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Evaluation of *in vitro* toxicity of polymeric micelles to human endothelial cells under different conditions

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ABSTRACT

Polymeric micelles have been extensively studied in the area of antitumor therapy, and more recently explored as nanocarriers for atherosclerosis. These applications of polymeric micelles in biomedicine will increase their contact with human blood vessels. However, few studies have considered the interactions between polymeric micelles and endothelial cells, especially in a complex system. This study used human umbilical vein endothelial cells (HUVECs) as an *in vitro* model for endothelial cells to investigate the toxic effects of methoxy-poly(ethylene glycol)-poly(D,L-lactide) (MPEG-PLA) based micelles. In addition, an endoplasmic reticulum stress inducer thapsigargin (TG), and a pro-atherogenic stimulus palmitate (PA), were used to co-expose HUVECs to further mimic the responses of diseased endothelial cells to micelle exposure. Overall, up to 200 μg/mL micelles did not significantly induce cytotoxicity, reactive oxygen species (ROS), release of inflammatory mediators in terms of interleukin 6 (IL-6), IL-8 and soluble vascular cell adhesion molecule 1 (sVCAM-1), or adhesion of THP-1 monocytes to HUVECs. TG and PA significantly induced cytotoxicity and THP-1 adhesion as well as modestly promoted the release of IL-6, but did not affect ROS or release of sVCAM-1 and IL-8. Co-exposure of micelles did not significantly enhance the effects of TG and PA to HUVECs, and ANOVA analysis indicated no interaction between concentrations of micelles and the presence of TG/PA. Taken together, these data indicated that micelles are not toxic to HUVECs under different conditions *in vitro*.

1. Introduction

With their unique advantages, a number of engineered nanoparticles (NPs) has been produced for biomedical uses, but their potential adverse effects should be carefully assessed to ensure the safe use [1]. Polymeric micelles, formed by amphiphilic copolymers with a hydrophilic shell and a hydrophobic core, are among one of the most commonly produced engineered NPs and have been extensively studied for anticancer therapy [2,3]. Recently, it was also shown that polymeric micelles may be used for atherosclerosis imaging and therapy [4,5]. As a consequence, the applications of polymeric micelles in biomedicine will inevitably increase their contact with human blood vessels, but few studies have concerned their side effects to relevant cells in the circulation system [6]. One of such relevant cells that should be used to understand NP-blood vessel contact is endothelial cell (EC), as suggested by a recent review [6]. The ECs cover the surface area and regulate the blood vessel tone, thrombogenicity, homeostasis, monocytes recruitment, and hormone transport [7]. Furthermore, they also serve as the first contact cells with engineered NPs when the NPs enter the blood [6]. During the early development of atherosclerosis, inflammatory and oxidative stimuli activate ECs, which express adhesion molecules and release inflammatory mediators. Circulating monocytes then adhere to the activated ECs, differentiate into macrophages, engulf excessive lipids and finally lead to plaque progression [7,8]. Whether polymeric micelles will induce adverse effects to ECs needs to be carefully assessed to ensure their safe use in nanomedicine. This should be done not only in 'healthy' ECs, but also diseased ECs, as the response of ECs to NPs could be different due to the diversity of EC types [6]. There may be also a need to assess the

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toxicity of polymeric micelles to ECs in a complex system where the nutrients are present, as the nutrients may also define the nanotoxicological responses [9,10].

The present study investigated the toxicity of micelles to human vein ECs (HUVECs) under different conditions. The HUVECs were used because they have been extensively used as an *in vitro* model for human ECs to understand the interactions of engineered NPs with blood vessels [6]. The empty micelles without any associated drugs were used because a recent study showed that drug-loaded micelles may induce drug-related hematological toxicity [11]. The cytotoxicity was determined by three different methods, namely water soluble tetrazolium-1 (WST-1), neutral red uptake and lactate dehydrogenase (LDH) assay. Oxidative stress was indicated by the measurement of intracellular reactive oxygen species (ROS). The inflammatory response was indicated by the measurement of release of inflammatory mediators, including interleukin 6 (IL-6), IL-8 and soluble vascular cell adhesion molecule 1 (sVCAM-1), as well as the adhesion of THP-1 monocytes to HUVECs. Meanwhile, to induce the injury of HUVECs and mimic the response of ECs under atherosclerotic conditions, thapsigargin (TG) or palmitate (PA) was used to co-expose HUVECs. TG is a classical endoplasmic reticulum (ER) stress inducer, and elevated ER stress has been suggested to play a pivotal role in the pathology of atherosclerosis [12,13]. A recent study showed that ZnO NPs could induce ER stress to HUVECs, which may be related with the cardiovascular effects [14]. PA is one of the basic fatty acids present in human blood. Elevated circulating PA may induce endothelial dysfunction and thus links excessive nutrients to the development of atherosclerosis [13,15]. A recent study showed that the presence of PA may enhance the vascular health effects of multi-walled carbon nanotubes to HUVECs *in vitro*, which indicated an interaction between saturated fatty acids and NPs [16].

2. Materials and methods

2.1. Cell culture

HUVECs (passage 1; purchased from ScienCell Research Laboratories, Carlsbad, CA) were cultured in supplemented endothelial medium and used at passage 3–6 as our previously described [17]. For each experiment, 1×10^4/well (on 96-well plates) or 4×10^4/well (on 24-well plates) HUVECs were seeded on 0.2% gelatin solution (ScienCell Research Laboratories, Carlsbad, CA) pre-coated plates and grown for 2 days prior to exposure. THP-1 monocytes (ATCC) were cultured in RPMI 1640 medium (sodium pyruvate and HEPES added; Gibco, USA) supplemented with 10% FBS and 1% penicillin-streptomycin (P/S) solution in a CO_2 incubator at 37 °C. The cells were used within 3 month to keep their best characteristics.

2.2. Preparation of polymeric micelles

The amphiphilic copolymer methoxy-poly(ethylene glycol)-poly(D,L-lactide) (MPEG-PLA) was synthesized as our previously described with slight modifications [18]. Briefly, 0.5 g lactide monomer (LA) was reacted with 1.0 g methoxy-poly(ethylene glycol) (MPEG) in 1 mL CH_2Cl_2 under nitrogen gas condition, catalyzed by 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD)-CH_2Cl_2 ([M]/[Cat] = 100). Here we used a non-metallic reagent TBD as the catalyst to reduce the possible metallic contaminations in micelles [19,20]. After 48 h reaction under room temperature, the polymer/CH_2Cl_2 solution was precipitated by absolute ether, followed by vacuum drying to remove CH_2Cl_2. To make the micelle solution, MPEG-PLA was dissolved in acetone, followed by drop wise addition of the MPEG-PLA acetone solution into MilliQ water during slowly constant stirring. The cloudy micelle solution was further constantly stirred for 0.5 h, and then rotary evaporated overnight under room temperature to remove acetone. Finally, the stock solution of micelles was made as 2 mg/mL in MilliQ water.

The critical micelle concentration (CMC) of the polymeric micelles was determined by using a fluorescent probe pyrene. A stock solution of pyrene was made as 6 mg/mL in acetone. A working solution was then prepared by adding 0.1 mL of pyrene solution into 1000 mL MilliQ water with constant stirring for 2 h, after which the working solution was placed at room temperature for 2 days to allow the acetone to evaporate. Micelles in MilliQ water were diluted to a series of concentrations from 0.0001 mg/mL to 1 mg/mL and mixed with equal concentrations of pyrene solution (final volume 10 mL). Micelle–pyrene solutions were briefly sonicated in ultrasonic water bath for 15 min and then placed for 12 h under room temperature to allow partition of the pyrene into the micelles. Emission was done from 230 to 360 nm, with 392 nm as the excitation wavelength. The maximum absorption of pyrene shifted from 335.91 to 339.05 nm on micelle formation. The ratio of absorption of encapsulated pyrene (339.05 nm) to pyrene in water (335.91 nm) was plotted as the logarithm of polymer concentrations. The inflection point of the curve was taken as the CMC.

2.3. Characterizations

^{1}H nuclear magnetic resonance (NMR) spectra of the synthesized MPEG-PLA were measured by a Bruker AV-400 NMR spectrometer at room temperature, with $CDCl_3$ as solvent. The molecular weight of MPEG-PLA was measured by gel permeation chromatography (GPC) using a PL-GPC 120 instrument (UK), with tetrahydrofuran (THF) as eluent at a flow rate of 1.0 mL/min.

The hydrodynamic size distribution of the polymeric micelles was measured using 1 mg/mL particles suspended in MilliQ water by Zetasizer Nano ZS90 (Malvern, UK). The hydrodynamic size was measured for three times, and mean ± S.D. (standard deviation) was calculated. The stability of the micelles solution was tested as changes of hydrodynamic size distribution after stored at room temperature for 10 and 20 days. The micelles were used within 20 days for all the experiments in this study as they slowly precipitated after 20-day storage at room temperature.

2.4. Cytotoxicity

The cytotoxicity was measured by using WST-1, neutral red uptake and LDH kits according to manufacturer's instruction (Beyotime, China). WST-1 reagent can indicate the mitochondrial activity as it could be converted to a yellow formazan by mitochondria in living cells. For the assay, HUVECs seeded on 24-well plates were exposed to 0 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL micelles, with or without the presence of 500 nM TG or 200 μM PA (from Sigma-Aldrich, USA). After 24 h exposure, the cells were rinsed once with Hank's solution, and then incubated with 10% WST-1 reagent for 2 h. The absorbance was read at 450 nm with 690 nm as reference by an ELISA reader (Synergy HT, BioTek, USA).

Neutral red is a dye which could be incorporated into intact lysosomes of living cells. For the assay, HUVECs on 24-well plates were incubated with various concentrations of micelles with or without the presence of TG or PA for 24 h as indicated above, rinsed and then incubated with 10% neutral red for 2 h. After rinsed once again, the neutral red incorporated into lysosomes was dissolved in the lysis solution provided by the kit, and the absorbance was read at 540 nm with 690 nm as reference.

LDH is an enzyme in cytoplasm, which could be released into extracellular fluid with the break of membrane. The LDH assay was