

The Size-dependent Cytotoxicity of Amorphous Silica Nanoparticles: A Systematic Review of in vitro Studies

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Xuemeng Dong¹
Zehao Wu¹
Xiuping Li¹
Liyan Xiao¹
Man Yang^{1,2}
Yang Li^{1,2}
Junchao Duan^{1,2}
Zhiwei Sun^{1,2}

¹School of Public Health, Capital Medical University, Beijing 100069, People's Republic of China; ²Beijing Key Laboratory of Environmental Toxicology, Capital Medical University, Beijing 100069, People's Republic of China

Abstract: With the increasing production and application of engineered amorphous silica nanoparticles (aSiNPs), people have more opportunities to be exposed to aSiNPs. However, the knowledge of its adverse health effects and related mechanisms is still limited, compared with the well-studied crystalline micron-sized silica. Since small differences in the physical-chemical properties of nanoparticles could cause significant differences in the toxic effect, it is important to distinguish how these variations influence the outcoming toxicity. Notably, particle size, as one of the essential characterizations of aSiNPs, is relevant to its biological activities. Thus, the aim of this systematic review was to summarize the relationship between the particle size of aSiNPs and its adverse biological effects. In order to avoid the influence of complicated in vivo experimental conditions on the toxic outcome, only in vitro toxicity studies which reported on the cytotoxic effect of different sizes aSiNPs were included. After the systematic literature retrieval, selection, and quality assessment process, 76 eligible scientific papers were finally included in this review. There were 76% of the studies that concluded a size-dependent cytotoxicity of aSiNPs, in which smaller-sized aSiNPs possessed greater toxicity. However, this trend could be modified by certain influence factors, such as the synthetic method of aSiNPs, particle aggregation state in cell culture medium, toxicity endpoint detection method, and some other experimental conditions. The effects of these influence factors on the size-dependent cytotoxicity of aSiNPs were also discussed in detail in the present review.

Keywords: silica nanoparticles, particle size, cytotoxicity, cell death, nanotoxicology, influence factors

Introduction

Silica particles often occur in crystalline and amorphous forms. Compared with crystalline silica, natural amorphous silica is generally considered as less harmful, since the toxicological potential of silica has so far been linked to its crystallinity.¹ Synthetic amorphous silica nanoparticles (aSiNPs), an attractive engineering nanomaterial, was commonly referred to amorphous silicon dioxide (SiO₂) with particle size ≤100 nm. Contrary to its micron-sized particles, aSiNPs possesses many excellent physical-chemical properties,² and is penetrating into many aspects of people's lives and productions.³ According to the Consumer Products Inventory (CPI), there were over 100 commercial products which contained aSiNPs, including foodstuffs, toothpastes, cosmetics, paints, electronic devices, even drugs and vitamins.⁴ Due to its multiple utilizations, aSiNPs has become the second largest

Correspondence: Zhiwei Sun; Yang Li
Beijing Key Laboratory of Environmental Toxicology, Capital Medical University, Beijing 100069, People's Republic of China
Tel/Fax +86 010 83911507;
+86 010 83911868
Email zwsun@ccmu.edu.cn;
li_yang@ccmu.edu.cn

engineering nanomaterial in terms of annual output.⁵ The large-scale production and widespread application of aSiNPs have increased the risk of human exposure. Thus, the adverse health impact of this kind of nanomaterial on human beings deserves more attention and should be carefully addressed.

The human body can be exposed to aSiNPs through several routes such as inhalation, oral ingestion, transdermal penetration and parenteral injection intentionally or unintentionally.⁶ In the last two decades, numerous studies in vivo and in vitro have evaluated the toxicity and biosafety of aSiNPs, and have demonstrated its potential adverse effects on human health. Several researches in vivo showed that after entering the experimental animal body, aSiNPs could distribute in almost all organs, and cause inflammation as well as tissue damage through direct or indirect ways.^{7,8} Further in vitro mechanism studies reported that aSiNPs could accumulate in cells by both active endocytotic pathway and passive diffusion, and distribute within endocytotic vesicles or freely in cytoplasm and organelles.⁹ Meanwhile, aSiNPs could exert cytotoxicity and genotoxicity in many kinds of cell lines, which was considered through the oxidative stress, endoplasmic reticulum stress, autophagy dysfunction or pro-inflammatory response induced by the particles.^{10,11}

It is well accepted in nanotoxicology that physical-chemical properties of nanomaterials, such as particle size, surface area, morphology, porosity, aggregation state, crystallinity and other characterizations, should be taken into account when assessing the potential toxicity of nanomaterials in biological systems. As to aSiNPs, a large number of studies have confirmed that these properties could influence the adsorption, distribution, excretion as well as cellular internalization of the particles, which were further associated with the toxic effect in vivo and in vitro.¹²⁻¹⁴ Notably, particle size, as an essential characterization of aSiNPs, is relevant to its biological activities. In nanoscale, the extreme small size confers specific large surface area of nanoparticles, which makes the surface atoms or molecules increase exponentially. Therefore, aSiNPs of smaller size possesses larger surface area and higher surface reactivity, which may render it more active chemically and biologically.

The possible biological effect related to aSiNPs exposure of different particle sizes have been widely studied. Researchers indicated that smaller size could facilitate the cellular uptake, tissue penetration and systemic distribution of aSiNPs.^{13,15} In addition, plenty of studies also demonstrated

the size dependent toxic effect of aSiNPs—the smaller the particle size was, the greater the toxicity produced.^{10,16} However, due to the complexity of experimental conditions and biological systems, there were still some researchers who reported that larger aSiNPs possessed higher toxicity or that no significant difference was observed between aSiNPs with different particle sizes.^{17,18} Herein, we provided a systematic literature review to summarize the relationship between the particle size of aSiNPs and its adverse biological effect. In order to avoid the influence of complicated experimental conditions in vivo, we only included in vitro studies of cell experiments to analyze the toxic intensity of aSiNPs with different sizes. We also explored the reasons why some studies did not conclude a size-dependent cytotoxicity of aSiNPs, and several influence factors of this trend were extracted and discussed in detail in this review.

Methodology

Inclusion Criteria

In this review, we would like to summarize the relationship between particle size of aSiNPs and its potential cytotoxicity. Firstly, the influence of other characteristics, such as crystallinity, shape, porosity, surface modification and so on, should be eliminated. Hence, only studies using amorphous non-mesoporous sphere silica nanoparticles without any surface modification were included. Secondly, compared with in vitro cell experiment, the internal environment of experimental animals is more complex and more difficult to control. As known, the ADME process and even the toxicity of aSiNPs can be influenced obviously by different exposure routes and complex internal environment, such as the presence of protein, the change of pH value, the difference of ionic strength and so on. Moreover, it was also difficult to trace the nanoparticles in vivo. Thus, in order to better summarize the size-dependent toxicity of aSiNPs and explore the influence factors of this trend, only in vitro studies assessing cytotoxicity of aSiNPs were included. Finally, we chose cell death as the toxicity endpoint to compare the toxic effect of aSiNPs with different particles, which due to the detection method of cell death is simple, convenient, and repeatable. Overall, according to the purpose of this review, studies should meet the following inclusion criteria otherwise they would be excluded:

- (i) Use amorphous non-mesoporous sphere silica nanoparticles without any surface modification;

- (ii) Use different sizes of aSiNPs;
- (iii) Primary size of aSiNPs should be reported;
- (iv) In vitro cytotoxicity studies;
- (v) Toxicological endpoints should include the detection of cell death or cell viability;
- (vi) Papers should be published in English.

Literature Search

A comprehensive literature search was performed to identify studies describing the cytotoxicity of aSiNPs based on the Office of Health Assessment and Translation (OHAT) approach for systematic review and evidence integration. We searched two databases for articles published and indexed from January 1, 2000 to July 30, 2020. In the PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) and Web of Science (<https://webofknowledge.com/>) databases, the following search strategy was used: (TS = “Silica nanoparticle” OR TS = “SiO₂ nanoparticle” OR TS = “silicon dioxide nanoparticle” OR TS = “silica nanomaterial” OR TS = “SiO₂ nanomaterial” OR TS = “Nano silica” OR TS = “Nano SiO₂”) AND (TS = “Cytotoxic*” OR TS = “Toxic*” OR TS = “Adverse effect”). Therefore, we could retrieve as many articles as possible concerning the toxic effect of aSiNPs. The article retrieval process was summarized in a flow chart as shown in Figure 1.

Quality Assessment and Information Extraction

Quality assessment of included studies was based on the guideline for assessing quality of in vitro studies as suggested

by Samuel et al.¹⁹ The quality of each study was assessed from 11 items concerning the basic information and experimental design, which were scientific background description, study purpose description, study model justification, study design description, cell culture condition description, endpoint measurement description, endpoint outcome description, statistical method description, dose/concentration response consideration, result interpretation, and discussion, as well as research funding. Each item was scored 1 and the overall score was 11. Studies were graded as poor quality if they met ≤ 4 items, fair if they met 5 to 8 items, and good if they met ≥ 9 items.

Information extraction was performed independently by two reviewers using a predesigned data extraction form. For each study, the following data were extracted:

- (i) Basic information of the article: first author and year published;
- (ii) ASiNPs characterization: primary particle size and aggregation state;
- (iii) Study design: cell type, exposure dose, and exposure time;
- (iv) Methodology: detection method of cell viability;
- (v) Result: relationship between particle size and cytotoxicity.

Results

Search Result Description

We retrieved a list of 4038 and 1543 articles published in PubMed and Web of Science, respectively, using the

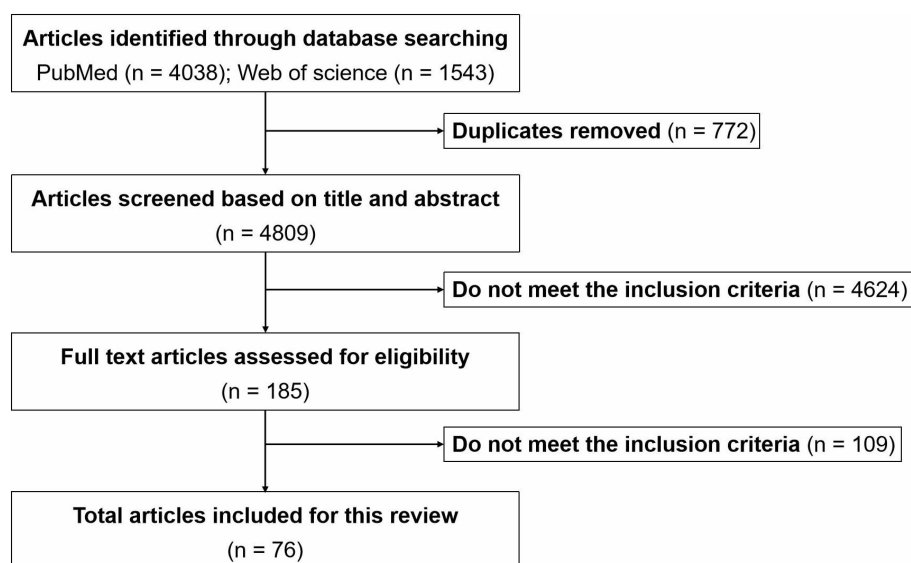


Figure 1 Flow chart of systematic selection of articles.

approach described in the methodology section. As shown in Figure 1, after removing duplicates and screening the titles and abstracts, 185 relevant articles were left. Then, another 109 articles that did not meet the inclusion criteria were removed through full text retrieval. Finally, 76 eligible articles reporting the cytotoxicity of aSiNPs with different sizes were selected for this systematic review. All 76 papers had good quality rating and the quality assessment result was shown in Table 1. The information descriptions of included articles were manifested in Table 2, which contains article source, type of cell line, primary size of aSiNPs, exposure dosage, exposure time, detection method of cell death/cell viability, and relationship between particle size and cytotoxicity. As summarized in Figure 2, 76% of the studies concluded that smaller aSiNPs possessed greater cytotoxicity, while 17% of the studies obtained the opposite conclusion, and the remaining 7% studies did not show any significant difference in the toxicity of aSiNPs with different sizes.

Size-dependent Cytotoxicity of aSiNPs

Size-dependent Cytotoxicity of aSiNPs on Cell Lines from Respiratory System

Gonzalez et al²⁰ examined the cytotoxic activity of aSiNPs with three different sizes in human alveolar carcinoma cell line (A549). The ED₅₀ of 16 nm, 60 nm and 104 nm aSiNPs, determined by the MTT assay after 24 h exposure, was 45.9, 48.9, and 165.9 µg/mL, respectively, indicating a size-response cytotoxicity of aSiNPs. Fede et al²¹ also attempted to study the role of size in aSiNPs (9 and 18 nm) toxicity in A549 cells. Results obtained showed that two sizes of aSiNPs caused very similar cytotoxicity, while the smaller aSiNPs was more effective in inflammatory and apoptosis processes. In the study of Gonzalez et al,²² A549 cells were exposed to a series of different sizes of aSiNPs (ranged from 12–174 nm), and a size-dependency of toxicity was observed. The smallest aSiNPs (12 nm) induced the highest cytotoxicity, especially in the absence of serum. Tokgun et al²³ demonstrated that among 6, 15, and 30 nm sizes of aSiNPs, 6 nm-sized nanoparticles had strongest cytotoxic effects on A549 cells, and this cytotoxicity came from dead receptor-mediated induction of apoptosis. Liu et al²⁴ examined the cell viability of A549 cells after exposing to SiO₂ particles at various concentrations (5, 10, 20, 30, 40, 60, 80, and 100 µg/mL). Results of CCK-8 assay showed that exposures of 10–20 nm aSiNPs resulted in significantly decreased viability of A549 cells, while 5 µm SiO₂ particles did not affect the cell viability obviously. Leibe et al¹⁰

analyzed the effects of two aSiNPs on A549 cells, which were 12 nm aSiNPs produced by flame synthesis and 50 nm aSiNPs produced by Stöber synthesis. As observed by the LDH release assay, the cytotoxicity induced by pyrogenic 12 nm aSiNPs was more pronounced than colloidal 50 nm aSiNPs. In addition, both two kinds of aSiNPs reduced the number of viable cells and provoke cell death only in the absence, but not in the presence of serum.

Wottrich et al²⁵ showed that aSiNPs (60 nm and 100 nm) induced dose-dependent and size-related reduction in cell viability of A549 cells and differentiated macrophage-like THP-1 cells. Meanwhile, smaller aSiNPs caused higher IL-6 and IL-8 release in co-culture system of A549 and macrophages (Mono Mac 6 or differentiated THP-1). In the research of Napierska et al,²⁶ co-culture of A549 cells and differentiated THP-1 cells were also introduced to elucidate the toxicity and related mechanism of aSiNPs (2, 16, 60, and 104 nm). A size-dependent cytotoxic response to aSiNPs was observed in both cell lines, and TC₅₀ increased with particle size. In addition, the release of pro-inflammatory mediators by endothelial cells after inhalation of aSiNPs could be a possible mechanism of its respiratory toxicity. Panas et al²⁷ explored the toxic effects of 12 nm and 25 nm aSiNPs in A549 cells and RAW264.7 mouse macrophage cell line in the presence and absence of serum. Both two particles decreased cell viability of A549 and RAW264.7 cells, and induced pro-inflammatory significantly in the absence of serum. Cytotoxicity of 12 nm aSiNPs was more prominent than that of 25 nm. Conversely, the toxicity was completely suppressed in the presence of serum.

Li et al^{28,29} investigated the possible cytotoxicity of three sizes of aSiNPs (40, 60, and 200 nm) in human bronchial epithelial BEAS-2B cells. These particles inhibited the cell viability in a size-dependent manner, the smaller aSiNPs produced higher toxic effect. Låg et al³⁰ studied the toxicity of 10 nm and 50 nm aSiNPs using two different human bronchial epithelial cell lines (BEAS-2B and HBEC3-KT). As measured by the LDH release assay, the cell viability was reduced approximately 50% after exposing to 50 µg/mL of 10 nm aSiNPs for 20 h in both cell types and 10–25% after exposing to 200 µg/mL 50 nm aSiNPs. Thus, 10 nm aSiNPs was far more cytotoxic than 50 nm aSiNPs. Kasper et al³¹ examined the cytotoxicity of different-sized aSiNPs (30, 70, and 300 nm) on lung epithelial cells line NCI H441 as well as endothelial cell line ISO-HAS-1, and illustrated a clearly increased toxicity of the smaller-sized aSiNPs. McCarthy et al³² studied

Table 1 Quality Assessment of Included Studies

Source	Scientific Background Described	Study Purpose Described	Study Model Justified	Study Design Described	Endpoint Specific Defined	Cell Culture Described	Statistical Method Corrects	Endpoint Measurement Described	Concentration Response Described	Result Interpreted and Discussed	Study Founded Described	Score	Study Quality
Wotrich ²⁵ (2004)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Lin ⁷¹ (2006)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Yu ⁵⁹ (2009)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Thomassen ³⁷ (2010)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	10	Good
Parik ⁸⁴ (2009)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Waters ⁴⁶ (2009)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Napierska ² (2009)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Wang ⁶¹ (2009)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Morishige ⁷⁹ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Ye ³⁹ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Parik ⁸⁵ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Kim ⁸⁶ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Yuan ⁶⁴ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Akhtar ⁷² (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Yang ⁵⁴ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Ye ⁴⁰ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Gonzalez ²⁰ (2010)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Nabeshi ⁵⁶ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Nabeshi ⁴⁷ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Ariano ⁶⁵ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Bauer ⁷⁵ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Lu ⁷⁷ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Li ⁴² (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	10	Good
Corbalan ³⁵ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Wang ⁶³ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Al-Rawi ⁶⁹ (2011)	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	10	Good
Uboldi ⁸² (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	10	Good
Gong ⁵⁵ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Napierska ²⁶ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	10	Good
Passagne ⁶⁸ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Kim ⁴⁸ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
McCarthy ³² (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Xu ³³ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Gualtieri ⁷⁴ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	10	Good
Panas ²⁷ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good

(Continued)

Table 1 (Continued).

Source	Scientific Background Described	Study Purpose Described	Study Model Justified	Study Design Described	Endpoint Specific Defined	Cell Culture Described	Statistical Method Corrects	Endpoint Measurement Described	Concentration Response Described	Result Interpreted and Discussed	Study Founded Described	Score	Study Quality
Gazzano ⁸⁰ (2012)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	10	Good
Stepnik ⁸¹ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Napierska ³⁸ (2012)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Gehrke ⁴⁴ (2013)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Kasper ³¹ (2013)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Freese ¹⁷ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓	✓	10	Good
Kim ⁶⁶ (2014)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	10	Good
Kusaka ⁵⁰ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Vo ⁷⁰ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Horie ⁷³ (2014)	✓	✓	✗	✓	✓	✓	✓	✓	✓	✓	✓	10	Good
Mendoza ⁵¹ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Docter ⁷⁸ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Nemmar ³⁶ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Kojima ⁵³ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Xue ⁴¹ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Fede ²¹ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Gonzalez ²² (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Nagakura ⁵⁷ (2014)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Tarantini ⁴³ (2015)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Kim ⁸³ (2015)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Tokgun ²³ (2015)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Guichard ³⁴ (2016)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Li ²⁸ (2016)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Saikia ¹² (2016)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Manganeli ⁶² (2016)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Nakanishi ⁵² (2016)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Gong ⁵⁸ (2017)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Nishijima ¹⁸ (2017)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Vicente ⁶⁰ (2017)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Premshkharan ⁴⁹ (2017)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Liu ²⁴ (2017)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Läg ³⁰ (2018)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Wang ¹⁵ (2018)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Fritsch-Decker ⁴⁵ (2019)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good
Kim ¹³ (2019)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11	Good

Table 2 In vitro Studies on Cytotoxicity of Amorphous Silica Nanoparticles (aSiNPs) with Different Sizes

Source	Cell Type	Particle Primary Size	Aggregates/ Agglomerates	Exposure Dose	Exposure Time	Assay(s)/ Method(s)	Size-dependent Cytotoxicity
Wottrich ²⁵ (2004)	A549 THP-I Mono Mac 6	S-60, 100 nm	–	10–200 µg/mL	24 h	LDH activity assay	Cytotoxicity: 60 nm >100 nm
Lin ⁷¹ (2006)	A549	15, 46 nm	√	10–100 µg/mL	24, 48, 72 h	LDH activity assay and Sulforhodamine B assay (SRB)	Not significant
Yu ⁵⁹ (2009)	HEL-30 cells	S-30, 48, 118, 535 nm	–	0–200 µg/mL	24 h	MTT assay and LDH activity assay	Cytotoxicity: 30 nm >48 nm >118 nm >535 nm
Thomassen ³⁷ (2010)	EAHY926 J774	Lu-11, 14, 15 nm Ly-2, 26, 34, 36 nm S-18, 60, 104, 335 nm	No substantial aggregation, except for the Ludox-Sil-15, Stober-Sil-104, Sober-Sil-18, and Lys-Sil-2 particles	0–2000 µg/mL	24 h	MTT assay and WST-1 assay	The smaller the particle size, the greater the cytotoxicity.
Park ⁸⁴ (2009)	D3	S-10, 30, 80, 400 nm	No substantial aggregation, except for the 10 nm particles	0.3–100 µg/mL	24 h, 10 days	WST-1 assay	Not significant; cytotoxicity: 30 nm >10 nm >80 nm and 400 nm
Waters ⁴⁶ (2009)	RAW 264.7	P-10, 50, 150, 300, 500 nm Lu-7, 16 nm	Moderate degree of aggregation of 10-nm particles	0–1000 µg/mL	24 h	MTT assay	The smaller the particle size, the greater the cytotoxicity.
Napierska ² (2009)	EAHY926	L-14, 15 nm S-16, 19, 60, 104, 335 nm	–	100 µg/mL	24 h	MTT assay and LDH activity assay	The smaller the particle size, the greater the cytotoxicity.
Wang ⁶¹ (2009)	HEK293	20, 50 nm	–	0–1000 µg/mL	24 h	MTT assay	Cytotoxicity: 20 nm >50 nm
Morishige ⁷⁹ (2010)	TPH-I	30, 50, 60, 300, 1000 nm	–	100 µg/mL	24 h	Standard methylene blue assay	Cytotoxicity: 300, 1000 nm >30, 50, 70 nm
Ye ³⁹ (2010)	H9c2(2–1)	S-21, 48 nm	–	0.1–1.6 mg/mL	12, 24, 36.48 h	LDH activity assay and MTT assay	Cytotoxicity: 21 nm >48 nm
Park ⁸⁵ (2010)	HaCaT	7 nm 10–20 nm	√	0–300 µg/mL	48 h	MTT assay	Not significant
Kim ⁸⁶ (2010)	SH-SY5Y	Lu-15.3, 16.9 nm	–	10, 100, 1000 ppm	48 h	MTT assay	Not significant

(Continued)

Table 2 (Continued).

Source	Cell Type	Particle Primary Size	Aggregates/ Agglomerates	Exposure Dose	Exposure Time	Assay(s)/ Method(s)	Size-dependent Cytotoxicity
Yuan ⁶⁴ (2010)	PC12 HEK293	S-20, 50, 80,140, 280, 760 nm	–	20–2000 µg/mL	24 h	MTT assay	Cytotoxicity: 20 nm >50 nm >80 nm >140, 280, 760 nm
Akhtar ⁷² (2010)	A549	S-10, 80 nm	√	50–400 µg/mL	48 h	MTT assay and LDH activity assay	Not significant
Yang ⁵⁴ (2010)	HaCaT	15, 30, 365 nm	–	2.5–80 µg/mL	24 h	CCK-8 assay and FITC apoptosis detection	Cytotoxicity: 15 nm>30 nm>365 nm
Ye ⁴⁰ (2010)	L-02	21, 48, 86 nm	–	200–1000 µg/mL	12, 24, 36, 48 h	MTT assay and LDH activity assay and V-FITC/PI apoptosis assay	Cytotoxicity: 21 nm >48 nm >86 nm
Gonzalez ²⁰ (2010)	A549	S-16, 60, 104 nm	–	10–500 mg/mL	24 h	MTT assay	Cytotoxicity: 16 nm >60 nm >104 nm
Nabeshi ⁵⁶ (2011)	HaCaT	70, 300, 1000 nm	–	10–1250 µg/mL	24 h	LDH activity assay	Cytotoxicity: 70 nm >300, 1000 nm
Nabeshi ⁴⁷ (2011)	RAW264.7	70, 300, 1000 nm	–	10, 30 µg/mL	5 days	WST-8 assay	Cytotoxicity: 70 nm > 300, 1000 nm
Ariano ⁶⁵ (2011)	GT1-7	S-50, 200 nm	–	15–292 µg/mL	24, 72 h	Count the cell number	Cytotoxicity: 50 nm >200 nm
Bauer ⁷⁵ (2011)	HUVECs	S-16, 41, 80, 212, 304 nm	–	1000, 15,000, 30,000 NP/cell	24, 48 h	MTT assay and LDH activity assay	Cytotoxicity: 304 nm >212 nm >16, 41, 80 nm
Lu ⁷⁷ (2011)	HepG2 L-02	7, 20, 50 nm	–	20–640 µg/mL	48 h	MTT assay	Not significant; cytotoxicity: 20 nm >7 nm >50 nm
Li ⁴² (2011)	HepG2	19, 43, 68, 498 nm	Moderate degree of aggregation of 19 nm particles	12.5–200 µg/mL	24 h	CCK-8 assay and LDH activity assay and annexin V-FITC/PI apoptosis assay	Cytotoxicity: 19 nm >43 nm>68 nm >498 nm

(Continued)

Table 2 (Continued).

Source	Cell Type	Particle Primary Size	Aggregates/ Agglomerates	Exposure Dose	Exposure Time	Assay(s)/ Method(s)	Size-dependent Cytotoxicity
Corbalan ³⁵ (2011)	HUVECs	10, 50, 150, 500 nm	–	1–100 µg/mL	3, 7, 15, 30 h	LDH activity assay	Cytotoxicity: 10 nm >50 nm >150, 500 nm
Wang ⁶³ (2011)	PC12	20, 50 nm	–	0–300 µg/mL	12, 24, 36, 48 h	MTT assay	Cytotoxicity: 20 nm >50 nm
Al-Rawi ⁶⁹ (2011)	HeLa	70, 200, 500 nm	—	50 µg/mL	24 h	LDH activity assay and WST-I assay	Cytotoxicity: 70 nm >200 nm, 500 nm
Uboldi ⁸² (2012)	Balb/3T3	10–25, 5–30 nm 15, 35, 80, 90 nm	No substantial aggregation, except for the 10–25 nm and 5–30 nm particles	1, 10, 100 µg/mL	72 h	MTT assay	Not significant; Only 80 nm silica nanoparticles produced significant cytotoxicity.
Gong ⁵⁵ (2012)	HaCaT	15, 30, 100 nm	–	0–100 µg/mL	24 h	CCK-8 assay	Cytotoxicity: 15 nm >30 nm >100 nm
Napierska ²⁶ (2012)	A549 THP-I	5–2, 16, 60, 104 nm	–	5 µg/cm ²	24 h	LDH activity assay and MTT assay	Cytotoxicity: 2 nm >16 nm >60 nm >104 nm
Passagne ⁶⁸ (2012)	HK-2 LLC-PK I	20, 100 nm	–	5–500 µg/mL	24, 48, 72 h	WST-I	Cytotoxicity: 20 nm >100 nm
Kim ⁴⁸ (2012)	U937	15, 50, 100 nm	–	0–2000 µg/mL	24 h	CCK-8 assay	Cytotoxicity: 15 nm >50 nm >100 nm
McCarthy ³² (2012)	Calu-3	10, 150, 500 nm	–	0–100 µg/mL	2, 6, 18, 24 h	MTT assay	Cytotoxicity: 10 nm >150,500 nm
Xu ³³ (2012)	HFL-I	20, 80 nm	–	250–2000 µg/mL	48 h	MTT assay	Cytotoxicity: 20 nm >80 nm
Gualtieri ⁷⁴ (2012)	BEAS-2B	30, 50 nm	√	100 µg/mL	20 h	LDH activity assay	Not significant
Panas ²⁷ (2012)	RAW264.7 A549	12, 25 nm	√	10–200 µg/mL	24 h	LDH activity assay and Alamar Blue assay	Cytotoxicity: 12 nm >25 nm
Gazzano ⁸⁰ (2012)	MH-S	Pyr-7, 40 nm S-1000 nm	√	0–160 µg/cm ²	24 h	LDH activity assay	Not significant; cytotoxicity: 7, 40 nm >1000 nm
Stepnik ⁸¹ (2012)	3T3-L1 WI-38	Lu-21, 30 nm	Partial aggregation	40–800 µg/mL	72 h	LDH activity assay and WST-I assay	Cytotoxicity: 30 nm >21 nm

(Continued)

Table 2 (Continued).

Source	Cell Type	Particle Primary Size	Aggregates/ Agglomerates	Exposure Dose	Exposure Time	Assay(s)/ Method(s)	Size-dependent Cytotoxicity
Napierska ³⁸ (2012)	EA.hy926	S-16, 60 nm	–	25–50 µg/mL	1, 2.5, 4, 24 h	MTT assay and LDH activity assay	Cytotoxicity: 16 nm >60 nm
Gehrke ⁴⁴ (2013)	HT29	12, 40, 200 nm	No substantial aggregation, except for the 12 nm particles	0.1–500 µg/mL	24, 48, 72 h	SRB assay and WST-I assay and LDH activity assay	Cytotoxicity: 12 nm >40 nm >200 nm
Kasper ³¹ (2013)	H441 ISO-HAS-I	30, 70, 300 nm	Partly agglomerated of 30 nm particles	6–600 µg/mL	4 h	MTS assay and LDH activity assay	Cytotoxicity: 30 nm >70 nm >300 nm
Freese ¹⁷ (2014)	HUVEC	30, 70 nm	The 30 nm particles tended to agglomerate with time	60–150 µg/mL	24 h	MTS assay and LDH activity assay	Cytotoxicity: 70 nm >30 nm
Kim ⁶⁶ (2014)	U373MG	20, 100 nm	–	0–30 mg/mL	24 h	CellTiter-Glo luminescent assay	Cytotoxicity: 20 nm >100 nm
Kusaka ⁵⁰ (2014)	BMDMs	30, 100, 300, 1000, 3000, 10,000 nm	–	0.03–0.3 mg/mL	2 h	LDH activity assay	Cytotoxicity 30, 100, 300 nm >1000 nm >3000, 10,000 nm
Vo ⁷⁰ (2014)	RTgil-WI RTgut-GC RTL-WI RTBrain FHML2-6 FHMT- WIGFB3C GFSk-SI GloFish ZEB2J EelBrain HEW	S-16, 24, 44 nm	–	1.8–1800 µg/mL	24 h	Alamar Blue assays	Cytotoxicity: 16 nm >24 nm >44 nm
Horie ⁷³ (2014)	A549 HaCaT	S-7, 25, 34 nm	√	10–1000 µg/mL	24, 48 h	LDH activity assay and MTT assay	Not significant
Mendoza ⁵¹ (2014)	PBMC cells	S-10, 100 nm	10 nm particles in culture medium tend to aggregate	50–4000 ppm	24, 48 h	PI staining with flow cytometer	Cytotoxicity: 10 nm >100 nm
Docter ⁷⁸ (2014)	Caco-2	20, 30, 100 nm	√	6–600 µg/mL	4, 24 h	MTT assay	Not significant; 30 nm silica particles produced significant cytotoxicity

(Continued)

Table 2 (Continued).

Source	Cell Type	Particle Primary Size	Aggregates/ Agglomerates	Exposure Dose	Exposure Time	Assay(s)/ Method(s)	Size-dependent Cytotoxicity
Nemmar ³⁶ (2014)	HUVEC	50, 500 nm	–	0.1–100 µg/mL	24 h	CellTiter-Glo [®] Luminescent Cell Viability Assay	Cytotoxicity: 50 nm >500 nm
Kojima ⁵³ (2014)	KUP5	30, 70, 300 nm	The particles of SNP70 and SNP300 appeared to be loosely aggregated in DMEM cell culture medium	1–30 µg/cm ²	24 h	WST-I assay	Cytotoxicity: 30 nm >70 nm >300 nm
Xue ⁴¹ (2014)	BRL	20 nm, 0.5–6 µm	20 nm silica nanoparticle aggregates were visible	0.005–1 mg/mL	18, 24 h	Thiazolyl blue tetrazolium bromide assay and LDH activity assay and MTT assay	Cytotoxicity: 20 nm >0.5–6 µm
Fede ²¹ (2014)	A549	Lu-9, 18 nm	–	0.01–0.05 mg/mL	2 h	MTS-assay	Cytotoxicity: 9 nm >18 nm
Gonzalez ²² (2014)	A549	Lys-12, 40 nm, S-28, 59, 139, 174 nm	–	0–1056 µg/mL	40 h	Peak flow green flow cytometry-based cytotoxicity measurement	Cytotoxicity (absence of serum): L-12 nm >L-40 nm; S-28 nm >S-59 nm >S-139 nm >S-174 nm
Nagakura ⁵⁷ (2014)	HaCaT	30, 70, 300 nm	30 nm particles approximately uniformly dispersed in the medium	0–50 µg/cm ²	24 h	WST-I assay	Cytotoxicity: 30 nm >70 nm >300 nm
Tarantini ⁴³ (2015)	Caco-2	15, 55 nm	15 nm particles formed aggregates	0–256 µg/mL	24 h	XTT assay	Cytotoxicity: 15 nm >50 nm
Kim ⁸³ (2015)	A549 HepG2 NIH/3T3	S-20, 40, 60, 80, 100, 150, 200 nm	√	10–500 µg/mL	24, 72 h	LDH activity assay and MTT assay	Not significant
Tokgun ²³ (2015)	A549	S-6, 15, 30, 55 nm	–	0.1–1000 µg/mL	72 h	CytoToxGlo kit and TUNEL analysis	Cytotoxicity: 6 nm >15 nm >30, 55 nm

(Continued)

Table 2 (Continued).

Source	Cell Type	Particle Primary Size	Aggregates/ Agglomerates	Exposure Dose	Exposure Time	Assay(s)/ Method(s)	Size-dependent Cytotoxicity
Guichard ³⁴ (2016)	V79	Pyr 20: 19±5 nm	√	1.25–100 µg/cm ²	24 h	WST-I	Cytotoxicity: Col 15 >Pyr 20=Pre 20 >Pyr 25/70=Col 40/80.
		Pyr 25/70: 71 ±25/25±8 nm					
		Pre 20: 19±3 nm					
		Col 15: 15±4 nm					
		Col 40/80: 79 ±3/38±5 nm					
Li ²⁸ (2016)	BEAS-2B	5–40, 60, 200 nm	–	6.25–100 µg/mL	6, 12, 24 h	MTT assay and LDH activity assay	Cytotoxicity: 40 nm >60 nm >200 nm
Saikia ¹² (2016)	RAW 264.7	5–50, 500 nm	–	4–500 µg/mL	24 h	CCK-8 assay and LDH activity assay	Cytotoxicity: 50 nm >500 nm
Manganelli ⁶² (2016)	HEK293	20, 50 nm	–	25–200 µg/mL	12, 24, 36, 48 h	MTT assay	Cytotoxicity: 20 nm >50 nm
Nakanishi ⁵² (2016)	mBMDCs	30, 70, 300 nm	–	1–20 µg/cm ²	24 h	LDH activity assay	Cytotoxicity: 30 nm >70 nm >300 nm
Gong ⁵⁸ (2017)	HaCaT HaCaT-shPARP-I	15 nm, 1–5 µm	–	0–200 µg/mL	24 h	CCK-8 assay and LDH activity assay and MTT assay and flow cytometric analysis	Cytotoxicity: 15 nm >1–5 µm
Nishijima ¹⁸ (2017)	TPH-I	10, 30, 50, 70, 100, 300, 1000 nm	–	12.5–200 µg/mL	6, 12, 24 h	LDH activity assay	Not significant
Vicente ⁶⁰ (2017)	K17 HDF	Lu-20 nm 70, 200, 500 nm	70 nm silica nanoparticle aggregates were visible	5–200 µg/mL	24 h	LDH activity assay and WST-I assay and PI staining and nuclei counting	Cytotoxicity: 20 nm >70 nm ≥200, 500 nm
Premshakaran ⁴⁹ (2017)	THP-I	50 nm, 2 µm	The particles of 2 µm appeared to be loosely aggregated in cell culture medium	1–100 µg/mL	22 h	MTT assay	Cytotoxicity: 50 nm >2 µm

(Continued)

Table 2 (Continued).

Source	Cell Type	Particle Primary Size	Aggregates/ Agglomerates	Exposure Dose	Exposure Time	Assay(s)/ Method(s)	Size-dependent Cytotoxicity
Liu ²⁴ (2017)	A549 A549-shNrf2 A549-shRNA	10–20 nm, 1–5 μ m	–	0–100 μ g/mL	24 h	CCK-8 assay and annexin V FITC apoptosis detection kit	Cytotoxicity: 10–20 nm >1–5 μ m
Läg ³⁰ (2018)	BEAS-2B HBEC3-KT	10, 50 nm	10 nm particles showed polydispersity in media	0–200 μ g/mL	20 h	LDH activity assay	Cytotoxicity: 10 nm >50 nm
Wang ¹⁵ (2018)	HUVECs	5–10, 25, 50, 100 nm	A degree of aggregation of four particles	1–25 μ g/mL	24 h	CCK-8 assay and flow cytometry assay	Cytotoxicity: 10 nm >25 nm >50 nm >100 nm
Fritsch-Decker ⁴⁵ (2019)	HCT116	12, 70, 200, 500 nm	12 nm NPs were already aggregated to 232 nm in medium without FBS	0–100 μ g/mL	24, 48 h	LDH activity assay and Alamar Blue assay	Cytotoxicity: 12 nm >70 nm >200, 500 nm
Kim ¹³ (2019)	HepG2 A549 SW480	5–20, 30, 40, 50 nm	Particles aggregates formed in PBS solution containing FBS	5–500 μ g/mL	24 h	WST-1 assay	Cytotoxicity: 20, 30 nm >40, 50 nm
Lee ¹⁶ (2019)	HUVECs	5–20, 30, 40, 50 nm	–	5–25 μ g/mL	24 h	FITC-annexin -V & propidium iodide (PI) assay	Cytotoxicity: 20 nm >30, 40, 50 nm
Kamikubo ³ (2019)	Rat hippocampal cells HEK293.	10, 30, 100, 400, 1500 nm	–	0.0001–5 mg/mL	2 h	CTG assay and MTT assay and propidium iodide (PI) assay	Cytotoxicity: 10 nm >30 nm >100 nm >400 nm >1500 nm
Du ⁶⁷ (2019)	N9 bEnd.3 HT22	5–50, 100, 300 nm	–	25–200 μ g/mL	24 h	MTS assay	Cytotoxicity in N9 cells: 50 nm >100 nm >300 nm
Li ²⁹ (2019)	BEAS-2B	5–40, 60, 200 nm	–	25 μ g/mL	24 h	CCK-8 assay	Cytotoxicity: 40 nm >60 nm >200 nm
Leibe ¹⁰ (2019)	RAW 264.7 A549	12, 50 nm	Large numbers of 50 nm particles agglomerates in the absence of serum	10–100 μ g/mL	24 h	LDH activity assay and Alamar Blue assay	Cytotoxicity: 12 nm >50 nm
Wang ⁷⁶ (2020)	HUVECs	20, 100 nm	–	0–200 μ g/mL	24 h	CCK-8 assay and LDH activity assays	Cytotoxicity: 100 nm >20 nm

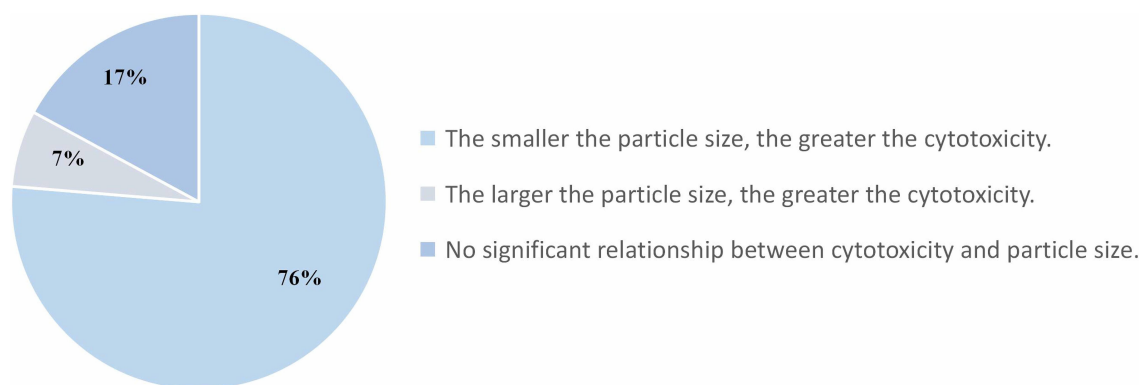


Figure 2 Fan-shaped diagram of the relationship between cytotoxicity of aSiNPs and its particle size.

Napierska et al² reported that exposures to aSiNPs (14–335 nm) would result in cytotoxic damage and a decrease in cell survival in human umbilical vein endothelial cell line (EAHY926) in a dose-related manner. Concentrations leading to a 50% reduction in cell viability (TC₅₀) declined gradually with the nanoparticle size decreased. Furthermore, the smaller particles also appeared to affect the exposed cells faster within just a few hours. The cytotoxicity of a series of aSiNPs (2–335 nm) in EAHY926 cell line and J774 differentiated monocyte-macrophage mouse cell line was investigated using MTT and WST1 assays, respectively. When cells were exposed to the same mass of aSiNPs, larger particles were less toxic compared to smaller ones.³⁷ Napierska et al³⁸ studied the cytotoxicity response of EAHY926 cells to aSiNPs of two sizes (16 and 60 nm). Results of MTT and LDH assays showed that cytotoxic effects could be detected after four hours exposure to 16 nm aSiNPs at the concentration of 50 µg/mL. However, no significant cytotoxicity was found for 60 nm aSiNPs at any dose and any time of exposure. Ye et al³⁹ exposed rat embryonic ventricular myocardial cell line H9c2 (2–1) to 21 and 48 nm aSiNPs, and assessed cell viability, oxidative stress and cell cycle arrest. Consequently, both aSiNPs produced significant decreases in viabilities in dose-dependent manners. Furthermore, silica nanoparticles of smaller size possessed higher cytotoxicity.

Size-dependent Cytotoxicity of aSiNPs on Cell Lines from Digestive System

In the study of Ye et al,⁴⁰ the normal human hepatic cell line (L-02) was exposed to 0.2, 0.4, and 0.6 mg/mL of aSiNPs (21, 48, and 86 nm) for 12, 24, 36, and 48 h. As indicated by MTT and LDH assays, 21 nm aSiNPs showed

significantly higher cytotoxicity than the large sizes of aSiNPs. Xue et al⁴¹ exposed buffalo rat liver (BRL) cell line to nano- and micro-sized silica particles (20 nm and 0.5–6 µm). Both particles exhibited significant toxicity as determined by MTT, LDH leakage and mitochondrial membrane potential assessments. Moreover, greater effects were found for the silica nanoparticle samples than for the micrometer particle samples. Li et al⁴² compared the potential toxicity induced by aSiNPs with different sizes (19, 43, 68, and 498 nm) on cultured human hepatoma cell line (HepG2). Results of CCK-8 and LDH release assay suggested that the cytotoxicity of aSiNPs strongly depended on the particle size, and smaller particles possessed higher toxic effects. Kim et al¹³ studied the cytotoxicity of different sizes of aSiNPs (20, 30, 40, and 50 nm) in HepG2 cells and two other human carcinoma cell lines (A549 and SW480). The result of cell viability manifested that aSiNPs smaller than 30 nm in size were lethal to the cells, while the cytotoxicity of 40 and 50 nm aSiNPs was slight.

Tarantini et al⁴³ introduced human intestinal cell line (Caco-2), which is a well characterized model of the intestinal epithelium and commonly used in toxicity studies. Following exposures to aSiNPs (15 and 55 nm), a statistically significant dose-dependent decrease in cell viability was observed in the 15 nm aSiNPs treated group, in contrast, 55 nm aSiNPs only affected viability of Caco-2 cells at the highest tested dose. Gehrke et al⁴⁴ assessed the toxic effects of 12 nm, 40 nm, and 200 nm aSiNPs in human colon carcinoma cell line (HT29). Size-dependent cytotoxicity was confirmed using the SRB assay, WST-1 assay and LDH activity assay. Fritsch-Decker et al⁴⁵ tested the effects of aSiNPs with nominal diameters of 70, 200, and 500 nm on the viability of human colon cancer

epithelial cell line (HCT116). Administration of particles with increasing diameters but at the same mass concentration (10 or 50 $\mu\text{g}/\text{mL}$) resulted in an inverse relationship of particle size and cytotoxicity, larger particles were less toxic compared to smaller particles.

Size-dependent Cytotoxicity of aSiNPs on Cell Lines from Monocyte Macrophage System

Waters et al⁴⁶ exposed RAW 264.7 cells to aSiNPs (7–500 nm) with mass concentrations from 0 to 1000 $\mu\text{g}/\text{mL}$, and observed the dose–response and size-dependent relationship for cytotoxicity. In addition, whole genome microarray analysis of the early gene expression changes induced by 10 and 500 nm aSiNPs manifested that the magnitude of changes for the majority of affected genes correlated more tightly with particle surface area than either particle mass or number. In other words, it was also identified that the changes of gene expression were particle size specific. Nabeshi et al⁴⁷ investigated the cytotoxicity of 70, 300, and 1000 nm aSiNPs in RAW264.7 cells using WST-8 cell proliferation assay. As shown, 30 $\mu\text{g}/\text{mL}$ of 300 and 1000 nm aSiNPs treatment for five days did not induce any toxic effect, while 70 nm aSiNPs treatment induced obvious cytotoxicity. Saikia et al¹² exposed RAW 264.7 cells to 50 nm and 500 nm aSiNPs, and measured the LC_{50} values of each aSiNPs in the presence and absence of proteins. Results manifested that with the decrease of particle size, the cytotoxicity of aSiNPs increased by an increase in the surface area. Furthermore, the presence of the protein corona reduced the toxicity of both aSiNPs, which was probably due to the reduction of particle uptake efficiency.

Kim et al⁴⁸ assessed the cytotoxicity of aSiNPs (15, 50, 100 nm) on human macrophage cells (U937) by the CCK-8 assay. As shown, the cell viability decreased depending on the particles size, and 15 nm particles triggered cell death more significantly than did 50 nm or 100 nm particles. The LD_{50} of 15 nm aSiNPs was 1.29 mg/mL and that of 50 nm aSiNPs was 1.5 mg/mL. In the study conducted by Premshkharan et al,⁴⁹ the MTT assay was performed to explore the non-cytotoxic exposure levels of engineered 50 nm and 2 μm aSiNPs on differentiated THP-1 macrophages. For 2 μm silica particles, dosage of 50 $\mu\text{g}/\text{mL}$ and below did not differ apparently from the control, whereas for 50 nm aSiNPs, doses of 10 $\mu\text{g}/\text{mL}$ and below did not differ significantly from the control. Thus, 50 nm aSiNPs was more toxic than 2 μm particles. Kusaka et al⁵⁰ addressed the relationship between the size of aSiNPs (30–10,000 nm) and the cytotoxicity as well as

inflammatory activity in primary mouse macrophages (C57BL/6N mouse bone marrow-derived macrophages, BMDMs). The size-dependent induction of cytotoxicity and IL-1 β secretion was observed, the toxic activity of 30–1000 nm aSiNPs was apparently higher than that of 3000 nm and 10,000 nm silica particles.

In the research of Mendoza et al,⁵¹ the effects of 10 or 100 nm aSiNPs on human peripheral blood mononuclear cells (PBMC) were examined. Ten nanometers of aSiNPs were more cytotoxic and induced more oxidative damage than 100 nm particles. Nakanishi et al⁵² examined LDH release as well as IL-1 β and IL-18 production after incubating mouse bone marrow dendritic cells (mBMDCs) with aSiNPs of different sizes (30, 70, and 300 nm). An obviously size-dependent toxic and inflammation induction effect was confirmed, in which the greatest cytotoxicity was seen with 30 nm aSiNPs. Kojima et al⁵³ further investigated the mechanism of inflammatory response in mouse Kupffer cell line (KUP5) induced by aSiNPs of 30, 70, and 300 nm. The cell viability decreased apparently with the particle size. In addition, 30 nm aSiNPs led to the greatest increase of IL-1 β secretion, while the effect of 70 nm aSiNPs was similar to that of 300 nm particles.

Size-dependent Cytotoxicity of aSiNPs on Cell Lines from Epidermis

Yang et al⁵⁴ evaluated the effects of aSiNPs (15, 30, and 365 nm) on cell viability, cell cycle and apoptosis in human epidermal keratinocyte cell line (HaCaT). The IC_{50} values of 15 nm, 30 nm and micro-sized silica particles were 23.0 $\mu\text{g}/\text{mL}$, 27.3 $\mu\text{g}/\text{mL}$ and 34.8 $\mu\text{g}/\text{mL}$, respectively. Furthermore, the smaller SiO_2 particle size was, the higher apoptotic rate the cells underwent. Gong et al⁵⁵ explored the adverse effects of aSiNPs (15, 30, and 100 nm) on HaCaT cells as well. As observed in CCK-8 assay, exposure to aSiNPs decreased the cell viability in a concentration- and size-dependent manner, and the IC_{50} values of 24 h exposure were 19.4 ± 1.3 , 27.7 ± 1.5 and 35.9 ± 1.6 $\mu\text{g}/\text{mL}$ for 15, 30, and 100 nm aSiNPs, respectively. The tendency of smaller nanoparticles showing higher toxicity was found. The nanoscale silica particles used by the two research groups were both 15 and 30 nm, and the IC_{50} values of each size of nanosilica were similar.

Nabeshi et al⁵⁶ illustrated the size-dependent cytotoxicity of aSiNPs (70, 300, and 1000 nm) on HaCaT cells. Results of LDH release, intracellular ROS level and DNA oxidative damage indicated that the smallest particles produced greatest toxic effects. Then, they further studied the

cytotoxic effects and inflammatory response caused by 30, 70, and 300 nm aSiNPs, and a clearly size-dependent manner of aSiNPs could be also concluded in cell viability and IL-6 production.⁵⁷ Gong et al⁵⁸ reported that exposure of HaCaT cells and HaCaT-shPARP-1 cells to 15 nm aSiNPs resulted in significantly decreased cell viability in a dose-dependent manner. Meanwhile, flow cytometric analysis showed the apoptotic rate in HaCaT-shPARP-1 cells induced by nanosized SiO₂ (15 nm) was significantly higher than by microsized SiO₂ (1–5 μm).

In the study of Yu et al,⁵⁹ mouse keratinocytes cells (HEL-30) were exposed for 24 h to various concentrations (0–200 μg/mL) of aSiNPs with different sizes (30, 48, 118, and 535 nm). Results of LDH and MTT assay showed dose- and size-dependent cytotoxicity of 30 and 48 nm nanoparticles, while larger sizes of aSiNPs (118 and 535 nm) exhibited less toxicity compared with smaller nanoparticles. Vicente et al⁶⁰ investigated the toxicological profile of aSiNPs (20, 70, 200, and 500 nm) in human keratinocytes (K17) and human dermal fibroblasts (HDF), and described that the ultra-small particles (20 nm) was the most toxic aSiNPs, whereas particles larger than 70 nm demonstrated to be nontoxic for both dermal cells in a wide range of concentrations.

Size-dependent Cytotoxicity of aSiNPs on Cell Lines from Kidney or Nervous System

Wang et al^{61,62} showed that exposure to 20 or 50 nm aSiNPs at dosage levels between 20 and 100 μg/mL decreased cell viability of cultured human embryonic kidney cells (HEK293) in a dose-dependent manner. Median lethal dose (LD₅₀) of 24 h exposure was 80.2±6.4 and 140.3±8.6 μg/mL for 20 and 50 nm aSiNPs, respectively. The same research group evaluated the neurotoxicity of these aSiNPs in pheochromocytoma cells (PC12) as well. As determined by MTT assay, the cell viability decreased as the function of both dose and time. The LD₅₀ values obtained after 24 h exposure were 120±8 μg/mL for the 20 nm aSiNPs and 320±10 μg/mL for the 50 nm aSiNPs.⁶³ Yuan et al⁶⁴ studied the toxic effects of six sizes aSiNPs (20, 50, 80, 140, 280, and 760 nm) also using HEK293 cells and PC12 cells. Compared with microsized-SiO₂ (140, 280, and 800 nm), nanosized-SiO₂ (20, 50, and 80 nm) caused more severe cell damage, and it was obvious that the IC₅₀ values of aSiNPs increased with the particle size. Kamikubo et al³ investigated the toxic effects of aSiNPs (10, 30, 100, 400, 1500 nm) on primary cultured rat hippocampal cells and HEK29 cells. As determined by

the CellTiter-Glo 2.0 (CTG) Luminescent Cell Viability Assay system, aSiNPs with smaller sizes tended to cause higher toxicity on both types of cells at concentrations of 0.03–1 mg/mL. In addition, compared to HEK293 cells, rat hippocampal cells were more vulnerable to aSiNPs.

In the study of Ariano et al,⁶⁵ the responses of the well differentiated neuronal cell line (GT1-7) to 50 and 200 nm aSiNPs were compared. The 50 nm aSiNPs affected cell proliferation in a dose-dependent way. In contrast, 200 nm aSiNPs did not show any toxicity even at relatively high concentrations. Kim et al⁶⁶ determined the cytotoxicity of aSiNPs with mean sizes of either 20 nm or 100 nm in human glioblastoma cell line (U373MG). The results indicated that the 20 nm nanoparticles were more toxic than the 100 nm nanoparticles. Du et al⁶⁷ compared the neurotoxicity of commercial 50, 100, and 300 nm Stöber aSiNPs using bEnd.3, N9 and HT22 cells. The result of MTS assay indicated that three aSiNPs induced size- and dose-dependent cytotoxicity in N9 cells, with smaller particles inducing higher cytotoxicity. In contrast, no alteration of cell viability was detected in bEnd.3 and HT22 cells. Passagne et al⁶⁸ reported on the cytotoxicity of 20 nm and 100 nm aSiNPs in two renal proximal tubular cell lines (human HK-2 and porcine LLC-PK1). Results of a WST-1 assay indicated that both aSiNPs reduced cell viability in a dose- and time-dependent manner, and 20 nm aSiNPs was more cytotoxic than 100 nm aSiNPs.

Size-dependent Cytotoxicity of aSiNPs on Other Kinds of Cell Lines

Al-Rawi et al⁶⁹ evaluated the possible size-dependent toxicity of aSiNPs (70, 200, and 500 nm) in human cervical carcinoma cell line (HeLa). In the presence of serum, all three sizes of aSiNPs did not show any negative effect on the viability of HeLa cells. However, compared to 200 nm and 500 nm aSiNPs, 70 nm aSiNPs diminished the cell viability apparently when the serum was absent. In the study of Vo et al,⁷⁰ twelve adherent fish cell lines (RTgill-W1, RTgut-GC, RTL-W1, RTBrain, FHML2-6, FHMT-W1, GFB3C, GFSk-S1, GloFish, ZEB2J, EelBrain, and HEW) derived from six species (rainbow trout, fathead minnow, zebrafish, goldfish, haddock, and American eel) were used to investigate the toxic effects of aSiNPs (16, 24, and 44 nm). Toxicity produced by aSiNPs appeared to be size-, time-, temperature-, and dose-dependent as well as tissue-specific. Smaller particles (16 nm) were relatively more toxic than larger sized ones (24 and 44 nm).

Size-independent Cytotoxicity of aSiNPs on Cell Lines from Respiratory System

Lin et al⁷¹ found that 15 nm or 46 nm aSiNPs could reduce A549 cell viability in a dose-dependent manner. Nevertheless, the cytotoxicity of 15 nm and 46 nm aSiNPs were not significantly different, since the similar hydrodynamic sizes of these two nanoparticles. Akhtar et al⁷² performed MTT assay and LDH activity assays to explore the viability of A549 cells exposed to two sizes (10 and 80 nm) of aSiNPs. Due to the agglomeration of particles in cell culture medium, the toxicity did not differ significantly between this two aSiNPs. In the study of Horie et al,⁷³ aSiNPs (7, 25, and 34 nm) were dispersed directly in DMEM-FBS at concentrations of 10, 100, and 1000 µg/mL. There was no significant difference in the changes in mitochondrial activity of A549 and HaCaT cells induced by different sizes of particles. The authors found that the aSiNPs formed large secondary particles and accumulated onto cells by gravity sedimentation, therefore, the concentrations of the nanoparticles in dispersion did not correctly reflect the concentration to which the cells were exposed. In the research of Gualtieri et al,⁷⁴ 30 and 50 nm aSiNPs were examined for their cytotoxicity in BEAS-2B cells. However, little toxicity of both aSiNPs was generally observed, due to the formation of micrometer-sized agglomerates in the absence of bovine serum albumin in the culture medium.

Size-independent Cytotoxicity of aSiNPs on Cell Lines from Cardiovascular System

Bauer et al⁷⁵ exposed HUVECs to aSiNPs with diameters ranging from 16 nm to 304 nm. As assessed by the MTT reduction method, mitochondrial activity significantly decreased upon incubation of aSiNPs with diameters between 212 nm and 304 nm. In contrast, aSiNPs with a diameter of 80 nm or less showed no cytotoxicity within the same particle concentrations. The authors observed that larger sized silica particles accumulated around the nucleus and blocked the cell migration obviously, but the reason why smaller sized silica nanoparticles did not show obvious toxicity was not clearly stated. Freese et al¹⁷ investigated the cytotoxicity of aSiNPs (30 and 70 nm) in HUVECs. As measured by the MTS-assay and LDH activity assay, the cell viability of HUVECs treated with 70 nm particles under stretch conditions was significantly decreased up to 77% ($\pm 12\%$). By comparison, 30 nm particles did not decrease the cell viability, owing to the tendency of agglomeration over time. Wang et al⁷⁶ demonstrated that both 20 nm and 100 nm aSiNPs decreased the cell viability of HUVECs in

a concentration-dependent manner from the minimal toxic concentration (50 µg/mL), whereas 100 nm aSiNPs was found to be more toxic than 20 nm aSiNPs. An in vitro sedimentation, diffusion and dosimetry (ISDD) model was introduced to estimate the aSiNPs sedimentations onto the cell surface, and results suggested that 100 nm aSiNPs reached to the cell surface more efficiently than 20 nm aSiNPs over time, larger aSiNPs thus have a higher effective exposure concentration than smaller particles.

Size-independent Cytotoxicity of aSiNPs on Cell Lines from Digestive System

The adverse effects of 7–50 nm aSiNPs on HepG2 cells was studied by Lu et al,⁷⁷ whose results showed that the toxicity of 20 nm aSiNPs was much higher than that of 7 nm aSiNPs, while 50 nm aSiNPs did not have a significant cytotoxic effect at any concentration. In further comparison, it was found that 20 nm aSiNPs produced stronger oxidative damage than 7 nm aSiNPs. Docter et al⁷⁸ reported that compared with untreated control Caco-2 cells, exposure to 30 nm aSiNPs reduced the cell vitality significantly. In contrast, low toxicity was observed upon treatment with 20 nm or 100 nm aSiNPs. The authors implied that the adverse effects triggered by 30 nm aSiNPs were significantly ameliorated upon formation of the protein corona, which they found was efficiently established on all aSiNPs studied. As a potential explanation, corona formation reduced cellular uptake of 30 nm aSiNPs, which was, however, not significantly affected by particle surface charge in their model.

Size-independent Cytotoxicity of aSiNPs on Cell Lines from Monocyte Macrophage System

Morishige et al⁷⁹ evaluated the cytotoxicity of different-sized silica particles (30–1000 nm) on THP-1 cells. Silica particles with diameter of 300 and 1000 nm showed strong cytotoxicity which was depended on ROS level, whereas 30, 50, and 70 nm aSiNPs did not induce cell death obviously. Nishijima et al¹⁸ examined the effects of aSiNPs in THP-1 cells with a large overall size range (10, 30, 50, 70, 100, 300, and 1000 nm). The cytotoxicity in the dose range from 12.5 to 200 µg/mL at six and twelve hours was hardly detected. Meanwhile, dose-dependent cytotoxicity was observed in all treated groups at 24 h, at a dose greater than 100 µg/mL, while the effect of particle size on the toxicity was not significant. Further assessment of inflammatory cytokines showed there was a bell-shaped size-specific effect, where the silica particles with a diameter of 50 nm induced the greatest secretion of IL-

1 β and TNF- α and silica particles with smaller or larger diameters had progressively less effect (overall size range, 10–1000 nm). Gazzano et al⁸⁰ reported the toxicity of aSiNPs with different sizes (7, 40, and 1000 nm) on murine alveolar macrophages (MH-S). As manifested, except for 1000 nm silica particles, an increase in cytotoxic activity was shown on the other two samples. However, cytotoxic damage observed of 7 and 40 nm aSiNPs was similar, since the aggregation of 7 nm aSiNPs.

Size-independent Cytotoxicity of aSiNPs on Other Kinds of Cell Lines

Stepnik et al⁸¹ reported the potential toxic effects in murine (3T3-L1) and human (WI-38) fibroblast cell lines of commercially available aSiNPs. Results indicated that 3T3-L1 cells were more sensitive to aSiNPs than WI-38 cells, and 21 nm aSiNPs induced no, or marginal, cytotoxicity on WI-38 cells, whereas 30 nm aSiNPs was found to be cytotoxic at the higher concentrations. This could be the result due to the larger hydrodynamic diameter of 21 nm aSiNPs, as measured by dynamic light scattering (DLS), the hydrodynamic sizes of 21 nm and 30 nm aSiNPs in distilled water were 66 \pm 3 nm and 41 \pm 1 nm, respectively. Uboldi et al⁸² examined the cytotoxicity induced by 1–100 μ g/mL of aSiNPs with a size diameter ranging from 16 nm to 300 nm, and showed that the viability of Balb/3T3 mouse fibroblasts was slightly impaired only after exposing to 80 nm aSiNPs. The authors implied that the absence of toxicity might be due to the huge aggregate formation of aSiNPs and the sensitivity of the cell line studied. Kim et al⁸³ cultivated three types of cells, mouse embryonic fibroblasts (NIH/3T3), A549, and HepG2, with exposure of seven sizes aSiNPs (20, 40, 60, 80, 100, 150, 200 nm). The NIH/3T3 cells showed dose-dependent reduction in viability, and larger sized particles caused higher cytotoxicity. The following intracellular ROS measurement indicated that 100 and 200 nm aSiNPs induced the production of more ROS than 20 or 60 nm aSiNPs. For both the A549 and the HepG2 cells, there was no significant difference in viability reduction between different doses (\geq 50 μ g/mL) and sizes except for 60 nm aSiNPs, owing to the preferential endocytosis of this nanoparticle observed. Park et al⁸⁴ verified the developmental toxicity of four well-characterized aSiNPs (10, 30, 80, and 400 nm) in D3 embryonic stem cells. Addition of 80 nm and 400 nm aSiNPs to the cell culture medium did not affect the metabolic activity of D3 cells after exposure for either 24 h or 10 days. However, the treatments with 10 nm and 30 nm aSiNPs for 24 h remarkably increased the metabolic activity of D3 cells. In contrast, when the treatment was extended to 10 days,

exposure to high concentration of the same particles caused a reduction in metabolic activity to 60% and 57%, respectively. This might be the result of a second burst of nanoparticles being dispersed following the cell culture medium change after three, five, and seven days, finally leading to toxic concentrations. The initial stimulation of metabolic activity after 24 h of exposure may be an adaptive response of the cell to the nanoparticles. Another research group evaluated the dermal toxicity of aSiNPs with different sizes (7 and 10–20 nm) on cultured HaCaT keratinocytes (CHKs). Both particles decreased cell viability in a dose-dependent manner. However, no difference in cytotoxicity was found between two sizes of aSiNPs, due to the agglomeration of particles and the small difference in hydrodynamic size.⁸⁵ Kim et al⁸⁶ exposed human neuronal cells (SH-SY5Y) to similar sizes of aSiNPs (16.9 nm and 15.3 nm). As revealed by MTT assay, there was no significant difference in cytotoxicity with small difference in particle size.

Discussion

As known, differences in the physical–chemical properties of nanoparticles could cause significant variation in its potential toxicity.⁸⁷ The particle size, as an essential characterization of nanoparticles, could influence the particles' biological reactivity obviously. In general, smaller sized nanoparticles possesses larger surface area as well as higher surface activity, which may make the particles more reactive in biological systems.² Moreover, compared to larger sized nanoparticles, it is easier for smaller nanoparticles to penetrate the cell membrane and induce further damage inside the cells.⁴² However, due to the complexity of the biological system, the results of current reports on the size-dependent toxicity of aSiNPs are not completely consistent. In this systematic review, we explored the relationship between the particle size and cytotoxicity of aSiNPs in vitro, 76 scientific papers were included and the majority of studies tended to demonstrate that smaller sized aSiNPs possessed higher cytotoxicity. However, this size-dependent toxicity could also be influenced by other experimental factors, such as synthetic method of aSiNPs, aggregation state of aSiNPs, experimental conditions, toxicity endpoint detection method and so on.

Amorphous silica particles could be produced by thermal (pyrogenic) or wet routes (colloidal, precipitated and gel).⁸⁷ According to different synthesis conditions and raw materials, the wet route for colloidal silica can be further classified into the Stöber method, Ludox method, and Lysine method, etc. It was reported silanol and siloxane

were two main groups on the surface of aSiNPs, and the silanol group was more reactive than the siloxane group, moreover, the distribution of these active groups depended on the silica type and the method of synthesis.⁸⁸ Thus, it could be proposed that different synthetic methods varied the surface properties of aSiNPs, which would eventually lead to diverse biological reactivity.⁸⁹ However, Napierska et al²⁶ used two types of silica nanoparticles produced by either Stöber process or Lys-Sil process, with four different diameters (2, 16, 60, and 104 nm), and found the cytotoxicity was related to the particle size directly, which indicated that compared with the synthetic method, the higher surface area of smaller particles played a pivotal role in the enhancement of surface reactivity. Similar results were obtained in the study of Napierska et al,² in which little difference in toxicity was found between Ludox silica nanoparticles (diameters of 14 and 15 nm) and Stöber silica nanoparticles (16 nm). Guichard et al³⁴ used three different types of aSiNPs, which were pyrogenic powder, precipitated powder and precipitated colloid, with the particle sizes ranging from 20–80 nm. The results also showed that the toxicity was strongly associated with the particle sizes. Nevertheless, the authors further observed that pyrogenic and colloidal manufactured aSiNPs of the same size were able to induce DNA damage, while no DNA damage was detected for precipitated particles. Thus, although the toxicity intensity of similar sizes silica particles synthesized in different ways was sometimes similar, the mechanism of toxicity was slightly different. Thomassen et al³⁷ reported that the catalytic ability of hydroxyl radical formation varied with different synthesis methods of aSiNPs. Compared with the lysine method and Stöber method, aSiNPs synthesized using the Ludox method showed higher rate of hydroxyl radical formation and catalytic activity. In summary, the particle size and specific surface area of aSiNPs are still important factors affecting the toxic effects of aSiNPs, but the differences in particle surface chemistry caused by different synthesis methods would also affect the toxic pathways and mechanisms of the particles.

In some cases, aggregation of aSiNPs was observed in cell culture media, which could largely influence the cytotoxicity. Aggregation not only substantially alters the overall characteristics (such as size, shape and surface topology) but also potentially influences the biological outcomes of aSiNPs.²³ In particular, smaller size of aSiNPs possess extremely large surface area and a huger number of particles in the same mass, consequently having more tendency

to aggregate.⁶⁰ By the influence of electron charge and Zeta potential, the suspensions of nanomaterials may be unstable and subsequently promote the aggregation of particles.⁸⁴ Akhtar et al⁷² reported that the mean of hydrodynamic sizes of 10 nm and 80 nm aSiNPs in culture medium, were 352 nm and 380 nm, respectively. As a result, no significant difference in cytotoxicity between these two sizes of aSiNPs was detected. In the study of Lin et al,⁷¹ the hydrodynamic sizes of 15 nm and 46 nm aSiNPs in water suspension were 590 ± 104 nm and 617 ± 107 nm, respectively; and the decrease of cell viability of the group exposed to 15 nm aSiNPs was not significantly different from that of 46 nm aSiNPs. Due to aggregation, the size of nanoparticles was much larger than its original size which means only a small part of nonaggregated particles can penetrate the membrane of cells and produce cytotoxicity. Therefore, original particle size as well as hydrodynamic size should be mentioned in the experiment, which could facilitate the subsequent analysis and comparison of aSiNPs toxicity.

Differences in experimental conditions, such as exposure dose, exposure time, tested cell line, presence of serum, etc, have been proved to affect the toxicity of aSiNPs obviously. Lots of studies in this systematic review have demonstrated the dose- and time-dependent cytotoxic effects caused by aSiNPs. Tokgun et al²³ reported that the viability of A549 cells was dose-dependently reduced by all four sizes aSiNPs (6, 15, 30, and 55 nm), examined at 24 h post-treatment. Wang et al⁶¹ exposed HEK293 cells to 20 or 50 nm aSiNPs and observed that the exposure at dosage levels between 20 and 100 $\mu\text{g/mL}$ from 0 to 48 h decreased the cell viability in both dose- and time-dependent manner. In the study of Ye et al,⁴⁰ L-02 cells were exposed to 0.2, 0.4, and 0.6 mg/mL of aSiNPs (21, 48, and 86 nm) for 12, 24, 36, and 48 h, similar toxicity trends were confirmed as well. It is well known that the number of nanoparticles accumulated around cells or internalized by cells rose gradually with the increased exposure dose, thus contributing more toxic effects on tested cells.⁴⁰ In addition, the exposure time should be another influencing factor of experimental results. Since nanoparticles reached the cytosol of cells by penetration or endocytosis and it took time to induce cytotoxicity, the reduction in survival of cells showed gradually with time. Therefore, longer exposure time of aSiNPs usually causes higher toxicity, as irreparable cell damage accumulates kinetically.⁷⁰

Studies have shown that the toxic reaction of different cell lines to aSiNPs was related to the cellular uptake rate of particles and the reaction mechanism. Du et al⁶⁷

demonstrated that N9 cells (microglia) were more sensitive to the toxicity of aSiNPs than bEnd.3 cells (micro-endothelial cells) and HT22 cells (neurons), owing to the different uptake rate of aSiNPs. The authors observed that after crossing the blood–brain barrier, aSiNPs were mainly swallowed by N9 cells. However, HT22 cells, a representative model of neurons, were found to barely phagocytize aSiNPs and have minimal cellular uptake of particles. Further research showed that the uptake rate of aSiNPs was closely related to the cellular internalization mode of nanoparticles, and different cell types had different internalization processes. Vicente et al⁶⁰ found human keratinocytes (K17) showed more intense toxic reactions to aSiNPs than human dermal fibroblasts (HDF), with higher uptake rate of nanoparticles. Moreover, phagocytosis had been identified as the main internalization mechanism for K17, while slower caveolae-mediated endocytosis for HDF. Different cell lines have different reaction mechanisms to aSiNPs, which is also one of the reasons influencing cytotoxicity. Oxidative stress is considered to be an important mechanism of nanomaterial-induced toxicity, leading to the elevated production of ROS, which are directly involved in the regulation of cell survival and death.^{90,91} Passagne et al⁶⁸ used two proximal tubular cell lines (human HK-2 and porcine LLC-PK1) to investigate the toxic mechanism of aSiNPs. Compared with HK-2 cells, LLC-PK1 cells were found to be more susceptible to the toxic effects induced by aSiNPs, and the significantly increased intracellular ROS level was clearly related to the toxicity. After entering the cells, nanoparticles can also stimulate the cells to release cytokines, which are involved in inflammatory processes and further cell structure damage, even cell death.⁹² Låg et al³⁰ found that after exposing to aSiNPs, the survival rate of BEAS-2B was lower than that of HBEC3-KT. Moreover, BEAS-2B cells showed much higher cytokine release than HBEC3-KT cells, including both IL-6 and CXCL8, which played a central role in inflammatory process induced by particle components. In addition to the above mechanisms, there are also other reaction mechanisms closely related to the toxicity of aSiNPs, such as autophagy dysfunction. The reaction mechanisms may vary from cell to cell, which requires special attention in the analysis of toxic effect of nanoparticles.

The last experimental condition we would like to discuss was the presence of serum in aSiNPs suspension. Due to its high surface reactivity, aSiNPs always absorbs the serum protein leading to the formation of protein shield or

protein corona surrounds the particles. It has been well established that the presence of protein corona resulted in surface modification, which further contributed to changes in Zeta potential, aggregation state, cellular uptake, even toxicity.¹² Lesniak et al⁹³ reported that in the absence of serum, an increased amount of aSiNPs associated with A549 cells was detected and hence an enhanced uptake was postulated. Leibe et al¹⁰ analyzed the effects of two sizes of aSiNPs on A549 cells, and found that the cytotoxicity induced by 12 nm aSiNPs was more pronounced than that of 50 nm aSiNPs. In addition, both aSiNPs reduced cell viability only in the absence, but not in the presence of serum. Similar phenomenon that serum protein or protein corona reduced the toxicity of aSiNPs was also confirmed by several other investigations.^{27,69} From a mechanistic point of view, researchers suggested different hypotheses of this phenomenon: (1) the formation of protein corona reduced the surface energy of bare aSiNPs; (2) the protein corona decreased the cellular uptake of aSiNPs and thereby the cytotoxic effects were induced to a lesser extent compared to serum free conditions; (3) the presence/absence of protein led to different cellular targeting of aSiNPs.^{12,22} Moreover, for risk assessment with the possibility of threshold dose effects, it is essential to apply the most sensitive conditions. Therefore, serum free condition in addition to serum condition was advised for hazard identification because it demonstrated a higher sensitivity.²²

Detection method of toxicity endpoint may also influence the results' consistency in different research. The most common endpoint of cytotoxicity was cell viability reduction, which could be reflected by the decrease of mitochondrial activity of tested cells and the increase of lactate dehydrogenase (LDH) release in culture medium. Mitochondria are the energy center of cells, and the mitochondrial activity is closely related to the cell viability. LDH release is an indicator of plasma membrane rupture, which is proportional to the number of cells damaged or lysed.² Several researchers studied the cellular uptake of aSiNPs and pointed out that the transport of smaller particles was dominated by diffusion while larger particles were mainly transported by sedimentation or endocytosis,¹³ which means smaller aSiNPs could easily penetrate cells and induce oxidative reaction to mitochondrial, while larger aSiNPs tended to firstly induce membrane injury through mechanical friction or oxidative damage.⁹⁴ Moreover, it was reported that nanoparticles, owing to their high reactivity, would possibly absorb or interact with the experimental components, leading to

inaccurate or false results provided by some classical toxicity assays, including MTT and LDH activity assay.⁹⁵ Therefore, in order to ensure the accuracy of experimental results, different toxicity endpoint detection methods should be combined when detecting and comparing the toxic effects induced by aSiNPs with wide range of sizes.

The abovementioned were the main influence factors of size-dependent cytotoxicity of aSiNPs in vitro, which should be taken into consideration when performing relevant studies.

Conclusion

In the present review, most of the included studies tended to demonstrate that smaller sized aSiNPs with larger surface areas produced higher cytotoxicity. However, there were also some studies that did not reach similar conclusions. As for the influence factors of size-dependent cytotoxicity of aSiNPs in vitro, we mainly expounded from the following four aspects: (1) The synthetic method of aSiNPs could influence the distribution of active groups on the surface of aSiNPs, and further affect the toxic pathways or mechanisms. (2) Aggregation of particles would alter the overall characteristics (size, shape, surface topology, etc) and the cytotoxicity of aSiNPs significantly. Thus, both the original particle size and hydrodynamic size of nanoparticles should be measured in toxicology study. (3) In terms of the influence of different experimental conditions, we mainly discussed exposure dose, exposure time, tested cell line and the presence of serum. A majority of studies reported both dose- and time-dependent cytotoxicity of aSiNPs, since higher exposure dose and longer exposure time could increase the cellular uptake of nanoparticles and the interaction duration between particles and cells. The sensitivity of different cell lines to aSiNPs mainly depends on different cellular uptake rate of particles and different reaction mechanisms to the particles. In the presence of serum, aSiNPs could absorb the serum protein and form protein corona, which inhibited its cellular toxicity in most cases. (4) Detection method of toxicity endpoint could influence the toxic effect of aSiNPs from two aspects. Firstly, aSiNPs with different particle sizes might produce toxicity in different subcellular structures. Secondly, nanoparticles owing to its high reactivity would possibly absorb or interact with the experimental components, leading to inaccurate or false results. Therefore, two or more kinds of toxicity endpoint detection methods should be combined in nanotoxicology study.

In addition to the above influence factors, the current studies on toxicity of aSiNPs still have some limitations, which should also be taken into account in future experimental research. Most included investigations in this review did not use a specific guideline to carry out their experiments, causing some diversity of experimental details and difficulties in quantitative comparison between studies. Thus, it is recommended to apply a certain guideline, which can largely improve the comparability of different studies and the control of systematic bias.⁹⁶ Another insufficiency was that some researchers used very high dosage in their toxicity and mechanism studies, which was far beyond the actual exposure dose of occupational population or general population. Hence, it is inappropriate and not recommended to use extremely high dosage in a nanotoxicology study in vitro. Lastly, although adverse effects of aSiNPs observed in vitro might indicate toxicity in vivo, follow-up investigations are needed to confirm this relationship. Moreover, evidence on population exposure data and potential effect of aSiNPs in human beings is still insufficient and needs to be explored further. Therefore, it is of great significance to conduct more studies on the toxicity of aSiNPs both in vitro and in vivo, and related adverse effects on human health should get more attention.

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Disclosure

The authors report no conflicts of interest in this work.

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