The uptake and effects on survival of nano silver and nano titanium dioxide in brine shrimp (*Artemia nauplii*)

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Prefactory note

As part of the veterinary training all students of Utrecht University have to complete a three months research project. This project has to contain a reading period, practical work as well as the writing of a report and giving a presentation. I have fulfilled my research project at the Norwegian School of Veterinary Science (NVH) in Oslo, where I joined a research group and studied the uptake and the effects on survival of silver and titanium dioxide nanoparticles in brine shrimp (*Artemia nauplii*). This project was a small pilot study of a larger project, since the effects and fate of these nanoparticles will also be studied in zebrafish larvae (*Danio rerio*). Originally I was to join in the water exposure experiment as well, but due to poor breeding of the fish this experiment had to be postponed. The reasons of this poor breeding is not really clear, but it might be due to stress, since the fish were send in the lab just a few weeks earlier.
Since I did get involved in the set up of the water exposure experiment and participated in setting the fish up for breeding, I will shortly describe the research plan of this experiment in the introduction of my report.

I think it is nice to mention that during my stay I also joined a week long training program for the work in the Aleström Zebrafish Laboratory. During this course I was taught among other about the zebrafish as a model organism, the laboratory structure, water handling, feeding, system water and daily maintenance, fish welfare, the technical installations, monitoring and documentation, and development, anatomy and pathology of the zebrafish. After this training week I received a certificate.
I also joined in the embedding of the artemia in plastic for electron microscopy, but since there was not enough time to have performed the TEM thoroughly I could not describe it in the results.

The main research group consisted of Camilla Almås, Marthe Røgeberg and Steven Verhaegen, under the supervision of Erik Ropstad.
On this specific project I have partly worked with another veterinary student from NVH, Vanessa Bettembourg, who was also writing a report about this study.

Contents

Abstract

Particles with a size below 100 nm are called nanoparticles. Nowadays these particles can be found in a wide range of consumer products and nanotechnology is a continuously growing area of research. Even though nanoparticles have a lot of convenient characteristics some have already proven to be quite toxic, especially to aquatic life forms.
In this report an exposure study of brine shrimp (*Artemia nauplii*) is described. Brine shrimp were exposed to 4 different concentrations of silver and titanium dioxide, both nano and bulk. The particles were characterised by use of a zetasizer and their actual size was determined.
To compare the effects of silver ions and nano silver an additional group of brine shrimp was exposed to silver nitrate. The control group was exposed to bovine serum albumin, since this was added to all solutions to keep the nanoparticles in suspension. After 6, 12 and 24 hours lethality was assessed.
For each substance the mean survival after 24 hours was calculated per concentration. These results were compared to the survival in the control group. In the silver and titanium groups the lowest concentrations showed the largest effect on survival, whereas in the silver nitrate the highest concentration was most lethal. The characterisation of the particles showed a wide spread in sizes and most particles were much larger than the advertised size. This was due to high aggregation of the particles. Because of this and the similar results for the nano and bulk in the lethality test no conclusions can be made about size dependent effects. However it can be concluded that agglomerates do cause a decrease in survival.

Keywords: nanoparticles, toxicity, artemia, zebrafish, silver, titanium dioxide

1. Introduction

Materials below a size of 100 nm are called nanoparticles. These particles provide a large surface area when used in consumer products, which can be very useful. In the past years nanotechnology has grown rapidly and nano materials are used in a wide range of products, such as sunscreens and paints. Although it is presumed that toxicity of these products to humans is little to none, the effects of nanoparticles in the environment is questionable. Nanoparticles from e.g. sunscreens can easily pollute the aquatic environment. Some products containing nanoparticles are even constantly exposed to the enviroment , such as pipelines. (Aitken et al., 2006; Hund-Rinke et al., 2006; Wiench et al., 2009)
It has been reported that nanoparticles can have significant effects on aquatic life forms, although some particles seem to be more toxic than others (Bar-Ilan et al., 2009)

In a collaborative project (NanEAU), the Gabriel Lippmann Institute (Luxembourg), the Napier University (Scotland) and the Norwegian School of Veterinary Science aim to "adapt and develop test protocols for the evaluation of toxicity of emerging nanoparticles (NPs) that are likely to end-up in our water columns and thereby potentially counteracting the sustainable use of water bodies for drinking water, recreation and adding an additional stress to the aquatic environment." In this project zebrafish (*Danio rerio*) are used as an experimental model to investigate the effects of nanoparticles.

Zebrafish are small and easy to house, have transparent embryos, are easy to breed and its early development is well characterised. Also the genome of zebrafish is well characterised. Therefore they are a good model to study effects on reproduction and development of aquatic life forms. (Aleström et al., 2006; Bar-Ilan et al., 2009; Kimmel et al., 1995)

As part of this project, the Norwegian School of Veterinary Science is setting up a research project to investigate the effects of nano silver (Ag) and nano titanium dioxide (TiO2) on zebrafish larvae. Several studies have shown that nano silver has obvious toxic effects to aquatic life forms (Asharani et al., 2008; Bar-Ilan et al., 2009; Laban et al., 2010; Wu et al., 2009)
Also titanium oxide has been used in multiple researches with aquatic life forms. (Federici et al., 2007; Wiench et al., 2009; Zhu et al., 2008)
Silver nitrate (AgNO3) will also be included in the study in order to compare the effects of

silver nanoparticles to the effect of silver ions, which are known for their toxic effects. (Laban et al., 2010; Ratte et al., 1999; Wood et al., 1999)

The final aim is to expose the zebrafish larvae through brine shrimp containing these nanoparticles. Brine shrimp *(Artemia*) are small aquatic crustaceans, which in nature can be found in saltwater lakes. Their eggs are metabolically inactive and can survive for many years while in a dry, oxygen deprived environment, sustaining very low temperatures. When the eggs are put in salt water with a temperature of 28º C they hatch after approximately 24 hours. (Baxevanis et al., 2004; Vanhaecke et al., 1981) After this the newly hatched larvae (about half a millimeter in size) can be filtered from salt and fed to zebrafish as live feed.
To come to this specific experiment, first a dose response study on these artemia was done in order to investigate the uptake and fate of the nanoparticles in artemia and the effects on survival. In the near future a dose response water exposure on zebrafish larvae will be performed, in order to investigate the uptake of the nanoparticles and the effect on survival on zebrafish larvae when only exposed through water. After this the exposure through live feed will be performed.
Exposure will start at six days post fertilisation (6 dpf) and will end at 20 dpf. A study at NVH has shown this is a critical timeframe in zebrafish development in which there is a significant drop in survival in normal situations. (ZEBPOP) Studies on toxicity of nanoparticles during this specific period of zebrafish life are unknown, and therefore this seems an interesting timeframe to use for nanoparticle exposure.

2. Materials and methods

2.1 Laboratory conditions

All experiments were performed at the Aleström Zebrafish Laboratory at the Norwegian School of Veterinary Science. This laboratory has an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accreditation and the capacity of housing 15.000 fish. The lab consists of three rooms: a main room, a clean room, containing four systems, and a quarantine room, containing one system, an artemia hatchery and a larvae bench. All experiments were performed in the quarantine room.

2.1.1 Water conditions

The water used for all animal handling, called "system water", is water which is prepared in a 1000 L tank in the main room of the laboratory. This water is to have all parameters set to their ideal levels for the zebrafish. This means normal tap water is lead through several filters and made to reverse osmosis regulated water (RO-water). After this the ideal amounts of salt and minerals are added. HCl is used to adjust the pH to 7.5. Temperature is set to 28º C.

2.2 Artemia

The subspecies of artemia used in all experiments are *Artemia nauplii*. The eggs were purchased from Argentemia (grade 0, platinum label), in the United States.

The artemia were hatched overnight (24 h) in a 1 L bottle, containing 1 gram of artemia eggs, 40 grams of salt and 1 L of system water.

The chosen subspecies of artemia for this project, *Artemia nauplii*, are a smaller kind of artemia than *Artemia salini*, which are usually used in the Aleström laboratory to feed the zebrafish with. *Artemia nauplli* are < 450 μm, whereas *Artemia salini* are 500-525 μm. These smaller artemia were chosen regarding the upcoming zebrafish experiment. Since zebrafish larvae have a small mouth opening the hope is that all larvae would be able to eat the artemia from the start of the experiment with exposure through feed.

2.3 Exposure groups

Of both silver and titanium two sizes were used: nano (20 nm) and bulk (200 nm). Silver nitrate (AgNO3) was included in the project, so the effects of silver ions could be compared with the nanosilver effects, since it has been shown silver nitrate dissolves almost immediately. (Laban et al., 2010) Bovine serum albumin (BSA) was added to the nanoparticle stocks, in order to keep them in suspension. Therefore the control group was exposed to system water with just BSA, since it is assumed this has no significant effect on survival. (Asharani et al., 2008)

The silver nanoparticles were purchased from PlasmaChem in Poland. The titanium dioxide was purchased from Sigma-Aldrig in the United States.

2.3.1 Characterisation of the nanoparticles

The characterisation of the system water, stock solutions and dilutions was performed at the Norwegian Radium Hospital, Rikshospitalet University Hospital in Oslo, using a Malvern Instruments' Zetasizer Nano series. This instrument can define size, zeta potential and weight of

particle sizes between 0.6 nm – 6 µm. The zeta potential is an important characteristic of nanoparticles, since it has a major effect on the behaviour of the particles in a suspension. A zeta potential lower then -30 mV, or higher then 30 mV is wanted, since these potentials will lead to lesser aggregation of the nanoparticles. The size was measured with NIBS technology and Dynamic Light Scattering (DLS).
An absolute measurement of molecular weight was performed by using Static Light Scattering and the sensitivity from an avalanche-photodiode detector and fiber detection optics.
Per substance the zetasizer measured among others the zeta average and the polydispersion index (PdI). Also graphs showing the size distribution in intensity and volume uptake were created. The zeta average gives the mean particles size and the PdI tells something about the spread in size distribution. The higher the PdI, the more difference there is between particle sizes. Since the zeta average is a mean, a higher PdI makes it less reliable. A PdI below 0.1 makes the zeta average very useful as mean particle size, but with a higher PdI the different peaks of intensity and volume uptake in the distribution analysis should be examined closer to determine the sizes more precise.

2.4 Design lethality test

2.4.1 Preparation of the stock solutions

All stock solutions were prepared the same morning as the exposure started. The BSA stock was made by adding 7.5 grams of BSA to 50 ml of autoclaved milli-Q water.
One milligram of nanoparticles was weighed out in eppendorf tubes, using a microgram scale. A static control ionizer was placed on the scale, to prevent loss of nanoparticles to electrostatic charge. After this 850 µL autoclaved milli-Q water was added and after a brief vortex the solutions were sonicated on ice with a probe sonicator, for approximately 3 minutes. The sonicator was set on 20 kHz and 130 W, 30% output. 50 µL of the BSA stock and 100 µL of system water was added (150 µL system water to the AgNO3) and the solutions were diluted in 99.9 mL system water containing 4 grams of salt (Instant Ocean ®). A list of the stock solutions can be seen in table 1.

Table 1: Stock solutions of the different exposure groups

ID	Exposure	Stock conc
1	Control BSA	150 mg/ml
2	Control AgNO3	1 mg/ml
3	Ag 20 nm	1 mg/ml
4	Ag 200 nm	1 mg/ml
5	TiO2 20 nm	1 mg/ml
6	TiO2 bulk	1 mg/ml

2.4.2 Exposure protocol

The morning prior to the start of exposure artemia were set up for hatching in a 1 L flask containing 1 gram of artemia eggs, 40 grams of salt and 1 L of system water. The flask was placed in a 28º C water bath and was aerated through a tube connected to a pump. A cotton pad was put on top to prevent evaporation.

The next morning the stock solutions of nanoparticles were prepared and diluted, and 24 well plates were filled with the given concentrations. Exposure groups are given in table 2. In the control group BSA was added, since this was used to keep the nanoparticles in dispersion. The highest used dose of BSA was also used in the control.

Per exposure group one plate was used, containing 1 well with the control solution and 4 replicates (wells) per concentration.

Table 2: Exposure groups artemia lethality test.

ID	Exp. group	NP size	Concentration
1	Control BSA		10 mg/L
2	AgNO3		0,01 mg/L
3	AgNO3		0,1 mg/L
4	AgNO3		1 mg/L
5	AgNO3		10 mg/L
6	Ag	20 nm	0,01 mg/L
7	Ag	20 nm	0,1 mg/L
8	Ag	20 nm	1 mg/L
9	Ag	20 nm	10 mg/L
10	Ag	200 nm	0,01 mg/L
11	Ag	200 nm	0,1 mg/L
12	Ag	200 nm	1 mg/L
13	Ag	200 nm	10 mg/L
14	TiO2	20 nm	0,01 mg/L
15	TIO2	20 nm	0,1 mg/L
16	TiO2	20 nm	1 mg/L
17	TiO2	20 nm	10 mg/L
18	TiO2	bulk	0,01 mg/L
19	TiO2	bulk	0,1 mg/L
20	TiO2	bulk	1 mg/L
21	TiO2	bulk	10 mg/L

Hatched artemia were now transferred into the wells using a 200 µL pipette, while trying to put as less extra water in the wells as possible. It was aimed to place 10 artemia in each well, and the estimated number of artemia per well was recorded.

The wells were put on a shaker which was set on 250 shakes per minute (Edmund Bühler (700

Tubinger)), to prevent the particles from sinking to the bottom.
The movement of the shaker and relative high room temperature (around 25º C) provided a water temperature in the wells around 28º C, which is ideal for artemia. The temperature was recorded with a digital thermometer in a separate temperature control plate.

2.4.3 Lethality assessment

At 6, 12 and 24 hours after start of exposure lethality was assessed by use of a light microscope. The assessment was performed as a blinded test: one person collected the plates and recorded the findings of the second person. A third person served as a control and assessed a few random wells after the original assessment.
Every time the total number of artemia was determined and single artemia were recorded as dead (no movement shown for at least 5 seconds), immobile (some occasional limb movement, no swimming) or alive (showing normal movement, swimming). After the assessment dead artemia were removed from the wells. At the end of each experiment artemia from the nano silver 1 mg/L group were collected in a glutaraldehyde buffer, to use for transmission electron microscopy (TEM) later. This will give a qualitative assessment on the uptake of the particles by the artemia, which is crucial for the following zebrafish experiment. Unfortunately this microscopy has yet to be performed.

2.4.3.1 Additional observations

Aspects of the water were also recorded, to detect any signs of sedimentation when possible. The colour of the gut region in the artemia was also assessed, because this might give an indication on particle uptake.

2.5 Statistical analyses

The statistical analyses were performed using the JMP 8.0 software. A bar chart presenting the mean percentage of survival after 24 hours was made.
To investigate effects on survival of the different concentrations and substances, their effects were compared to the BSA control using a Chi-Square test (logrank), derived from Kaplan Meier survival curves. Subsequently, Cox's proportional hazards models were run, to take not only the experiment groups, but also the experiment number and their interaction into account. P-values below 0.05 was considered as statistically significant.

3. Results

3.1 Characterisation of the nanoparticles

Our measurements of the nanoparticles showed a broad distribution of size and the PdI was always above 0.1. The size distribution by intensity and volume were therefore more useful as an indicator of the size.

The nano silver mainly ranged in size from around 25 to over 600 nm. The silver bulk differed from around 300-700 nm. The nano titanium dioxide showed sizes between 80 to over 700 nm, and the bulk was measured between 100 to over 5000 nm. This showed that there was a lot of variation between the particle sizes within the solutions, which is a result of high aggregation of the particles. Overall the results showed that the more the nanoparticles were diluted, the more they aggregated.

Also the stock solutions showed greater sizes then expected, but when they were compared to the dilutions, which were the final solutions used for the exposure, they seemed to be more or less in the same size range.

All the measured sizes are shown in table 3. The different peak means are given as well as the percentage of particles which appear within these peaks. As an example on which these peak means of intensity and volume are based, the size distribution graphs of nano silver can be seen in figure 1.

Appendix I also shows the size distribution graphs of bulk silver, nano titanium dioxide and bulk titanium dioxide.

Table 3: Results zetasizer. The zeta average (Z-Ave) is the mean diameter of the particles in the solutions. The polydispersion index (PdI) is an indication about the spread in size distribution. The peak means (Pk 1,2,3 Mean) are based on graphs displaying the distribution sizes. The peak means based on intensity (Int) give the sizes based on light refraction. The peak means based on volume give the sizes based on volume uptake per certain size. Sizes are given as the diameter in nanometers (d.nm). The percentages are the percentages of particles which appear within the corresponding peaks.

Sample Name	Z-Ave	PdI	Pk 1 Mean Int	Pk 2 Mean Int	Pk 3 Mean Int	Pk 1 volume	Pk 2 volume	Pk 3 volume
	d.nm		d.nm + %	d.nm	d.nm	d.nm + %	d.nm + %	d.nm + %
Ag 20nm 10	178.2	0.33	222.6 (90.9%)	33.36 (5%)	4870 (4.1%)	226.5 (32.9%)	29.23 (49.9%)	4991 (17.2%)
Ag 20nm 1	153.8	0.52	202.1 (86.2%)	4813 (7%)	31.65 (6.9%)	192.7 (20.8%)	4994 (17.7%)	25.43 (61.5%)
Ag 20nm 0.1	415.4	0.5	322.4 (78.4%)	55.44 (21.6%)		331.3 (38.5%)	50.98 (61.5%)	
Ag 20nm 0.01	832.2	0.46	611.1 (100%)			630.7 (100%)		
Ag 200nm 10	460.9	0.49	372.6 (93.5%)	88.87 (6.5%)		394.1 (89.6%)	83.81 (10.4%)	
Ag 200nm 1	1259	0.87	284.5 (100%)			287.2 (100%)		
Ag 200nm 0.1	927.3	0.61	570.4 (100%)			591.1 (100%)		
Ag 200nm 0.01	868.1	0.49	696.4 (100%)			740.5 (100%)		
TiO2 20nm 10	228.5	0.16	251.1 (100%)			196.6 (100%)		
TiO2 20nm 1	239.2	0.23	298.5 (100%)			117.3 (74.8%)	431.6 (25.2%)	
TiO2 20nm 0,1	374.2	0.41	390.8 (91%)	86.66 (9%)		428.2 (33.6%)	79.95 (66.4 %)	
TiO2 20nm 0,01	990.1	0.42	708 (100%)			737.7 (100%)		
TiO2 bulk 10	570.8	0.32	565.4 (98.6%)	5507 (1.4%)		651.5 (38.2%)	5555 (61.8%)	
TiO2 bulk 1	392.6	0.46	337 (95.3%)	5386 (4.7%)		153.4 (3.5%)	399.4 (9.2%)	5467 (87.3%)
TiO2 bulk 0.1	534.8	0.38	535.5 (91.4%)	107.2 (6.2%)	5457 (2.4%)	623.3 (21.4%)	99.08 (11.8%)	5520 (66.8%)
TiO2 bulk 0.01	1475	0.76	1265 (100%)			1369 (100%)		
Ag 20nm stock	160.5	0.27	222.1 (100%)			35.25 (52.1%)	236.2 (47.9%)	
Ag 200nm stock	430.1	0.3	511.6 (96.7%)	4952 (3.3%)		85.53 (1.1%)	619.1 (77.4%)	5059 (21.5%)
TiO2 20nm stock	230.1	0.11	260 (100%)			207.3 (100%)		
TiO2 bulk stock	187.8	0.24	251.4 (100%)			45.05 (66%)	124.7 (28.7%)	407.8 (5.3%)

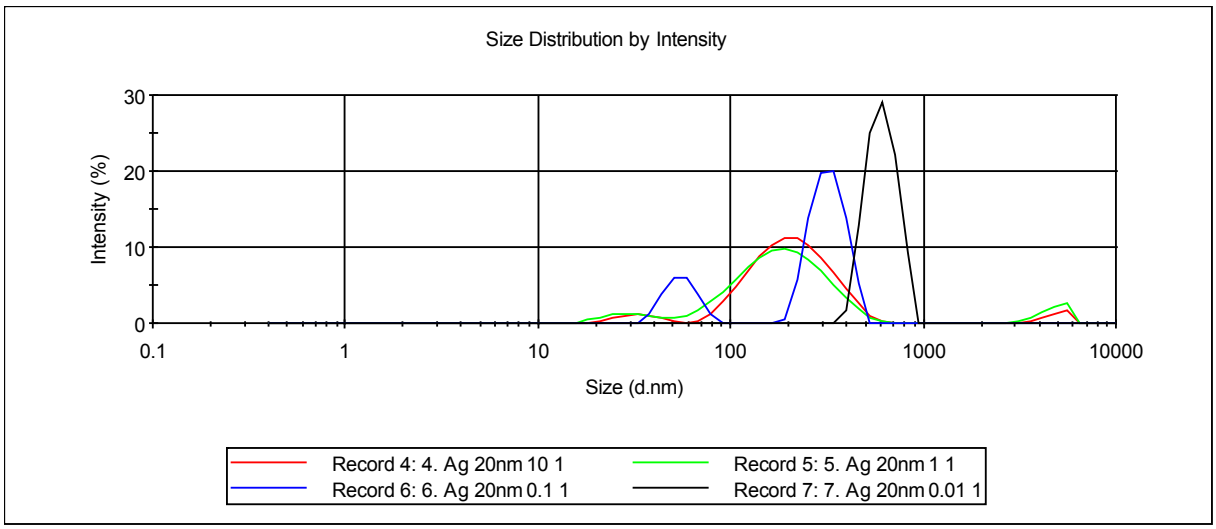

Figure 1a: Size distribution by intensity of the nano silver.

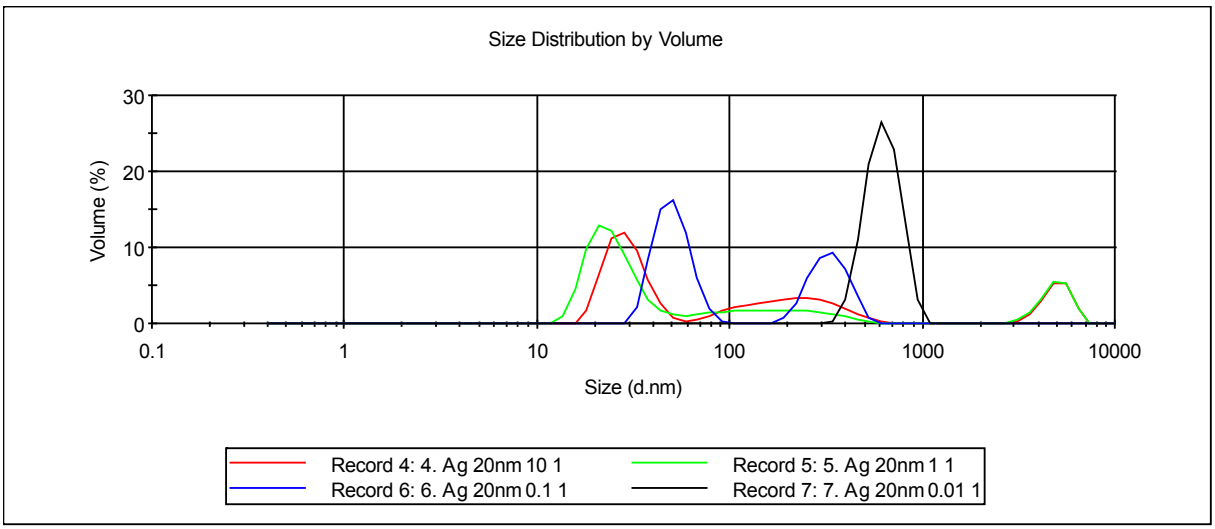

Figure 1b: Size distribution by volume of the nano silver.

Figure 1. The size distribution of the nano silver, measured by intensity of light refraction and by percentage of volume uptake, plotted per size. The exact values on which the graphs are based are shown in table 3. The graphs of the silver bulk, nano titanium and bulk titanium can be found in appendix I.

3.2 Lethality assessment

After all three experiments the mean percentage of survival after 24 hours was calculated per replicate (well).

In the BSA control the mean percentage of survival after 24 hours was 97.05 %, SE ± 1.65. The exposure group with the largest decrease in survival was the 10.0 mg/L concentration of silver nitrate. This group showed only 61.8 % survival after 24 hours ($p < 0.001$ logrank, Chi-Square). In

both silver and titanium dioxide, nano and bulk, the lowest concentration of 0,01 mg/L induced the largest effects.

With the calculated percentages bar charts were created for each substance, see figure 2. For the precise values of all the percentages and standard errors, see also table 4.

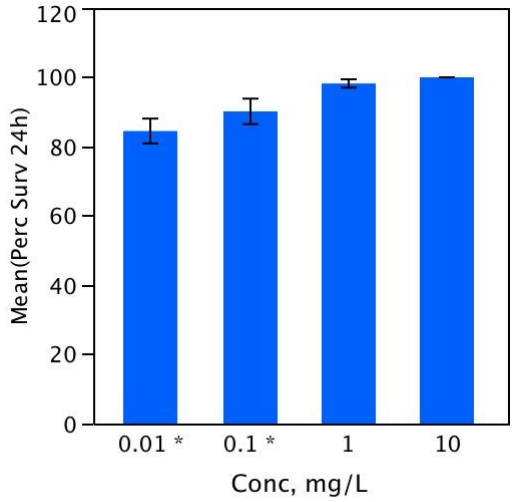

Figure 2a: Ag 20 nm
(p 0.0004, p 0.0256)

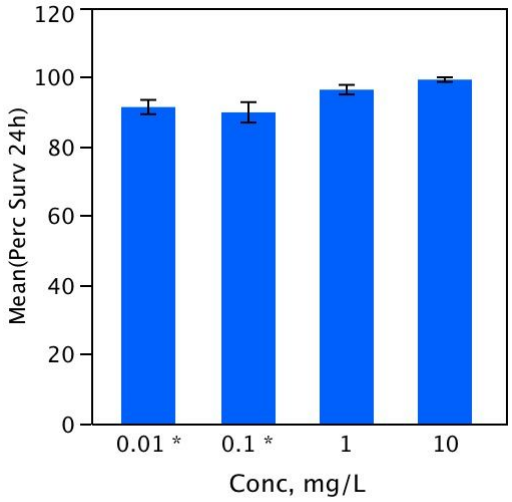

Figure 2b: Ag 200 nm
(p 0.0380, p 0.0126)

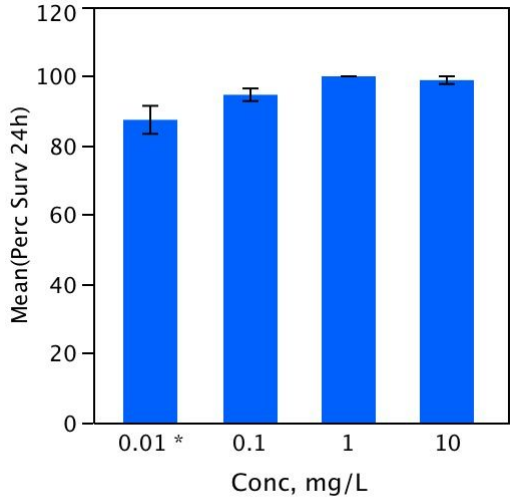

Figure 2c: TiO2 20 nm
(p 0.0015)

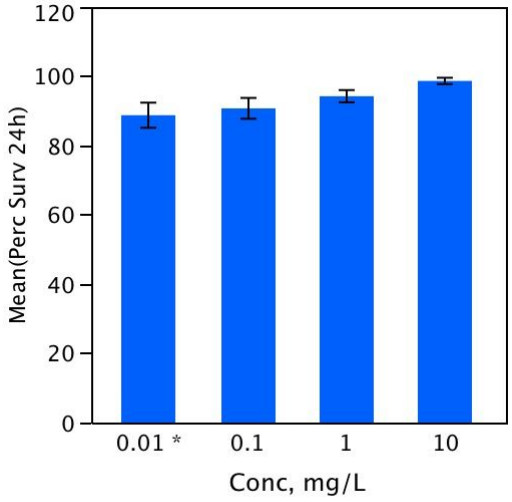

Figure 2d: TiO2 bulk
(p 0.0028)

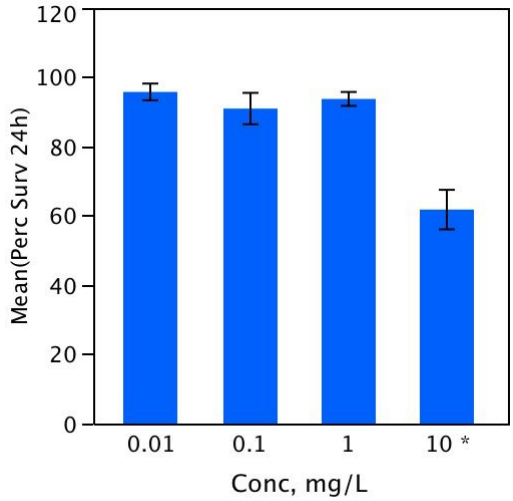

Figure 2e: AgNO3 (p <0.0001)

Figure 2: The mean percentages of survival after 24 hours per concentration, showing also the standard errors. A star is added to the concentrations that have shown a significant ($P \leq 0.05$) drop in survival compared to the BSA control, when Kaplan Meier survival analyses were used. P-values of the significant concentrations are found in the description of the individual charts.

Table 4: The mean (SE) percentages of survival after 24 hours of exposure.

Exp. Group	Conc, mg/L	Mean (% survival 24 h)	SE
Con BSA	10	97.05	1.65
Ag 20 nm	0.01	84.49	3.59
Ag 20 nm	0.1	90.16	3.68
Ag 20 nm	1	98.21	1.21
Ag 20 nm	10	100	0
Ag 200nm	0.01	91.41	2.07
Ag 200nm	0.1	89.88	2.95
Ag 200nm	1	96.43	1.38
Ag 200nm	10	99.33	0.67
AgNO3	0.01	95.77	2.41
AgNO3	0.1	90.97	4.54
AgNO3	1	93.75	2.01
AgNO3	10	61.8	5.7
TiO2 20 nm	0.01	87.43	4.04
TiO2 20 nm	0.1	94.67	1.82
TiO2 20 nm	1	100	0
TiO2 20 nm	10	98.89	1.11
TiO2 bulk	0.01	88.76	3.63
TiO2 bulk	0.1	90.74	3.01
TiO2 bulk	1	94.25	1.77
TiO2 bulk	10	98.67	0.91

Using the Cox's proportional hazards model, which accounted for both exposure group and experiment number, the same groups were found significantly different from the controls as in the Kaplan Meier analysis. The proportional hazards analysis was even more sensitive and gave an extra significant group; the TiO2 bulk 0.1 mg/L group showed a significant effect on survival when compared to the control (p=0.0492).
When looked at experiment effects two groups, the nano titanium 0.01 mg/L and the bulk titanium 0.01 mg/L, the experiment number seemed to have a significant effect on the survival results (p=0.009, and 0.0493, respectively). In the bulk titanium 0.01 mg/L the experiment number and exposure group showed a significant interaction (p=0.0051).

3.3 Additional observations

Apart from the survival effects, some additional observations were made during the experiments.
After 24 hours the wells containing the highest concentration of titanium dioxide the water appeared to have a white, milky colour. This was probably just due to the colour of the titanium itself. The wells containing the highest concentration of nano silver clearly showed sedimentation of particles on the bottom after 24 hours.
In the groups exposed to titanium dioxide (nano and bulk) after 24 hours the gut region of the artemia appeared as a white stripe, which was more clear in the higher concentrations. In the nano silver the gut region appeared as a black stripe, though this was only clearly observed in the highest concentration.

4. Discussion

4.1 Lethality of the nanoparticles

A slight drop in survival (mean survival after 24 hours 97.05%) could be observed in the BSA control group. This is probably due to the lack of aeration and feeding during the experiment, and it is therefore very unlikely that this can be prescribed to the BSA. This makes the BSA control group a good reference to compare the other exposure groups with.
When looked at the different concentrations, in the lower concentrations of the silver and titanium dioxide a significant greater drop in survival could be noted. For both silver and titanium the nano and bulk showed no difference in effect. In the titanium just the 0.01 mg/L concentration gave a significant drop in survival, but for the silver both the 0.01 and 0.1 mg/L

concentrations were significant.
The higher toxicity of the lower doses was quite unexpected. Even though there have not been any previous studies on the effects of these particles on artemia, one would expect that an increased concentration would give an increased effect. A previous study on daphnia, also a small crustacean, showed an increasing effect on mortality with increasing dosage (0.2 – 10 mg/L) of 30 nm titanium dioxide. (Lovern et al., 2006) In the same study they also found that titanium dioxide ranging from 100 – 500 nm (dosage 50 – 500 mg/L) had very little effect on survival and it clearly did not show any dose-response increase. Another study with titanium dioxide exposed daphnia (dosage 1 – 3 mg/L) did also not show any clear dose-response increase. (Hund-Rinke et al., 2006)
In other dose-response studies on fish, both nano silver and nano titanium caused increasing effects with higher concentrations.(Bar-Ilan et al. 2009, Federici et al. 2007, Laban et al. 2010, Wu et al. 2009).

Why exactly our results show a more toxic effect with lower concentrations remains unclear. For both silver and titanium no difference was observed between the nano and the bulk. Also, since the characterisation showed a wide spread in particle size and agglomeration in every substance, it is impossible to compare sizes and further studies on possible size dependent effects have to be performed. We can conclude however, that agglomerates do seem to have a toxic effect on artemia. In a previous study on daphnia no size dependent effect was found. (Wiench et al., 2009). In this same study did also found that agglomerates also caused a decrease in survival. Other studies however did find a size dependent effect, with smaller particles being more toxic. (Gaiser et al., 2009; Hund-Rinke et al., 2006; Lovern et al., 2006)

4.2 Lethality of silver nanoparticles compared to silver ions

With the silver nitrate exposure, the lethal effect is caused by silver ions. Silver ions clearly cause a drop in survival in a dose response way: the highest dose shows the largest effect ($p < 0.0001$). In the nano silver however, the highest drop in survival is shown in the lower concentrations, while the highest concentration shows a 100% survival. This proves that the lethal effect of nano silver is not caused by any silver ions which might have detached from the nanoparticles in the suspension, but by the particles itself.

4.3 Lethality assessment

With the assessment of survival of the artemia, not only alive and dead individuals were

recorded, but also the immobile ones. It showed however, that it sometimes was hard to assess these immobile individuals, since their immobility sometimes seemed to be temporarily, or it was shown in different degrees. The use of a control assessor made it clear this assessment was too subjective and not useful for the survival analysis.

4.3.1 Additional observations

The observations of white or black gut regions within the artemia has not been described in literature before. Since the colours are similar to the titanium (white) and silver (black) particles, it is highly possible that this effect is due to uptake of the particles. Because these observations were done with just a light microscope it cannot be said with certainty that these coloured regions were caused by the nanoparticles. The TEM however will give a qualitative assessment on particle uptake in the gut region.

4.4 Cox's proportional hazards model

When Cox's proportional hazards model was run to take both the experiment number and experiment group into account, the same groups were found significantly different from the controls as in the Kaplan Meier analysis. Because the Kaplan Meier analysis does not account for experiment variation, the proportional hazards analysis is more sensitive. This explains why in the proportional hazards analysis an extra experiment group was found to have a significant effect on survival; the bulk titanium 0.1 mg/L.

The experiment number seemed to have a significant effect on the results in the nano titanium 0.01 mg/L and bulk titanium 0.01 mg/L.

This might have been due to an error made in the first experiment. With the preparation of the stock solutions it was forgotten to add 100 µL system water to the stocks (150 µL to the AgNO3). Even though this is a slight dilution error this might have been the reason for a difference in the experiment numbers. On the other hand all the other groups did not show a significant difference with their experiment numbers.

In the bulk titanium 0.01 mg/L the experiment number and exposure group showed a significant interaction. This means that the response relationship between the survival, the dependent variable, and one of the independent variables, the exposure group or experiment number, is not the same at all levels of the other independent variable.

4.5 Characterisation of the nanoparticles

Characterisation of the nanoparticles showed some typical findings concerning the size. The

results showed that the higher the dilution, the more the particles were aggregated. The reason for this remains unknown, since it is hard to find a clear explanation for it.
A strong deviation of the true size from the advertised size and strong aggregation of nanoparticles has been reported before. (Adams et al., 2006; Wiench et al.,2009) However, the strong aggregation described by Wiench occurred only with uncoated particles, whereas coated particles remained visibly dispersed.
Another reason could be the high salt concentration of the water. However, the aggregation seemed to increase in the more diluted solutions, and it also appeared in the stock solutions, to which no extra salt was added. This makes it less likely that salt is the cause of the aggregation, though it could still be a contributing factor; maybe the salt interacted with the BSA coating.
A more simple explanation might be that the solutions were not sonicated long enough. Also the stock solutions were made more then 3 hours prior to the measuring. This might have given the particles the opportunity to sink to the bottom and aggregate.

4.6 Hatching conditions artemia

Since the use a 40 grams of salt per liter concentration for artemia hatching in the zebrafish laboratory always gives a good hatching rate, this was also the used concentration for the artemia survival experiment. After some other hatchings (after the exposure experiment) however, it seemed a lot of newly hatched artemia were less mobile than usual and there were a lot of unhatched eggs. Better observation of the artemia packages showed then that the ideal salt concentration for *nauplii* is 28 grams of salt per liter, and this obviously showed better results. Even though the higher salt concentration was used for hatching during the artemia exposure experiment, there might be good chance this has not interfered much with the results of the survival. The selected artemia to put in the wells were all highly mobile at that point, and the drop in survival in the control was low. The exposure groups were also compared to the control group, with a mean of 97.05% survival, in stead of to a 100% survival.

5. Conclusion

Even though the advertised size of our particles were 20 and 200 nm, we found a great variety in size with the characterisation. This was probably due to agglomeration of the particles. Typical was the increasing agglomeration with increasing dilutions.
We found that under our circumstances the lowest concentrations of the silver and titanium

dioxide showed the largest effect on survival of brine shrimp, with no difference between the nano and the bulk. Especially because there was such a large distribution in size, it is hard to say anything about a possible size dependent effect. It can be concluded however that agglomerates can cause a significant decrease in survival of brine shrimp.
It can also be concluded that titanium dioxide had a toxic effect in the same range as silver. Why exactly the lower concentrations turned out to be more toxic in our experiments remains a point of discussion. Also no other studies showing these same results can be found.
In the silver nitrate group the highest concentration showed the largest effect on survival, whereas this same concentration of nano and bulk silver showed no significant effect. This proves that the toxic effect of the silver particles was not due to ion release.
An interesting observation was the colour change in the gut region in the titanium and nano silver. There is a good chance these colours are actual ingested nanoparticles and hopefully TEM will prove this hypothesis.

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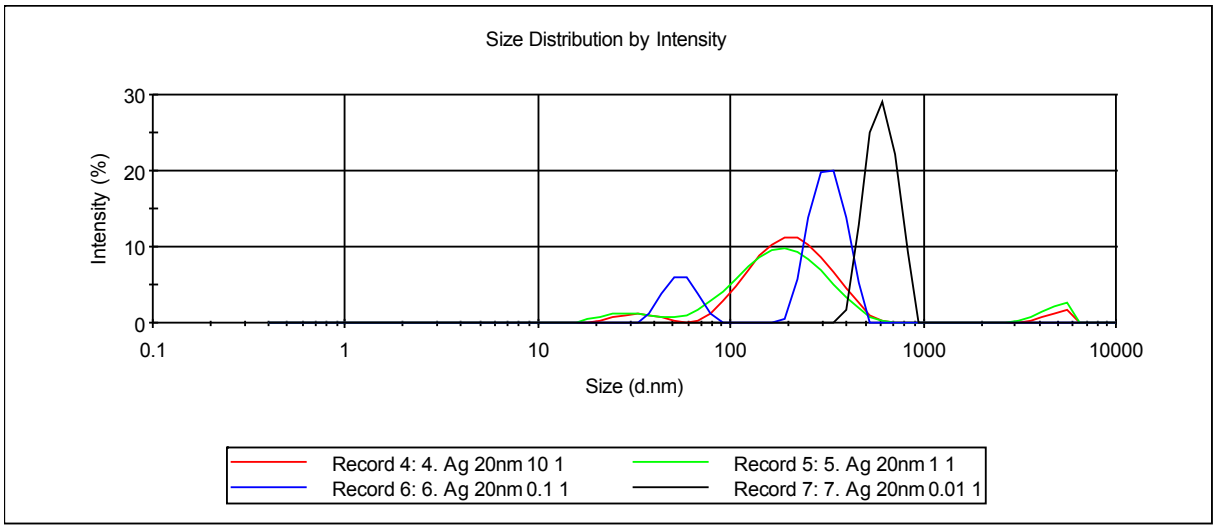
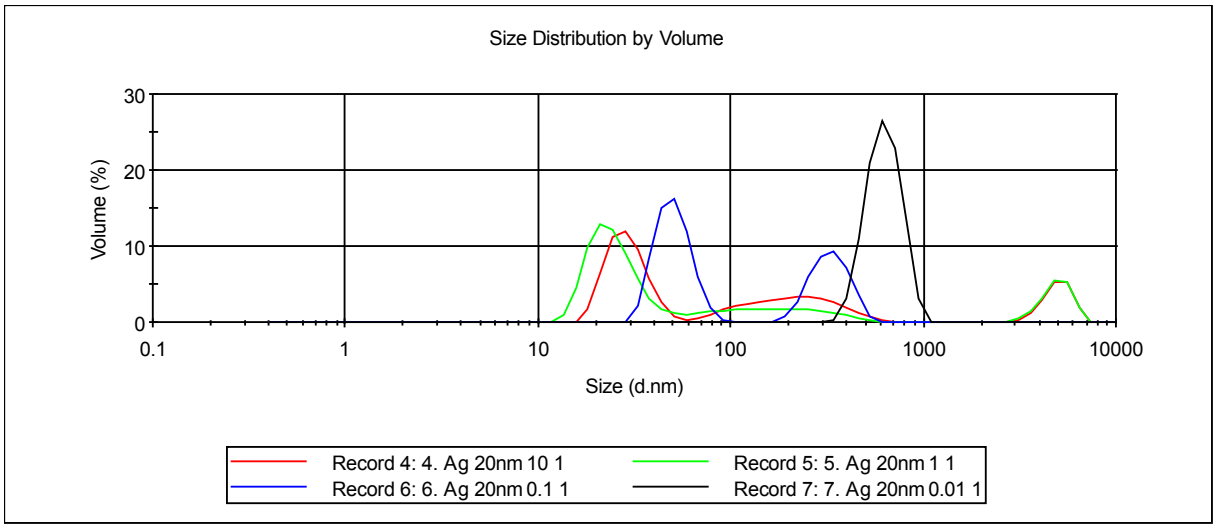
Appendix I: Size distributions by intensity and volume

Nano silver:





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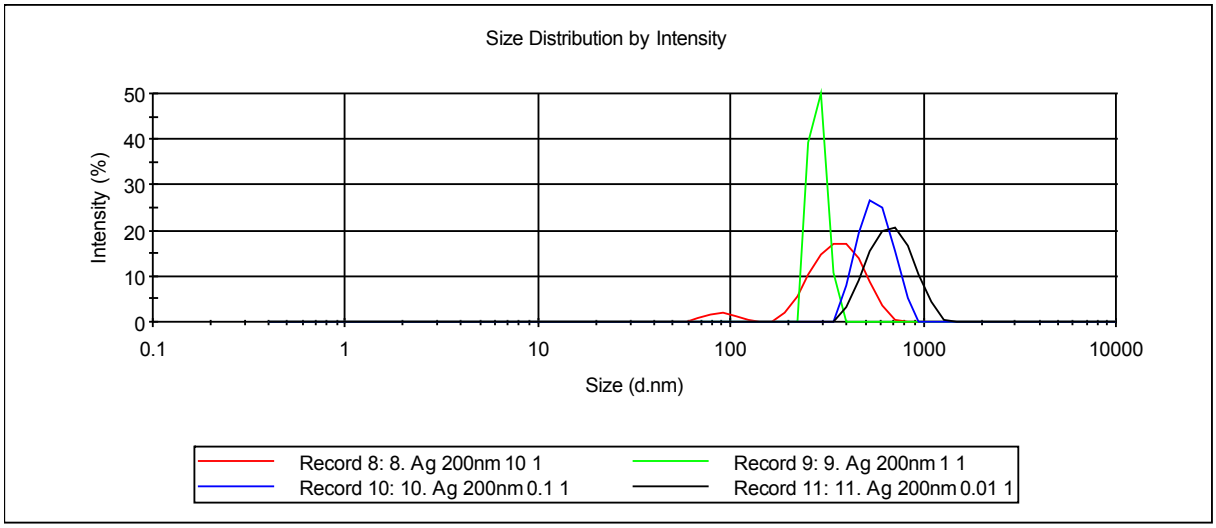
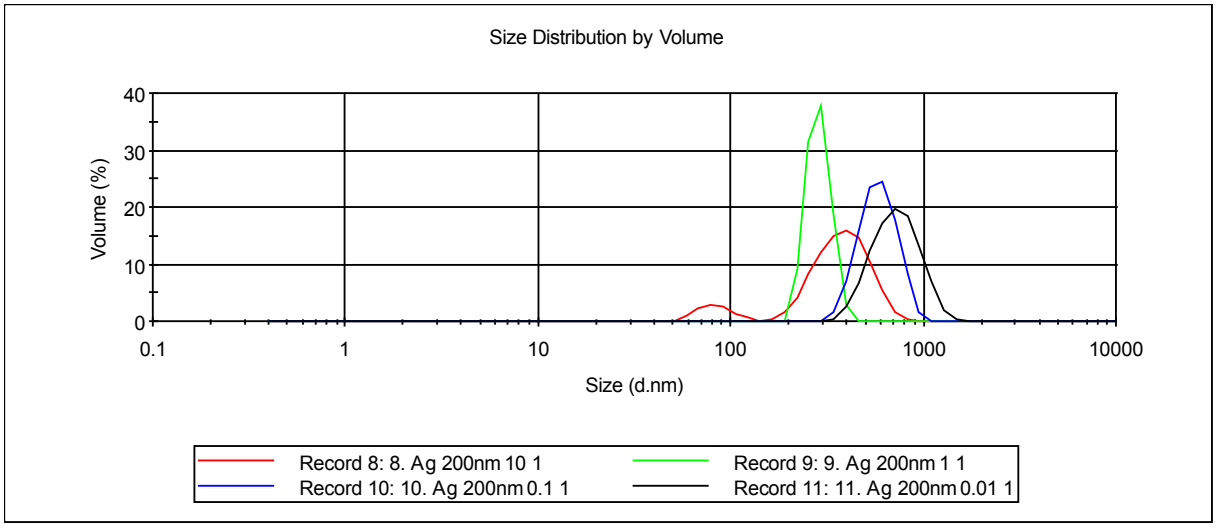
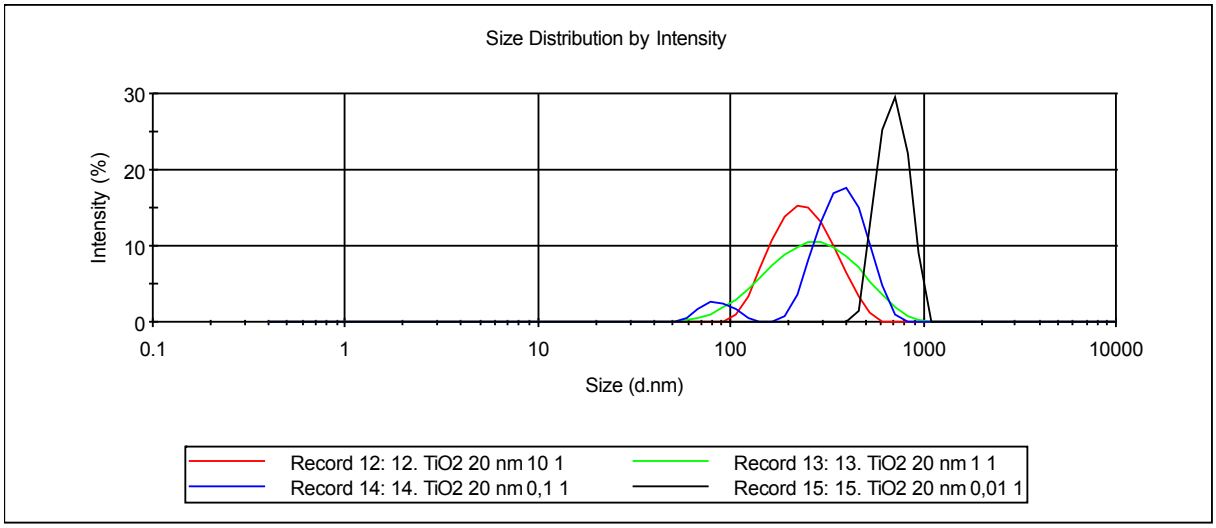
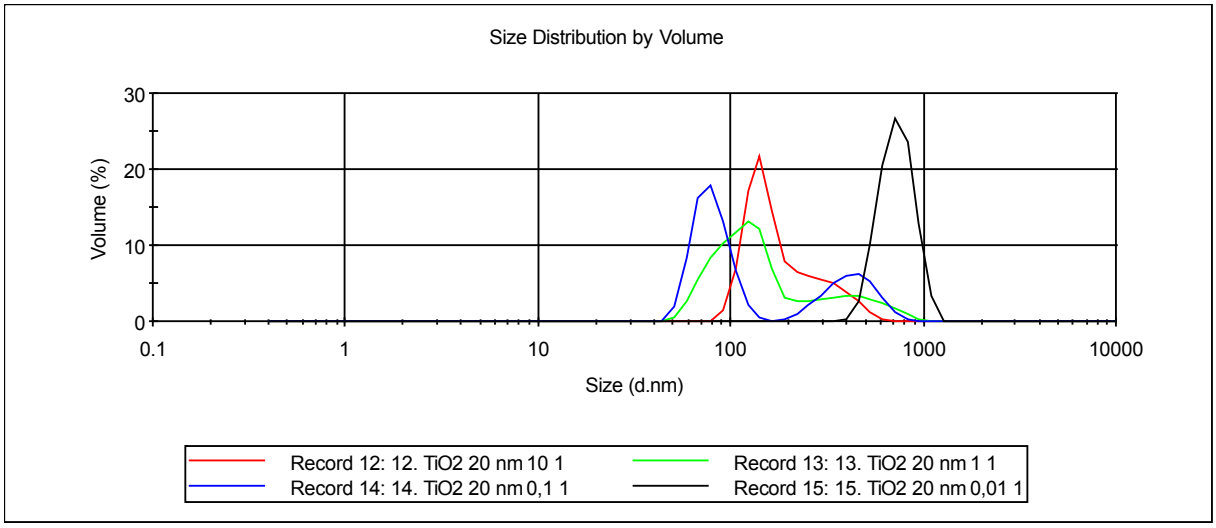

Size Distribution by Intensity
Intensity (%)
50
40
30
20
10
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 8: 8. Ag 200nm 10 1
Record 9: 9. Ag 200nm 1 1
Record 10: 10. Ag 200nm 0.1 1
Record 11: 11. Ag 200nm 0.01 1


Size Distribution by Volume
Volume (%)
40
30
20
10
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 8: 8. Ag 200nm 10 1
Record 9: 9. Ag 200nm 1 1
Record 10: 10. Ag 200nm 0.1 1
Record 11: 11. Ag 200nm 0.01 1

Nano titanium dioxide:





Bulk titanium dioxide: