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Communication

Characterization and *In Vivo* Toxicity Assay of Uncoated Silicon Nanoparticles

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Abstract: Silicon nanoparticles (SiNPs) are highly promising for biological and biomedical applications, including bioimaging, due to their unique opltical properties (i.e. strong fluorescence and very high photostability). Their low or negligible *in vitro* toxicity has been reported, but *in vivo* toxicity and biological effects of SiNPs are still uncertain. Uncoated SiNPs were dispersed in distilled water via sonication, and their rapid aggregation was observed (319.0 ± 2.4 nm particle size). *In vivo* toxicity was studied using *Danio rerio* embryos and larvae. Rapid aggregation in their incubation medium was observed; besides that, SiNPs at 25 mg/L or higher concentration induced swim bladder malformation and/or death of the fish. The estimated LC_{50} value for 7-day larvae was 180 mg/L. This is the first *in vivo* toxicity study of uncoated and unfunctionalized SiNPs. To achieve better stability in biological media and lower toxicity, SiNPs should be covered with hydrophilic layers, but their absorption by cellular membranes may be weaker in this case.

Keywords: uncoated silicon nanoparticles; aggregation; *in vivo* toxicity; LC_{50} for *Danio rerio* larvae

1. Introduction

Cancer is one of the leading causes of premature death worldwide [1]. Tumor development can be prevented at early stages if it is diagnosed early enough. Various nanosized materials have been introduced into the molecular diagnostics of cancer recently [2]. Nanomaterials are promising agents for that due to their ultrahigh surface-to-volume ratio which allows them to be stable and diffuse in aqueous biological media. The particles should be hydrophobic enough to be absorbed by cellular membranes and hydrophilic enough to be stable in ion-containing water.

Besides colloidal stability, nanomaterials should have some properties that would allow them to be used in tumor detection and characterization. If there are nanomaterials which can be used as contrast agents, fluorescence imaging would be helpful; fluorescence imaging is one of the most widespread methods of imaging in biological sciences [3].

Silicon (Si) nanoparticles (SiNPs) have been studied since late 1980s [4]. Currently, there are multiple industrial applications of SiNPs: luminescent display devices, semiconductors, solar energy cells, lithium batteries, etc [5]. It is possible due to their unique opltical properties (i.e. strong fluorescence and very high photostability) [6]. A strong and stable fluorescence peak of SiNP suspension at about 600 nm (depending on particle size) has been reported [7,8]. This Si-Si bond peak lost only about 30% of its intensity after 4 days of incubation at room temperature [7]. So, SiNPs are highly promising for biological and biomedical applications, including bioimaging and, for example, specific targeting cervical cancer cells or glioblastoma cells [3]. Such optical properties were also discovered for semiconductor nanocrystal quantum dots (QDs) consisting of CdSe/ZnS or CdSe/CdS/ZnS layers; they were also called promising for cancer imaging [9]. The structure of these particles shows that they are potentially strongly toxic for living matter. *In vivo* toxicity of Cd-based QDs caused mainly by Cd^{2+} release after hydrolysis in biological media has been reported [10,11]. Cd^{2+} is a very toxic heavy metal cation; besides chelation

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of biomolecules, it has a unique mechanism of toxic action. It blocks Ca^{2+} membrane transport channels by binding with channel proteins, which is caused by very close ionic radii of Ca^{2+} and Cd^{2+} [12]. SiNPs are quite chemically inert and potentially much less toxic and, therefore, more suitable for biomedical application.

SiNPs have been reported to be non-toxic *in vitro* [13]. The US Food and Drug Administration (FDA) has given approval for clinical trials to test SiNPs for food and medical applications [14], but there is lack of *in vivo* toxicity data for SiNPs, especially for the pure uncoated and unfunctionalized particles.

2. Materials and Methods

2.1. Chemicals

SiNPs were manufactured by Sigma-Aldrich, St. Louis, MO, USA. According to the manufacturer's datasheet, the reagent had 99.0% purity (oxygen content was up to 0.7% due to partial oxidation); the particles had spherical shape and 50 nm mean size. The reagents for reconstituted water preparation (see subsection 2.3) were manufactured by Spektr-Chem, Moscow, Russia; their purity was ≥ 98.0%.

2.2. Preparation and characterization of SiNP suspension

A stock 5 g/L suspension was prepared using the following technique. Two hundred and fifty milligrams of SiNP powder was weighed in a plastic glass, and 50 mL of distilled water was added. The mixture was shaken vigorously and sonicated three times in a Sonopuls HD 2200 ultrasonic homogenizer (Bandelin, Berlin, Germany) at 66% of power for 10 min in ice water with intermediate cooling for 5 min.

Particle size distribution was studied with N5 Submicron Particle Size Analyzer (Beckman Coulter, Brea, CA, USA) using 90° light scattering angle. The measurement was made in triplicate. The mean ± SD particle size value was calculated using the device software.

2.3. Animals for in vivo toxicity assay

Adult *Danio rerio* fish were purchased from local aquarium fish dealers in Moscow, Russia and held in the laboratory for 6–12 months before the experiment. The fish were held in aquaria in permanently filtrated and aerated reconstituted water (294 mg/L $CaCl_2{\cdot}2H_2O$ + 65 mg/L $NaHCO_3$ + 123 mg/L $MgSO_4{\cdot}7H_2O$ + 6 mg/L KCl in distilled water; 1 L of water per fish). The fish were fed with dry TetraMin Crisps flake feed (Tetra, Melle, Germany) twice a day + frozen brine shrimp (Aqua Logo, Moscow, Russia) once a day. Forty per cent of aquarium water was changed to newly prepared reconstituted water every 2 weeks. The water temperature was kept at 26 ± 1 °C. The aquaria were lit for 14 h per day. The fish maintenance procedure is described in details in [15].

A week before the experiment, 2 mature female fish were isolated in a special aquarium with the same conditions. The fish were fed 24 h before spawning, and the next feeding was only after spawning. After the last feeding, 5 mature males were moved into the aquarium with the isolated females. The aquarium bottom was covered by a plastic net with about 1.5 mm mesh size to prevent eating of eggs by the fish. Next morning the spawning occurred, the fish were moved back to basic aquaria, and the eggs were collected. The healthy fertilized eggs were selected at 16–32-cell blastula stage (1.5–2 h post-fertilization) using EZ4 D stereo microscope (Leica Microsystems, Wetzlar, Germany).

2.4. Toxicity assay procedure

Suspensions for incubation were prepared in wells of 24-well sterile microtiter plates (2 ml per plate) via dilution of the stock 5 g/L suspension by preliminarily aerated mixture of reconstituted water (see the previous section) and distilled water (1:4 volume ratio). The composition of reconstituted water is optimized for freshwater fish and recommended by OECD [15] for toxicity testing. Despite that, use of 5× diluted reconstituted water also does not make any adverse effects on fish embryos and larvae but significantly

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increases stability of suspensions of nanoparticles, according to our previous experience [16]. The selected eggs were placed into wells with the following SiNP suspensions: 0 (control), 10, 25, 50, 100, 250 and 500 mg/L, N = 24 eggs (1 plate) per each suspension. The classic static test was performed: the animals were incubated in the suspensions for 7 days at 26 ± 0.1 °C without medium replacement. Each 24 h the suspensions and the embryos/larvae were observed using the Leica EZ4 D microscope with built-in digital camera; any visible adverse effects including death were recorded. The assay was made in triplicate. The data were analyzed using STATISTICA 9.0 and Microsoft Excel software. The toxicity assay procedure is described in details in [15].

2.5. Ethical Approval

This study has been approved by the Ethics Committee of the Institute of Biomedical Chemistry and has been performed according to its protocol. The protocol is guided by the Directive 2010/63/EU of the European Parliament and the European Council "On the protection of animals used in the laboratory research".

3. Results

3.1. Particle size distribution of the stock SiNP suspension

The distribution is shown on Figure 1. The calculated particle size was 319.0 ± 2.4 nm. Despite intensive sonication, SiNPs aggregated immediately in aqueous medium (distilled water) due to their hydrophobic nature (the mean initial size was 50 nm).

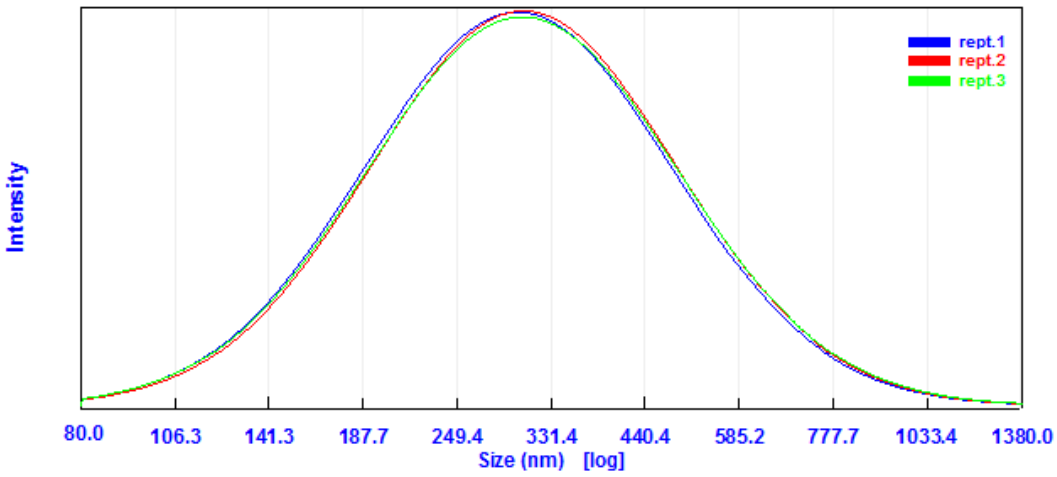

Figure 1. Particle size distribution of stock 5 g/L silicon nanoparticle (SiNP) suspension right after its preparation.

3.2. In vivo toxicity of SiNPs

Noticeable extent of aggregation and sedimentation of Si particles was observed even after 24 h of *Danio rerio* embryos incubation (Figure 2). Despite possible interference in diffusion of oxygen and metabolites through pores of embryo chorion by the sediment, there was no statistically significant toxicity at embryo stage (including hatching rate and time).

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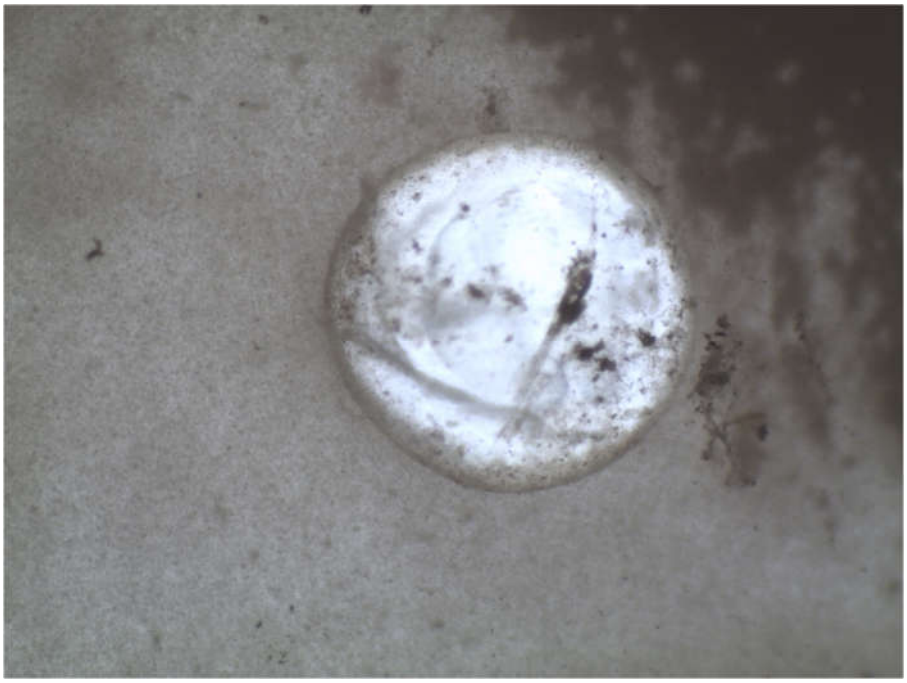

Figure 2. A *Danio rerio* embryo covered with Si sediment after 24 h of incubation in 250 mg/L SiNP suspension, 35 × magnification.

At larval stage (5–7 days of incubation), cases of void swim bladders and fish death were observed; the rate of these toxic effects was statistically significant (Table 1). However, there were no typical severe toxic effects i.e. edema, tail curvature, etc. A possible cause of swim bladder malformation was the influence of heavy hydrophobic Si sediment (2.33 g/cm^3 density, according to the manufacturer's datasheet) from the bottom of plate well (Figure 3), so it was hard for a larva to swim up and swallow some air.

Table 1. This is a table. Tables should be placed in the main text near to the first time they are cited.

Concentration of SiNPs (mg/L)	Survival rate (ratio to that in control)		Void swim bladders percentage among survivors	
	Mean ± SD, %	ANOVA between test and control groups	Mean ± SD, %	ANOVA between test and control groups
10	100 ± 0.0	-	0.0 ± 0.0	-
25	100 ± 0.0	-	20.8 ± 8.4	$p < 0.05$
50	100 ± 0.0	-	41.7 ± 8.4	$p < 0.01$
100	74.5 ± 7.9	$p < 0.05$	50.0 ± 12.6	$p < 0.01$
250	38.6 ± 8.3	$p < 0.05$	100 ± 0.0	$p < 0.01$
500	0.0 ± 0.0	$p < 0.01$	- [no survivors]	-

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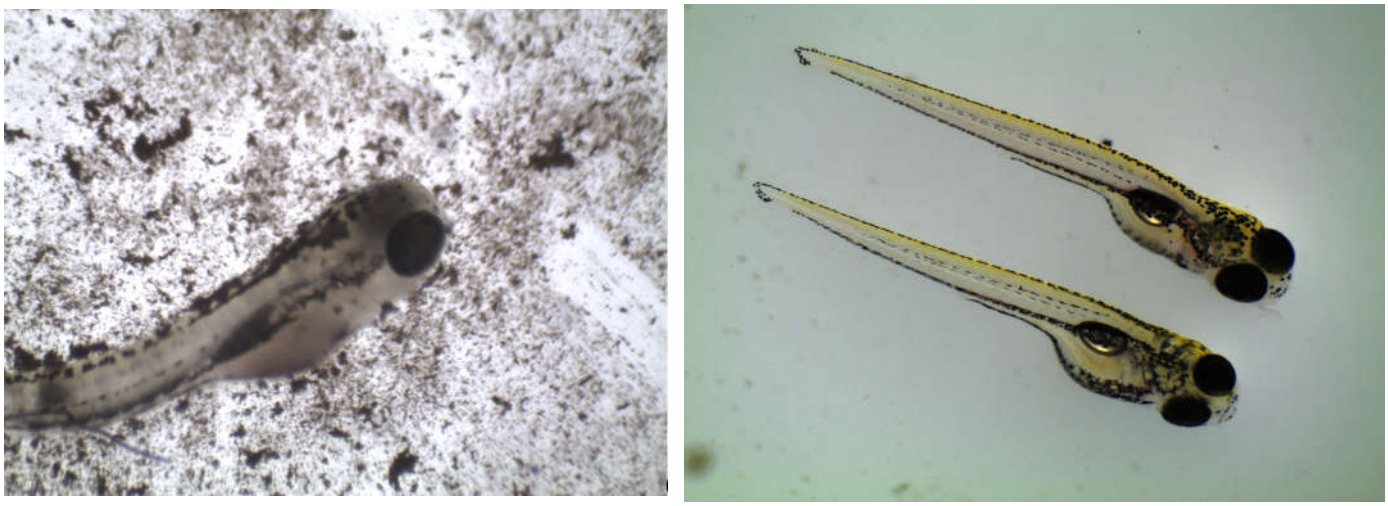

(**a**) (**b**)

Figure 3. (**a**) A *Danio rerio* larva with void swim bladder after 7 days of incubation in 25 mg/L SiNP suspension, 35 × magnification; (**b**) Healthy 7-day old larvae (control), 30 × magnification.

LC_{50} for 7-day larvae was 180 mg/L, the value was estimated graphically (Figure 4).

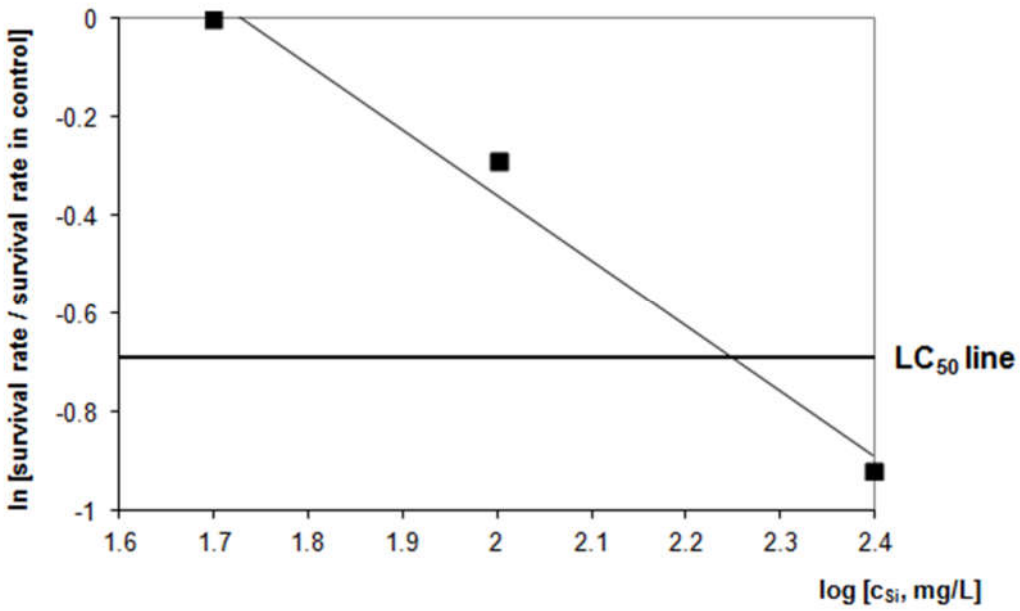

Figure 4. Graphical determination of LC_{50} of SiNPs for 7-day *Danio rerio* larvae. The value was calculated as 10 ^ [X-coordinate of intersection point of logarithmic linear regression line of survival rate (in form of ratio to that in control) vs. concentration and logarithmic LC_{50} line].

4. Discussion

Despite noticeable aggregation and sedimentation, SiNPs appeared to be significantly toxic *in vivo*. The acting concentration of SiNPs at the point of 50% death of larvae was quite lower than the estimated LC_{50} value. On the contrary, no *in vivo* toxicity of SiNPs was reported in the previous studies. It is necessary to consider that *in vivo* toxicity studies of only firmly coated SiNPs have been reported. Thus, intravenous injection of PEGylated micelles containing SiNPs (380 mg/kg) did not induce any toxic effects in mice [17]; carbohydrate-coated SiNPs were found to have no significant toxicity for *Xenopus laevis* frog embryos at 1000 mg/L [18]. In those studies, the coatings were quite hydrophilic and made the suspensions stable, but they were polymeric and chemically stable, so it is reasonable

to consider that Si itself did not interact with living matter. Intravenous injection of SiNPs functionalized with less firm non-polymeric structure ($H_2NC_3H_6$-) induced significant level of hemocyte apoptosis in *Bombyx mori* silkworm larvae at dosage of 3.9 μg of Si per animal [19]. Our study is the first *in vivo* toxicity study of uncoated and unfunctionalized SiNPs, and the toxicity was found significant too.

To achieve better stability in biological media and lower toxicity, SiNPs should be covered with hydrophilic layers, but their absorption by cellular membranes may be weaker in this case. If the particles are used for cellular bioimaging they should be hydrophobic enough to be absorbed by cellular membranes. The use of uncoated SiNPs for bioimaging applications appears to be questionable due to their significant *in vivo* toxicity. Perhaps it is reasonable to use other chemically inert hydrophobic nanoparticles; for example, gold nanoparticles (AuNPs) have weaker fluorescence than SiNPs do but toxicity of AuNPs is truly negligible [16,20].

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Conflicts of Interest: The authors declare no conflict of interest.

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