international organizations and researchers have been already carried out nano-toxicity assessments for various nanoparticles (metal, metal oxide, fullerenes, and carbon nanotubes) [9-12]. Those researches were shown that the small sized particles were possible to insert human body and induced toxicity [13], on the other hand, little toxicity of nanoparticles was reported by other researchers [14]. Poor characterization of the size of nanoparticles in suspension, different dispersion protocols, and different particokinetics in suspension for in vitro risk analysis [12], might be some of the reasons why such incoherence have been coming out.

2. SIZE IS IMPORTANT FACTOR FACTOR FOR NANO-TOXICITY ASSESSMENT

One of the most significant factors for recognition of the toxicity of nanomaterials is size. Commercial nanoparticles are commonly provided in dry powder form and the sizes of the primary nanoparticles are determined using microscopic techniques or the Brunauer, Emmett, and Teller (BET) method; however, nanoparticles are easily aggregated or agglomerated in a cell culture medium for *in vitro* toxicity assessment, because the high ionic nature of the solution and the electrostatic/van der Waals interaction between protein and nanoparticles results in the formation of secondary particles [15-18]. The hydrodynamic sizes of secondary nanoparticles in dispersion have a dramatic effect on cell response to exposure; therefore, not only the size of the primary nanoparticles, but also the size of the secondary nanoparticles, could be used as a characteristic parameter to determine the *in vitro* toxicity of nanoparticles in a cell culture medium [19].

The transport rate of particles to cells strongly affects the amount of uptake of particles by the cells; therefore, estimation of the transport processes of nanoparticles to cells for *in vitro* assessment is significant. It would be assumed the two transport modes of particles to cells for *in vitro* assessment; diffusion and gravitational settling (sedimentation) [20]. Although shape affects particle buoyancy, the settling convection increases due to local collections of particles, and the presence of proteins in the culture media can affect the settling rate. Diffusion processes should apply to nanosized materials, whereas submicron sized materials settle on the cells, because the diffusion rate of nanosized materials should be faster than that of micro-sized materials, and the sedimentation rate of micro and submicron sized materials is faster than that of nanosized materials. The determination of both secondary particle sizes and the transport mode of particles is therefore one of the key to prevent misinterpretation of *in vitro* toxicity assessment for nanomaterials.

3. CHARACTERIZATION BY DYNAMIC LIGHT SCATTERING

Dynamic Light Scattering (DLS) is widely used to determine the size of Brownian nanoparticles in colloidal

suspensions in the nano and submicron ranges [21-25]. When particles are dispersed in a liquid phase, they are in constant random motion, that is, Brownian motion, in which a given particle undergoes random position changes in time. The diffusion of spherical particles can be described by the Stokes-Einstein equation,

$$D = \frac{k_B T}{3\pi\eta d} \quad (3.1)$$

where k_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent, D is the diffusion coefficient of the particles, and d is the diameter of the particles. According to the Stokes-Einstein assumption, small particles move quickly by diffusion.

We have previously established a practical protocol for determination of the size of secondary particles and transport rate of particles to cells for *in vitro* toxicity assessments using the Dynamic Light Scattering (DLS) method [26-27]. The protocol of size determination of particles by DLS includes assessment of the DLS measurement reproducibility, change of the size of secondary nanoparticles during a period of *in vitro* toxicity assessment, and the difference in size of the secondary nanoparticles (determined using different DLS analytical procedures). Processes associated with particles in a suspension could be investigated by examining changes in the size and light scattering intensity of secondary nanoparticles during *in vitro* toxicity assessment using this protocol.

In previous study [27], various metal oxide particles with nanoscale primary particle sizes were characterized using the established protocol; however, all metal oxide particles in the culture medium resulted in submicron sized secondary particles, as confirmed by DLS measurement. Although these larger sized particles were expected to be settled faster by gravitation than by diffusion, less sedimentation was observed than theoretically expected in culture medium, which indicates that the effective densities of metal oxide secondary nanoparticles are lower than the corresponding true densities of pure metal oxide particles. Namely, stably dispersed secondary metal oxide nanoparticles with slow gravitational settling kinetics are induced by secondary nanoparticles consisting of small amounts of metal oxide particles and large amounts of protein, which results in lower particle densities than the pure metal oxide particles.

4. CHARACTERIZATION OF ADSORPTION ABILITY OF PROTEIN ON NANOMATERIALS BY FLOW FIELD-FLOW FRACTIONATION

The accurate assessment of the adsorption ability of the protein is significant for *in vitro* toxicity assessments since cell proliferation is strongly inhibited by a lack of protein caused by the influence of the adsorption ability of metal oxide nanoparticles [15, 16, 28], and the amount of bound protein strongly affects on the gravitational settling kinetics of secondary nanoparticles [27]. Furthermore, the adsorbed surfactants or protein molecules induced a high stability of nanomaterials in culture medium because of a steric interaction between protein molecules adsorbed on the nanoparticles [29, 30]. For example, the protective layer of protein prevents aggregation of the carbon nanoparticles, as has been reported previously for fullerene and carbon nanotubes [31, 32]. The observed zeta potentials of secondary nanoparticles (nanocarbon and metal oxide) in culture medium dispersion are between -15 to 0mV, again indicating that the stability of the secondary nanoparticles in the culture medium is maintained by steric interactions between the small amount of adsorbed protein molecules on the nanoparticles, while the effect of electrostatic interactions between adsorbed proteins was minimal. The studies of such protein adsorption to nanoparticles have therefore already begun to provide insights into the interaction between nanoparticles and proteins using techniques such as infrared spectroscopy, mass spectrometry, fluorescence spectroscopy, and size exclusion chromatography methods [33-37].

Flow field-flow fractionation (FFFF) is an elution technique wherein nanoparticles and macromolecules are separated by flow control in an aqueous solution.[38] In FFFF, the retention time, tr, of the nanoparticles can be predicted by Giddings' theory. According to this theory, the retention time, t_r, of the corresponding nanoparticles is represented by

$$t_r = \frac{\pi\eta d w^2}{2k_B T}\frac{V_C}{V_0} \quad (4.1)$$

where $k_B T$ is the thermal energy, η is the carrier elution viscosity, d is the diameter of the nanoparticle, w is the channel thickness, V_0 is the volumetric flow rate of the channel-flow, and V_C is the cross-flow rate. Thus, when the cross-flow and channel-flow rates are constant, size separation of the nanoparticles can be carried out, and the retention time is proportional to the size of the nanoparticles and molecules in the FFFF system [39]. FFFF can therefore be an effective method to separate free protein molecules from colloidal nanoparticles such as bound protein and fullerene molecules during a short elution time, i.e., 15 min for one measurement.

It is common knowledge that carbonaceous nanosized materials should have a high affinity for protein in the absence of surface modification, in the case of carbon black and nanodiamond, the calculated weight ratios between protein molecules and carbon black/nanodiamond nanoparticles were calculated to approximately 1:2 by FFFF measurement [30]. The observed ratios did not change depending on the concentration of the carbon nanomaterials, suggesting that the surfaces of carbon black and nanodiamond were fully covered at this ratio. For carbon black and nanodiamond, the increase in particle size corresponds to a thickness of the adsorbed BSA layer, it is called protein corona, of 20 – 30 nm. This result provided significant information that is expected to be useful for toxicity studies on nanomaterials.

Furthermore, it is possible to perform a structural analysis of the secondary nanoparticles with adsorbed protein in a cell culture medium using FFFF and Multi Angle Light Scattering (MALS) :Static Light Scattering (SLS) methods. Not only the size but also the shape of the resulting colloidal structure that forms by adsorption of protein in the culture medium with nanoparticles is also very significant factors for in vitro toxicity

assessment because nanoparticles with different geometric structures exhibit quite different cytotoxicity and bioactivity in vitro [40]. Since fitting to the scattering profile from a sample with a separated narrow size distribution of colloidal particles is preferable to determine reliable structure in the principle of SLS analysis, the FFFF-MALS system is effective methods to estimate the structure of secondary nanoparticle. For example, the structural analysis of the fullerene colloidal nanoparticles in culture medium was previously performed and it was found that the colloidal fullerene particles adopted a flexible polymeric structure [41]. On the other hands, the colloidal fullerene aqueous dispersion in water took a hard spherical structure [39]. Thus, this significant difference of the structures of nanoparticles in water and culture medium may provide a significant information in terms of *in vitro* toxicity assessments that may affect the inherent toxicity of nanoparticles.

5. CONCLUSION

The appropriate combination of the various characterization methods for nanomaterials should be taken into consideration by researchers performing accurate toxicity studies with nanosized materials and would be of benefit for the interpretation of the toxicity assessment results. Furthermore, the accurate selection of the characterization method of nanomaterials also plays an important role in producing a novel application of nanomaterials in research of functional / industrial materials.

AKNOWLEDGEMENTS

The financial support from the New Energy and Industrial Technology Development Organization (NEDO) is gratefully acknowledged.

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