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Amine modification of nonporous silica nanoparticles reduces inflammatory response following intratracheal instillation in murine lungs

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HIGHLIGHTS

- We investigated nonporous silica NPs both bare and modified with amine functional groups (3-aminopropyltriethoxysilane (APTES)) in order to evaluate the effect of surface chemistry on biocompatibility.
- No cytotoxicity was observed in a human lung cancer epithelial cell line (A549) for bare silica NPs or amine-functionalized silica NPs.
- Bare silica NPs elicited a significantly higher inflammatory response compared to amine-functionalized NPs.

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ABSTRACT

Amorphous silica nanoparticles (NPs) possess unique material properties that make them ideal for many different applications. However, the impact of these materials on human and environmental health needs to be established. We investigated nonporous silica NPs both bare and modified with amine functional groups (3-aminopropyltriethoxysilane (APTES)) in order to evaluate the effect of surface chemistry on biocompatibility. *In vitro* data showed there to be little to no cytotoxicity in a human lung cancer epithelial cell line (A549) for bare silica NPs and amine-functionalized NPs using doses based on both mass concentration (below 200 μg/mL) and exposed total surface area (below 14 m^2/L). To assess lung inflammation, C57BL/6 mice were administered bare or amine-functionalized silica NPs via intratracheal instillation. Two doses (0.1 and 0.5 mg NPs/mouse) were tested using the *in vivo* model. At the higher dose used, bare silica NPs elicited a significantly higher inflammatory response, as evidence by increased neutrophils and total protein in bronchoalveolar lavage (BAL) fluid compared to amine-functionalized NPs. From this study, we conclude that functionalization of nonporous silica NPs with APTES molecules reduces murine lung inflammation and improves the overall biocompatibility of the nanomaterial.

1. Introduction

Inorganic nanomaterials (<100 nm in size), including silica NPs, have been increasingly utilized in a wide variety of applications due to their unique material properties such as tunable size and ease of surface functionalization (Stöber and Bohn, 1968; He et al., 2008; Bharali et al., 2005a). There are well-established methods for the synthesis of silica NPs in addition to commercial availability that make them prime candidates for applications such as drug delivery (Cao et al., 2010; Stevens et al., 2010; Tang et al., 2012) and biomedical imaging (Benezra et al., 2011; Bradbury et al., 2013). Silica NPs are also commonly used as additives in many products including cosmetics, varnishes and printer toners (Lin et al., 2006; Nel et al., 2006).

Silica materials are composed of silicon dioxide (SiO_2) and are present in crystalline and amorphous forms. Quartz is the most common form of crystalline silica. Amorphous and crystalline silica

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NPs can enter the body through different routes, one of the most common being inhalation of free silica into the lungs which can potentially lead to pulmonary disorders (Rimal et al., 2005). The toxicity of crystalline silica has been studied for many years, especially in connection with chronic bronchitis, emphysema and silicosis (Hnizdo et al., 2002; Hnizdo and Vallyathan, 2003; Ross and Murray, 2004). While toxicity related to inhalation of larger, crystalline silica has been well documented (Hamilton et al., 2008; Reiser and Last, 1979; Saffiotti, 1992), more studies are needed to evaluate amorphous particles in the nanometer size range. Although silica NPs are used in many applications because of their small size and large surface area, these same properties could lead to increased toxicity in biological systems compared to larger silica particles (Nel et al., 2006; Oberdorster et al., 2005). For example, the small size of NPs allows for increased interactions with biological tissues and enhanced cellular uptake (Jiang et al., 2008; Verma et al., 2008; Nel et al., 2006). It is recognized that particles less than 1 μm in aerodynamic diameter penetrate more distally into lung tissue whereas larger particles (>5 μm) deposit primarily in the upper airways (Oberdorster, 2001; Sager et al., 2008). Previously, amorphous silica NPs were considered to be less toxic than crystalline silica; however, recent studies found that amorphous silica particles induce substantial lung inflammation (Park and Park, 2009). In addition to size, surface charge of the NPs could influence NP-cell interactions (Nel et al., 2009; Kairdolf et al., 2008). As the potential for human exposure increases, it is critical to evaluate the safety of nano-sized materials especially in occupational settings where engineered nanomaterials are manufactured or handled in bulk quantity (Seipenbusch et al., 2008). Furthermore, development of methodologies for production of safe silica particles that would produce less toxicity or less inflammation are very important. In the last several years, many efforts have been made in particle surface modification with the aim to decrease their potential toxicity (Shen et al., 2012; van Schooneveld et al., 2008).

Numerous *in vitro* studies have been published in the literature investigating the cytotoxicity of amorphous silica NPs on cultured cell lines (Lin et al., 2006; Nowak et al., 2014; Yang et al., 2010; Ye et al., 2010). While *in vitro* experiments are commonly used to predict the toxicity of engineered silica NPs *in vivo*, there are limitations to the interpretation of the results because of the oversimplified environment of cell culture experiments versus animal models where there is a more complex, physiological environment (Kim et al., 2014; Frohlich and Salar-Behzadi, 2014; Yildirimer et al., 2011). Although the evaluation of the side effects of silica NPs have been investigated previously, there is a lack of data in the literature where both *in vitro* cytotoxicity and *in vivo* inflammatory response studies have been performed using the same batch of silica NPs and reported together in a single study.

Many applications such as gene delivery (Csogor et al., 2003; Bharali et al., 2005b), DNA binding/transport (Kneuer et al., 2000), and biomedical imaging (Kumar et al., 2008) have been developed utilizing amorphous silica NPs where the surface is functionalized with amine groups in order to change the chemical properties of the material. It is important to investigate how the surface chemistry of silica NPs affects the interaction with biological systems and if there is an influence on toxicity as a result of amine functionalization. Here we present *in vitro* cytotoxicity and *in vivo* inflammatory response data in lung models evaluating 50 nm amorphous silica NPs with and without amine-functionalization. Cell cytotoxicity of bare silica NPs and amine-modified silica NPs was measured in human bronchoalveolar cells at three time points. Mice were treated with two different doses of particles via intratracheal instillation and markers of pulmonary inflammation in the lungs were evaluated 24 h after exposure. Silica NP treatments were compared to a crystalline form of silica (Min-U-Sil®5) which is known to be hazardous to human health (Working Group on the Evaluation of Carcinogenic Risks to Humans, 1997; Donaldson and Borm, 1998). Our studies provide evidence that surface chemistry plays an important role in silica NP-induced inflammation in murine lungs.

2. Materials and methods

2.1. Synthesis of silica NPs

Nonporous silica (or Stöber silica) was prepared following a modified procedure from the literature (Stober et al., 1968). In this synthesis no surfactant was used, and ammonia was used as the basic catalyst. In a glass vessel 120 mL anhydrous ethanol (Decon Labs, King of Prussia, PA) was combined with 6.0 mL aqueous ammonia (Sigma–Aldrich, St. Louis, MO) and stirred for 5 min. Tetraethyl orthosilicate (TEOS, 4 mL, Sigma) was used as the silicon source and added to the ammonia/ethanol mixture. The reaction mixture was stirred at room temperature for 24 h and then centrifuged at 11,000 × *g* for 30 min to obtain the products, which were washed three times with water and dried at 60 °C overnight.

2.2. Surface functionalization

Functional groups were covalently attached to the NP surface using a post-synthesis grafting method (Lehman et al., 2014). Functionalization with amine groups was carried out by refluxing a mixture of 4 g of aminopropyltriethoxysilane (APTES, Sigma) with 1.00 g of silica nanoparticles in 60 mL of toluene for 48 h. The reaction mixture was then centrifuged at 11,000 × *g* for 20 min, washed three times with 20 mL of dichloromethane and dried overnight at 80 °C.

2.3. Material characterization

NPs were characterized by nitrogen adsorption isotherms, thermogravimetric analysis, scanning electron microscopy (SEM) and zeta potential. Nitrogen adsorption experiments were conducted using a Nova 1200 Nitrogen Adsorption Instrument (Quantachrome). Approximately 100 mg of powder was dried at 120 °C under vacuum overnight. A seven-point BET isotherm and a 50-point adsorption/desorption isotherm in a liquid nitrogen bath were obtained, using pure nitrogen gas as the adsorbate. Surface area was calculated using the BET (Brunauer–Emmett–Teller) method. Functionalized NPs were evaluated by thermogravimetric analysis using a TA Q5000 TGA instrument with a heating rate of 5 °C/min. The sample was heated from room temperature to ~800 °C under a flow of nitrogen. Mass loss during the run was used to approximate the loading of the organic functional group. SEM was used to image the particles and determine average particle diameter. Particles were dispersed on to silicon wafers on aluminum stubs and then sputter coated K550 sputter coater (Emitech Ltd., Kent, U.K.) with gold and palladium for 3 min at 10 mA. Zeta potentials of particles were measured in water using a Zeta Sizer nano ZS (Malvern Instrument Ltd., Southborough, MA). Fine ground silica (Min-U-Sil®5 quartz, Berkeley Springs, West Virginia) was used as a positive control for *in vivo* experiments.

2.4. In vitro *cytotoxicity studies*

Adenocarcinoma human alveolar basal epithelial (A549) cells were cultured in RPMI-1640 (Gibco®, Life Technologies Corporations, Grand Island, NY) medium containing 10% fetal bovine serum (Atlanta Biologics, Lawrenceville, GA), 10 mM HEPES (Gibco®), 50 μg/mL gentamycin sulfate (IBI Scientific, Peosta, IA), 1 mM sodium pyruvate (Gibco®) and 1 mM Glutamax