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On-Chip Evaluation of Shear Stress Effect on Cytotoxicity of Mesoporous Silica Nanoparticles

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Abstract

In this work, nanotoxicity in the bloodstream was modeled and the cytotoxicity of sub-50 nm mesoporous silica nanoparticles to human endothelial cells was investigated under microfluidic flow conditions. Compared to traditional *in vitro* cytotoxicity assays performed under static conditions, unmodified mesoporous silica nanoparticles show higher and shear stress-dependent toxicity to endothelial cells under flow conditions. Interestingly, even under flow conditions, highly organo-modified mesoporous silica nanoparticles show no significant toxicity to endothelial cells. This paper clearly demonstrates that shear stress is an important factor to be considered in *in vitro* nanotoxicology assessments and provides a simple device for pursuing this consideration.

Keywords

microfluidic; nanoparticle; nanotoxicity; mesoporous silica

INTRODUCTION

Nanoparticles (NPs) have drawn significant attention in biological and medical sciences due to their promising characteristics for targeting, diagnosis, and drug delivery applications.^{1–4} Toward realizing these NP applications, there have been intense *in vitro* and *in vivo* studies on the effects of NPs on biological systems. Unfortunately, there is poor correlation between *in vitro* and *in vivo* NP toxicity results,^{5–6} and accordingly, there is a great need for a new *in vitro* platform that better models relevant *in vivo* systems, facilitating a more accurate and deeper understanding of cell-NP interactions and the implications for resulting toxicity. One likely cause of the discrepancy between *in vivo* and *in vitro* assays is the nature of the environment in which the assays are performed. *In vivo* environments are very complex and dynamic while *in vitro* environments are likely too simple and static. There are many ways to move *in vitro* assays toward a better mimic of the *in vivo* environment,^{6–13} and in this work, the dynamic nature of the *in vivo* environment is considered.

Fluid flow is a critical feature in our body and known to be related to a variety of cellular behaviors.^{14–16} Flow may be especially important in considerations of NP toxicity as, in addition to the generated forces on the cells (i.e. shear stress onto endothelium) by flow, it will likely influence cell - NP contact time. To date, a small collection of studies have investigated particle interaction with cells under flow conditions as an indicator of leukocyte adhesion to the endothelium^{17–23} but the direct effect of flow conditions on cell-NP interaction has largely gone unconsidered.

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Toward achieving physiological relevance in the study of cell-NP interaction under flow conditions, the cell type employed herein is human endothelial cells, the type of cells that line blood vessels. They are known to have flow-dependent behavior, such as morphology changes and activation,^{14–15} and will come in direct contact with injected NPs in the case of intravenous NP injection. The microfluidic system described herein enables human endothelial cell culture inside the device and investigation of NP-endothelium interactions under more *in vivo*-like, dynamic conditions.

While many different nanoscale materials are being used or considered for use in consumer and/or therapeutic products, the nanoparticles chosen for initial consideration herein are mesoporous silica (MS) NPs. These materials are a promising candidate for controlled and stealth drug delivery based on their materials characteristics, including size control, large internal surface area and easy surface modification.^{24–25} Prior to practical use in clinics with intravenous injection as a delivery method, however, the biocompatibility of MS NPs under dynamic flow conditions should be carefully examined and well characterized. To date, researchers have found that the MS NPs are nontoxic to mammalian cells at exposure concentrations less than 200 µg/mL,^{24,26–28} but all of these studies were performed under static conditions. Here, MS NPs are used as a model to study the shear stress effect on the cytotoxicity of NPs due to ease of uniform NP diameter control, incorporation of fluorescence or magnetic function, and surface modification via silane coupling reactions. In this paper, two types of fluorescent MS NPs with diameter less than 50 nm - unmodified fluorescein incorporated MS NPs (denoted as FMS) and organically modified FMS NPs - were synthesized and their cytotoxicity in human endothelial cells were studied in a microfluidic device.

RESULTS AND DISCUSSION

Herein, a microfluidic system is employed to investigate cell-NP interactions under controlled flow conditions (Figure 1). The microfluidic system consists of a simple channel, but is an integrated platform on which cell culture, exposure to experimental conditions, imaging, and a cell viability assay are performed. Figures 1a–c show the complete device, the design, and a schematic of the platform, respectively. The microfluidic channel is fabricated in polydimethylsiloxane (PDMS), and the uncomplicated design simplifies device production, fluid modeling and control, and device operation with the accurate control of flow. The shear stress levels targeted in this work range from 0 to 6.6 N/m², based on physiologically relevant shear stress levels in various vessels of the human body.^{16,29–30} The shear stress levels at 75 µL/min flow rate with varying channel dimensions were determined using the equation,

$$\tau = \frac{6\mu Q}{wh^2} \quad (1)$$

in which μ , Q , w and h represent dynamic viscosity of the media (0.00084 Pa·s), volumetric flow rate (75 µL/min), channel width, and channel height, respectively.²⁹ The calculated values for various devices used herein can be found in Table 1. While the equation above is generally accepted and used to estimate shear stress, this equation is obtained with an assumption that the channel width is infinite (i.e., that there are no side wall effects).³³ Accordingly, the numbers obtained using equation (1) were confirmed using computational fluid dynamics (CFD) (Figure 1d). CFD provides steady state modeling of fluid rheology with given channel dimensions, allowing quantitative analysis of fluid rheology parameters on all four sides of the channel. COMSOL Multiphysics 4.1 software was used to produce a

single phase laminar flow model with given channel dimensions and fluid properties at 37 °C. The model was simplified in length (set at 500µm rather than the actual channel length of 20 mm) to reduce the amount of time for computing, and the volumetric flow rate at the inlet was set at described in Table 1. The shear stress patterns in all channel geometries were the same as shown in Figure 1d; at the bottom channel surface, the effective zone (the zone where the shear stress level changes less than 1 unit from the reported value) of shear stress across all channel geometries was ~10 µm thick at minimum, indicating that the endothelial cells, which are sitting on the bottom surface of the channel, experience the intended shear stress level. All the modeled values reported herein are the maximum shear stress values from each model geometry. According to Table 1, the COMSOL modeling confirmed that accurate control of shear stress in a physiologically relevant range could be achieved in endothelial cell-coated device channels. The channels with 150 µm width showed slightly higher shear stress level in modeled results than in the calculated ones (e.g. 6.6 N/m² calculated versus 7.3 N/m² modeled for 5 the 150W80H case), and the geometries with 300 µm width (higher aspect ratio than 150 µm cases) showed more accurate shear stress control, indicating that high aspect ratio devices may be advantageous for controlling shear stress in a device in future work, but further optimization will be necessary to find the channel dimensions that provide physiologically relevant environments with fine control of shear stress. The unmodified FMS and organically modified FMS NPs were synthesized using our published and recently developed methods.^{4,34} All details of synthesis and characterization are described in the Supporting Information (SI). The transmission electron microscopy (TEM) images of FMS NPs and polyethylene glycol(PEG)/trimethyl silane(TMS)-modified fluorescent MS NPs (denoted as FMS@PEG/TMS) show that both FMS and FMS@PEG/TMS have similar diameter (~40 nm) (Figure 2a and b). In addition, the TEM images and XRD patterns (Figure 2c) reveal that the pore structure inside FMS@PEG/TMS NPs is not as ordered as FMS, confirming a high amount of organic silane modification on either the outer or interior surface of FMS NPs. The detailed pore structure of FMS and FMS@PEG/TMS is shown in Figure S2. Compared to the hydrodynamic diameter of FMS NPs (~200 nm) in serum-free cell culture medium (Dulbecco's modified Eagle's medium, DMEM), the FMS@PEG/TMS NPs have smaller hydrodynamic diameter (~60 nm), showing that unmodified FMS NPs agglomerate in a highly salted environment while the PEG/TMS modification prevents agglomeration of FMS NPs (Figure 2d). The incorporated fluorophore, fluorescein isothiocyanate (FITC), facilitated fluorescence imaging to assess NP association with the endothelial cells under controlled shear stress conditions. Figures 3a, 3e and 3b, 3f show bright field and fluorescence micrographs, respectively, obtained after 2 hour endothelial cell exposure to FMS NPs at different shear stress levels following a PBS wash to remove non- or weakly adherent NPs. The fluorescence imaging confirms that a portion of the presented FMS NPs were strongly associated with endothelial cells under both conditions, while no significant amount of FMS@PEG/TMS NPs was observed under the same flow conditions (Figures 3g and 3h).

To investigate the cytotoxicity of these associated FMS NPs to endothelial cells under various shear stress conditions, an *in vitro* viability assay (the methylthiazolyldiphenyl-tetrazolium bromide or MTT assay) was performed. The MTT assay is a well-established method used to assess cytotoxicity of both molecular toxins and NPs in many studies.³⁵ While the MTT assay has been shown to produce false positive results in the presence of some nanoscale materials,³⁶ previous work has clearly established that it is an appropriate choice for toxicity assessment of silica NPs.³⁷ A NP concentration of 200 µg/mL in serum-free cell culture media was chosen for comparison with previous work where significant toxicity was apparent in endothelial cells after 24 hour incubation in static conditions.^{24,26–28} A direct comparison of cytotoxicity was performed under static conditions and shear stress conditions, with shear stress ranging from 0.5 to 6.6 N/m². Within the healthy human body, arterioles (< 200µm in diameter) and capillaries (< 20µm in

diameter) experience an average of $\sim 5 - 6 \text{ N/m}^2$ shear stress, but these values can fluctuate depending on the person and condition.^{16,29–32} Thus, the shear stress values used here represent NP exposure conditions upon introduction into normal or abnormal arterioles or capillaries. Figure 4a shows the toxicity results obtained from both static and shear stress conditions. Endothelial cells were exposed to the unmodified FMS NP stream for two hours, and as shown in the figure, unmodified FMS NPs were significantly more toxic to endothelial cells under the two highest shear stress conditions ($88.9 \pm 0.9 \%$ and $70.4 \pm 3.3 \%$ viability at 3.3 and 6.6 N/m^2 , respectively) while no apparent toxicity was found under either the static ($96.5 \pm 1.5 \%$) or 0.5 N/m^2 ($103.4 \pm 0.7 \%$) shear stress conditions with the same exposure time and concentration. These results indicate that shear stress influences cytotoxicity of FMS NPs to endothelial cells, and FMS NPs do not show significant toxicity to endothelial cells under static or very low shear stress conditions. These results indicate that the dynamic nature of the environment (flow conditions) is one of the likely causes of the discrepancy between *in vivo* and *in vitro* nanotoxicity assays. It is a generally accepted concept that NP toxicity is dependent on both NP dose (amount) and exposure (time). Under the flow conditions, however, concentration cannot represent the dose because, at least in this work, the absolute number of NPs passing through the microfluidic channel is larger under flow conditions than static conditions, and therefore, the shear stress-dependency in the toxicity result might not be from the effect of shear stress. To investigate this possibility, the NP dose under flow conditions was estimated and varied by tuning NP concentration under static conditions and channel dimension and flow rate under flow conditions. Taking both flow velocity and NP diffusion length into account, NPs within $1 \mu\text{m}$ above the endothelial cell layer are estimated to have the potential to interact with the endothelial cells. Using this limit, the number of NPs that interact with endothelial cells under the 6.6 N/m^2 shear stress condition was calculated to be 1.1×10^{12} NPs, while the value for the static condition was calculated to be 5.2×10^{11} NPs (Table S1); so, the original experimental conditions led to endothelial cells under the highest shear stress condition interacting with approximately double the number of NPs as in the static condition. To account for this dose difference, three additional experiments were performed; two under static conditions but with $\sim$ two- ($475 \mu\text{g/mL}$, 1.2×10^{12} NPs) and four-fold ($950 \mu\text{g/mL}$, 2.3×10^{12} NPs) higher concentrations of NPs to achieve the same and higher dose as the $200 \mu\text{g/mL}$ exposure under the flow conditions (1.1×10^{12} NPs), and one where flow rates are varied to achieve the same dose as the $200 \mu\text{g/mL}$ (5.2×10^{11} NPs) exposure under static conditions (Table S2, S3). In the latter case, channel dimensions were altered to achieve the same 6.6 N/m^2 shear stress under the lower flow rates used; this shear stress was verified by COMSOL modeling (Table 1). Figures 4b and 4c summarize the cytotoxicity results with the number density of NPs described above (Table S2, S3), and the results confirm that the increased cytotoxicity of FMS NPs under 3.3 and 6.6 N/m^2 shear stress conditions was not from the different dose. Under static conditions, two hour exposure of endothelial cells to 5.2×10^{11} , 1.2×10^{12} , and 2.3×10^{12} NPs results in 96.5 ± 1.5 , 88.9 ± 4.1 , and $88.8 \pm 5.5 \%$ endothelial cell viabilities, respectively. Under flow conditions, on the other hand, 70.4 ± 3.3 , 73.1 ± 3.0 , and $75.9 \pm 4.0 \%$ endothelial cell viabilities were obtained from 1.1×10^{12} , 4.5×10^{11} , and 5.6×10^{11} NPs, respectively. While there is a possible, slight dose effect in cytotoxicity results under static conditions, this assessment clearly confirms that the difference in the toxicity results in the original assessments (static vs. flow) came from the shear stress rather than dose.

One potential explanation for this phenomenon is that increased shear stress induces activation of the endothelial cells. Several studies have reported changes in endothelial cell behavior under various shear stress conditions.^{14–15} In fact, clear morphological changes were observed in this work in endothelial cells under shear conditions of 6.6 N/m^2 compared to static conditions (data not shown). While the specific changes to the endothelial cell population is the subject of future work, it is already clear that flow plays a critical role in the interaction between NPs and cells, making *in vivo* cells vulnerable to silica NP exposure.

With the intention to realize MS NP applications in the human body, the toxicity of FMS NPs must be controlled. One strategy known to ameliorate silica NP toxicity is based on blocking the interaction between surface silanol groups and the cellular membrane. Specifically, modification with low molecular weight poly(ethylene glycol) successfully masks the surface silanol groups in static conditions; herein, we consider the role of this surface modification under flow conditions. The NP cytotoxicity for FMS@PEG/TMS NPs under 3.3 and 6.6 N/m² shear stress conditions were obtained and compared to the previously obtained unmodified FMS NP toxicity results under the same shear stress conditions. In each shear stress condition, the cytotoxicity results indicate that FMS@PEG/TMS NPs do not have significant toxicity (99.11 ± 0.3 and 97.22 ± 1.6 % viability at 3.3 and 6.6 N/m², respectively) to the endothelial cells, revealing that NP surface chemistry plays an important role in flow-mediated cell-NP interactions (Figure 4d). One possible explanation for this is that, while these endothelial cells were activated by shear stress, the PEG surface modification prevented non-specific association of the NPs, resulting in decreased interaction between endothelial cells and NPs, and thus, decreased toxicity.

CONCLUSIONS

In summary, this work systematically compares the cytotoxicity of FMS and FMS@PEG/TMS NPs under flow and static conditions. A simple microfluidic platform enabled this assessment under accurately and precisely controlled shear stress conditions. Unmodified FMS NPs showed clear flow-dependency in their toxicity to human endothelial cells that was not due to the dose of the NPs. In agreement with our previous result,⁴ under static conditions, FMS@PEG/TMS NPs showed no serious toxicity to endothelial cells; the same is true under flow conditions, confirming that the surface modification significantly removes the potential toxic activity of MS NPs. This platform will likely find use in other applications, in which precise shear stress control is necessary, as well as in further investigation of nanotoxicity in more realistic biological environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Complete device (14 single channels on a cover slip) (b) device design, (c) cross-section operation schematic, and (d) an example COMSOL simulation with channel dimensions of 300 μm , 80 μm , and 500 μm for width, height, and length, respectively. Flow rate is set 75 $\mu\text{L}/\text{min}$.

Figure 2. (a, b) TEM images and (c) XRD patterns of surfactant-removed FMS and FMS@PEG/TMS NPs. (d) Hydrodynamic size distribution of FMS and FMS@PEG/TMS measured by dynamic light scattering (DLS) at RT in cell culture medium (200 $\mu\text{g/mL}$).

Figure 3.

Bright field and fluorescence images of endothelial cells exposed to FMS (a, b, e, and f) and FMS@PEG/TMS (c, d, g, and h) NPs for 2 hours at 200 µg/mL concentration in 2 different shear stress conditions: a, c, e, and g are under a shear stress level of 3.3 N/m² and b, d, f, and h are under a shear stress level of 6.6 N/m². Compared to FMS NPs (e and f), FMS@PEG/TMS NPs associate significantly less with endothelial cells than unmodified NPs under the same conditions (g and h).

Figure 4.

MTT assay results after endothelial cell exposure to NPs for 2hrs. (a) Cytotoxicity results for FMS NPs in different shear stress conditions. Concentration of FMS NPs is 200 µg/mL (b) Dose effect in the cytotoxicity of FMS NPs under static conditions (c) Dose effect on the cytotoxicity of FMS NPs in the same (similar) shear stress conditions (d) Effect of PEG/TMS modification on the cytotoxicity of MS NPs. (*, **, and *** represent $p < 0.1$, $p < 0.01$, and $p < 0.0001$, respectively.)

Table 1

Summary of shear stress levels calculated and modeled in the given channel dimensions

Channel dimensions ^a	150W80H	150W65H	300W45H	300W80H	300W200H
Flow rate (μL/min)	75	50	45	75	75
Calculated shear stress (N/m ²)	6.6	6.6	6.2	3.3	0.5
Modeled shear stress (N/m ²)	7.3	7.1	5.6	3.2	0.6

^aW: Width (μm); H: Height (μm)