# In vivo nanotoxicity assessment: the role of size, surface coating, nanostructuration, and dose-metrics

G. Vecchio, A. Galeone, V. Brunetti, G. Maiorano, M. A. Malvindi, S. Sabella, R. Cingolani, and P.P. Pompa

IIT - Italian Institute of Technology, Center for Bio-molecular Nanotechnologies (CBN), via Barsanti 73010, Arnesano (Lecce), Italy, giuseppe.vecchio@iit.it

## ABSTRACT

The growing use of nanomaterials in commercial goods and as novel carriers for drug delivery is generating increasing questions about possible risks for human health and environment, due to the lack of an in-depth assessment of their potential toxicity. In this work, we investigated the *in vivo* effects of AuNPs and QDs of different sizes, surface coatings, and nanostructuration, on the model system *Drosophila melanogaster* upon ingestion. We observed that the nanoparticles induce clear adverse effects in treated organisms, such as a strong reduction of their life span and fertility, presence of DNA fragmentation and apoptosis, as well as a significant overexpression of the stress proteins. Interestingly, the toxic effects were found to be dependent on NPs dosage, surface coatings and nanoscale surface features.

***Keywords***: gold nanoparticles, quantum dots, *Drosophila melanogaster*, dose-metrics

## 1 INTRODUCTION

Nanomaterials possess unique chemical, physical, and mechanical properties, so they are increasingly used for a wide variety of applications. A current estimation suggests that there are over 1300 commercial goods and novel technologies which contain nanomaterials [1]. In such a context, the potential exposure for humans and environment is growing, thus leading to a dramatic augment of possible risks for human health [2-6]. Despite such nanotoxicology issues have crucial implications for public health, to date, no systematic protocols are present to guarantee an effective risk assessment. Furthermore, the large number of studies recently reported in the literature, though clearly demonstrating a general toxicity of NPs in living systems, often lead to controversial conclusions because of the different choices regarding the parameters that characterize the toxicology studies (cell lines, dose metrics, different source of NPs, NPs characterization, surface modification, etc.)[7-9].

In this context, it is imperative to define a rigorous strategy to study the complex interactions occurring between nanostructured materials and living systems, by means of an in-depth physical/chemical characterizzation of nanomaterials along with the use of reliable and well established *in vivo* experimental procedures. Such an approach may be useful to outline the correct experimental routes [8], providing important information for dosage, dose-metrics, and bio-kinetics of nanoparticles. In this regard, it is important to use nanomaterials that are highly characterized in terms of size, charge and shape, and that show peculiar features of monodispersion and purity. Moreover, to understand which are the main parameters responsible for the toxicity of some nanomaterials, the design and testing of nanoparticles presenting tunable coating, ligand organization at nanoscale, and/or nanoscale surface features may allow the establishment of effective relationships between the nanomaterial properties and the exerted biological outcomes. For instance, it has been recentely demonstrated that nanoparticles functionalized with the same ligands but exposing opposite charges elicited different effects on cells [10, 11]. On the other hand, a different nanoscale structuration of surface ligands was shown to strongly affect the internalizzation mechanisms, of NPs in the cells [12].

In this scenario, our aim was to assess the *in vivo* effects of nanoparticles with a well-esthablished metrology, namely differently sized gold nanoparticles presenting a size dispersion better than 6%, differently coated CdSe/ZnS quantum dots (QDs), and metal nanoparticles with different surface nanostructuration [12]. To evaluate the biological outcomes, we selected, as a model organism, the fruit fly *Drosophila melanogaster*. This organism is well suited for toxicology studies because of a wealth of genetic and biochemical similarities to mammalians [13, 14]. Importantly, *Drosophila* was also exploited to investigate the molecular biology of several human diseases [15-19].

## 2 RESULTS AND DISCUSSION

As first step, we studied the effects of 15 nm citrate-capped gold nanoparticles (AuNPs) on *Drosophila*. The fruit flies were nurtured with increasing concentration of AuNPs, and the organism lifespan and fertility were analyzed, finding dramatic consequences in both cases. Figure 1A reports the survival curves relative to AuNPs treated population compared to control experiments (Control and supernatant, SN). The evaluation of the effect of the SN is important to

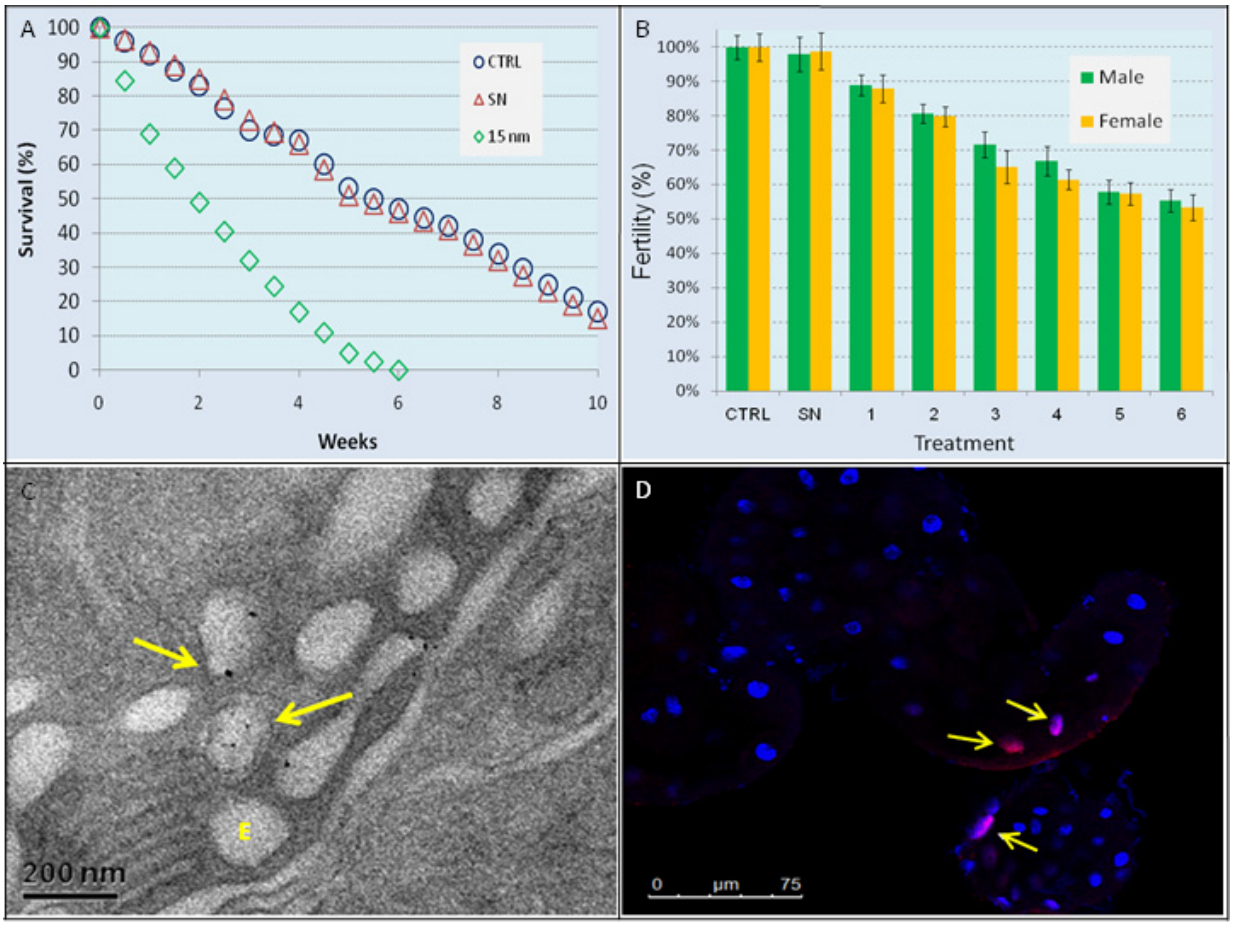


Figure 1: A) Lifespan curves of fly populations nurtured with 15 nm AuNPs treated food (7.8 µg/g dose) (green square) as compared to a control population bred with normal food (blue circle) and supernatant (red triangle) (results from 5 independent experiments); B) Male (green) and female (yellow) fertility tests of *Drosophila melanogaster* treated with increasing concentrations of AuNPs (1-6 correspond to $3.9x10^{-2}$, $7.8x10^{-2}$, 0.39, 0.78, 3.9 and 7.8 µg/g, respectively) as compared to control samples (CTRL) and supernatant treated samples (SN), (10 independent experiments, the error bars in the graphs indicate the standard deviation). C) Representative TEM image reporting enterocyte section of *Drosophila* gastrointestinal tissue treated with AuNPs. AuNPs are present both as aggregates and monodispersed particles (yellow arrow). D) Representative confocal microscopy image of *Drosophila* midgut in flies treated with 15 nm AuNPs. Nuclei are stained with Hoechst 33342 (blue) while cells containing DNA strand nicks are detected by TUNEL assay and fluorescence red (highlighted by the yellow arrows).

exclude any possible toxic contribution due to other components in the solution, such as reaction precursors, surfactants and/or possible contaminants. Experimental data indicate a strong reduction of animal survival (the half-life of the treated population was c.a. 50% as compared to the control and surnatant), demonstrating that the treatment with NPs caused a significant toxicity with a strong perturbation of the entire life span of the organisms. The reproductive performance of *Drosophila* was also dramatically affected by treatments with 15 nm AuNPs (Figure 2B). The toxic effect appeared to be dose-dependent and not sex-linked, suggesting a generalized mechanism of toxicity. AuNPs biodistribution in the *Drosophila* tissues was monitored by TEM (Figure 1C). Overall, we observed a quite homogenous distribution of the NPs into the deep layers of the enteric tissue. In particular, Figure 1C reports a representative enterocyte section in which several AuNPs can be seen, both as aggregates and monodispersed particles. Moreover, by performing TUNEL assay, we observed that AuNPs elicited DNA damage (Figure 1D). Such results further support the findings of significant toxicity of AuNPs in *Drosophila*.

To study the toxicity of different sizes of AuNPs (5, 15, 40, 80 nm), we used two parallel approaches. We analyzed the lifespan and the Reactive Oxygen Species (ROS) levels in *Drosophila melanogaster* upon NPs treatment, applying two different dose-metrics. In particular, we used (i) the same molar concentration of NPs for the different sizes, or (ii) a different concentration but the same surface area. To exclude possible interferences in the toxicity evaluation (for

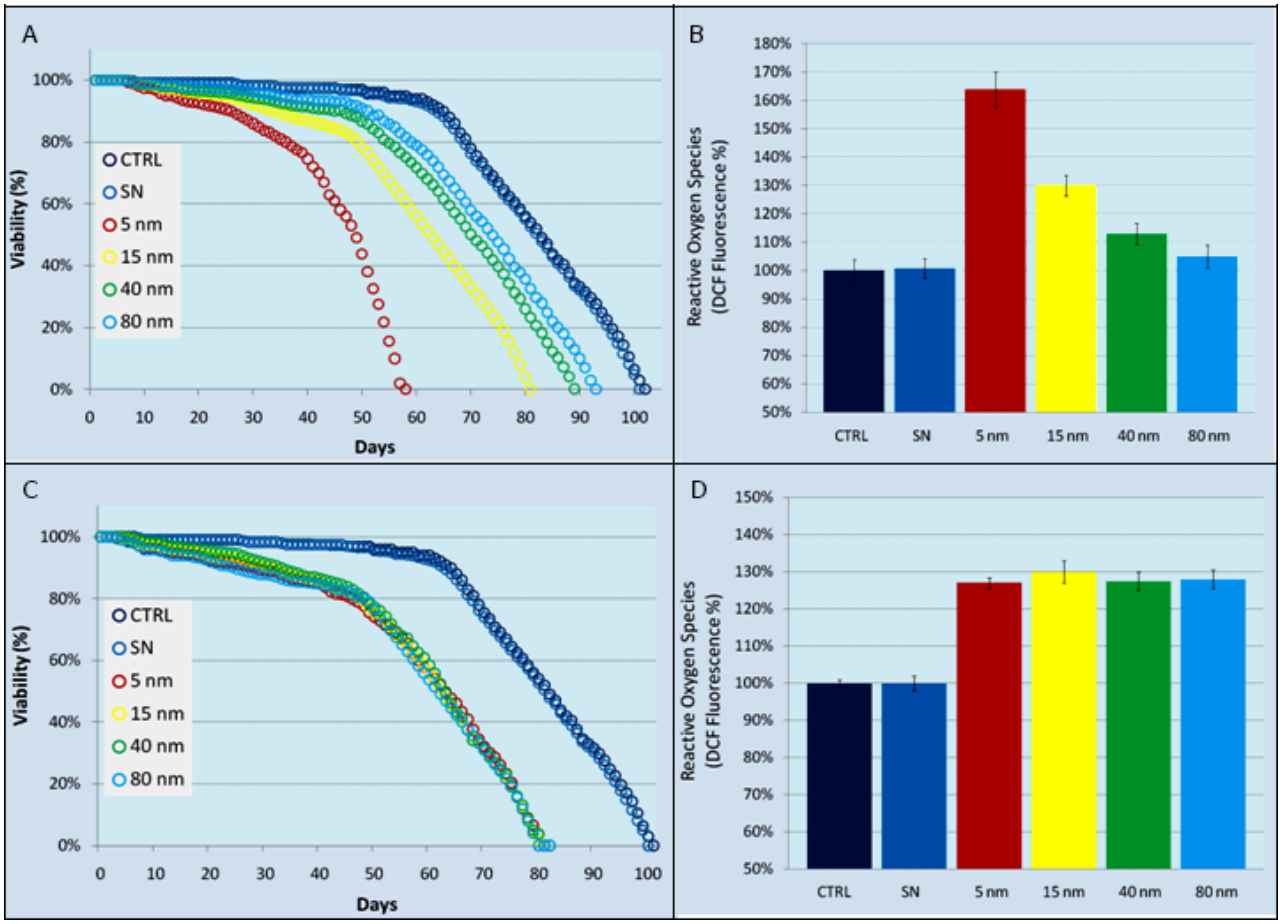


Figure 2: Lifespan curves and ROS quantification of *Drosophila* flies nurtured with differently sized AuNPs treated food. (A, B) Esperimental data relative to flies treated with different concentration, but same exposed surface area of AuNPs. (C, D) Data realtive to flies nurtured with same concentration of AuNPs but different exposed surface area.

Instance, presence of aggregates, or impurities), we used metrologically controlled and stable AuNPs of 5, 15, 40 and 80 nm, having a size dispersion lower than 6% [20, 21]. Figure 2 reports the lifespan curves and ROS determination in the two modalities, namely constant surface area of NPs (A and B) or constant molar concentration (C and D). In both cases, although with some differences, we observed a strong reduction of *Drosophila* lifespan, as well as a consistent increase in the production of ROS upon NPs ingestion. In particular, when using surface area based dose-metrics (Figure 2A and 2B), we can observe that the toxicity is not directly related to the surface area of the NPs. The smallest NPs showed the highest reduction of viability ($\tau_{50}$ = 48 days), followed by 15, 40 and 80 nm AuNPs ($\tau_{50}$ = 62, 70, and 74 days, respectively). Such effect was also confirmed by ROS data (Figure 2B) that showed a similar trend. However, it is important to note that, by using surface area based dose-metric, the concentration of 5 nm AuNPs is much higher than that of 80 nm particles (900 vs 3.5 pM). In fact, when treating *Drosophila* with the same number of NPs, but different sizes, the survival curves are basically overlapped in an unique curve (Figure 2C), indicating that the toxicity induced by AuNPs is mostly related to the total number of NPs ingested by the animal. As shown in Figure 2D, also the ROS generation confirms such finding.

Another parameter that we are currently investigating is the surface nanostructuration of NPs. We are, in fact, testing the toxicity effect on *Drosophila* of peculiar metal nanoparticles that show a different nanoscale ligand arrangement [12]. Interestingly, we observed that, whereas the disordered ligand assembled NPs negatively impacted the fly vitality and fertility, the ordered ones did not, behaving similar to the control (data not shown). Another important nanomaterial that we tested in vivo is CdSe/ZnS core/shell QDs. We evaluated whether the surface coating may play a role in determining the toxicity. To perform such study, we selected 3 differently coated QDs, exposing MUA, PC and PEG coating (Figure 3 top). Fruit flies were fed with 85 pM of the 3 different types of QDs and their entire lifespan was evaluated. We found out that PEG protected NPs are not toxic, producing a lifespan curve very similar to the control, whereas the MUA coated QDs resulted very toxic.

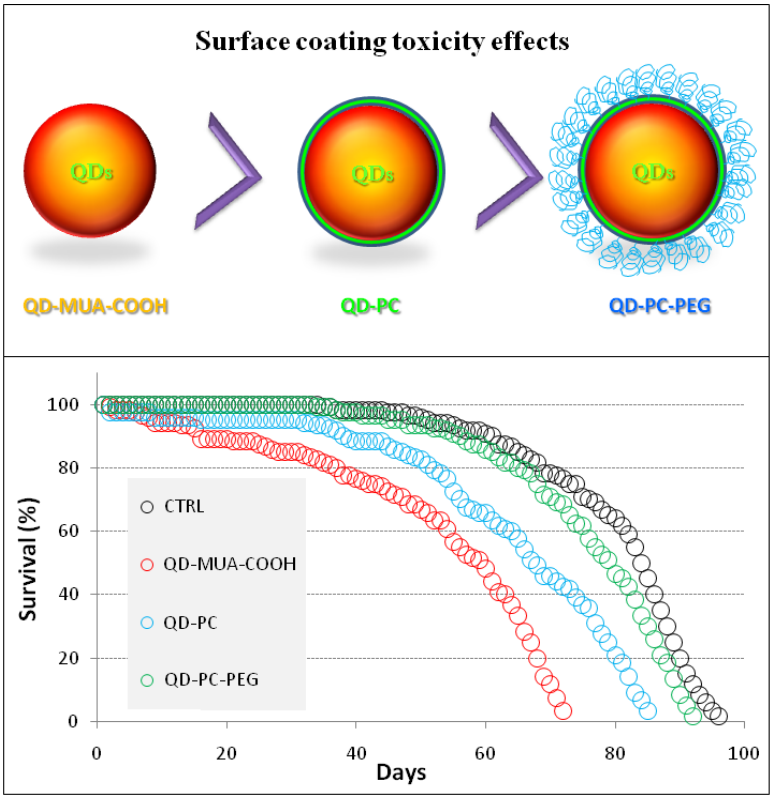


Figure 3: top) Cartoon of different QDs coated with MUA (Mercapto-Undecanoic Acid), PC (Poly(maleic anhydride-*alt*-1-octadecene), and PEG (di-amino- Polyethylene-Glycol); bottom) Lifespan of *Drosophila* nurtured with the same concentration of the differently coated QDs.

Such differences among the diverse coatings can be likely ascribed to the different chemical reactivity of the exposed functional groups, which in turn may influence the NPs uptake in the animal tissues, thus causing a different toxicity. Moreover, the different coating of QDs modify the progressive $Cd^{2+}$ ions release *in vivo,* that further contributes to exert other toxic effects.

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