In Vivo Nanotoxicology: Toxicoproteomics

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ABSTRACT

Proteomics applied to nanotechnology is now emerging as an attractive tool to address the still unanswered questions dealing with nano-biointeractions and NPs-induced toxicity in living systems [1-3]. In this work, we aim at developing a proteomics-based strategy to provide insights on the impact of NPs *in vivo* by using doses and administration routes relevant for risk assessment. We set up the analytical methodology to obtain whole protein mapping of blood and liver of rats treated with different classes of NPs (SiO_2 and QDs NPs) using 2D-DIGE and MALDI/TOF-TOF. Analysis of the protein expression profiles of the treated rats with respect to controls were found to be altered by NP treatments in blood and liver, activating specific biochemical pathways of cellular toxicity. Such variations were strongest after 24 hr of exposure, suggesting an early acute response. Nonetheless, no general physiological damage was observed in the rats during NP exposure (in agreement with recent reports).

Keywords: silica nanoparticles, quantum dots, proteomics, nanotoxicity, 2D-DIGE

1 INTRODUCTION

Nanotechnology, nanoparticles (NPs) and their applications in medicine have raised great enthusiasms in the last years, because of the more effective, sensitive and highly specific properties of NPs as compared to conventional drugs, organic dyes, and *in vitro* and *in vivo* diagnostic tools. Quantum Dots nanoparticles (QDs) show a number of novel physico-chemical characteristics that have allowed them to be considered highly innovative and effective diagnostic agents in nanomedicine. Many examples about their stronger efficacy are reported. In particular, especially PEGylated QDs were successfully used as optical imaging nanoprobes for the early detection of pancreatic cancer. Despite the clear advantages provided by the optical properties of QDs at nanoscale and their potential huge applications in diagnostics, concerns about their toxicity have been raised by toxicologists. *In vitro* and *in vivo* toxicity information on QDs are not currently enough to gain a clear understanding on how QDs interact with living systems. Moreover present results appear contradictory [5]. Recently, toxicity of QDs has been reported using *Drosophila melanoganster* as animal models. Authors demonstrated that chemical composition as well as the cellular release of toxic ions are the main parameters responsible of the induced detrimental effects [6,7]. *In vivo* toxicity of QDs has been also showed by Liu and co-workers [8] suggesting the occurrence of morphological and functional impairments to liver in mice. Nonetheless, no general physiological damage was observed in the monkeys during chronic NP exposure [4]. Moreover, although bioaccumulation of NPs strongly depend of the specific administration route used for the experiment, bio-distribution studies revealed a major accumulation of the QDs in liver, spleen, and kidney [9]. Importantly, the above mentioned applications of QDs in nanomedicine suggest that the only exposure route that would result as a potential risk to humans and their health is the intentional exposure *via* injection [10].

Amorphous and nanoporous silica nanoparticles (SiO_2 NPs) are another important NP class used in nanomedicine. Because of their proved biocompatibility [11], SiO_2 NPs are widely applied as novel drug delivery nanocarrier *in vivo*, presenting several advantageous properties as passive and active selective tissue targeting, payload capability and theranostic activity [12]. However, SiO_2 NPs are produced at industrial level so that unintentional (together to intentional) exposure should also be considered for risk toxicity assessment.

Ideally, proteomics allow to the identification of the entire set of proteins expressed by genes at a precise temporal cell state, revealing precious information on specific biological processes (e.g., disease processes or drug induced effects), molecular mechanisms for genes regulation, proteins activities, protein-protein interactions, and large scale mapping of subcellular and tissue protein distributions [2]. As such, proteomics has been demonstrated to be an effective analytical tool for toxicology, providing insights in the toxic molecular mechanisms induced by drugs or toxicants in cells or specific tissues. Toxicoproteomics applied to nanotechnology is now emerging as an attractive tool to address the still unanswered questions dealing with bio-nanointeractions [1-3] and NP-induced toxicity in living systems. Within this framework, we describe the development of a proteomics-based strategy to provide insights on the impact of QDs and SiO_2 NPs *in vivo* by using doses and administration routes relevant for their risk assessment (especially in view of their medical applications). In particular, we applied 2D-DIGE and bioinformatics to obtain whole protein mapping of blood and liver of rats treated with NPs at two different time of exposure (24 hour and 2 weeks). Statistical analyses show that the protein expression profiles of the treated rats with respect to controls were altered by NP treatment in blood and liver. Such variations were found strongest after

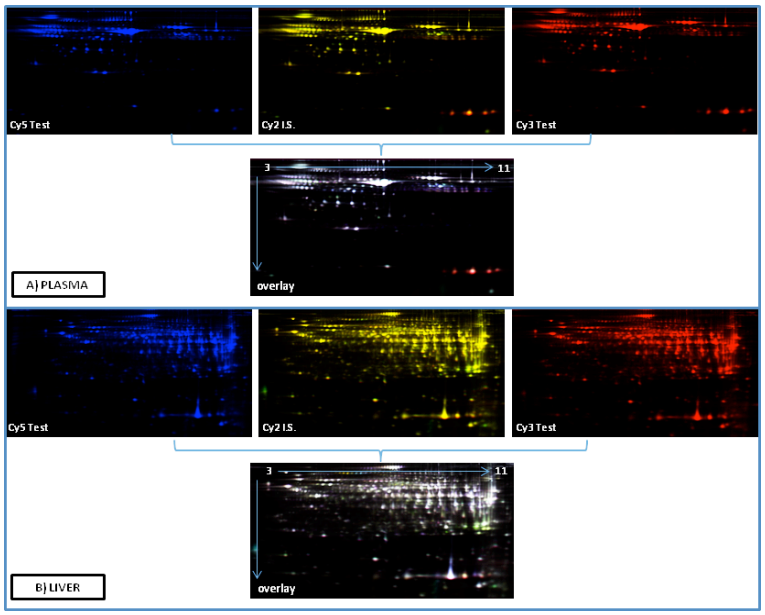

Figure 1: Representative 2D-DIGE gel images of whole blood (A) and liver (B) proteome. Merged images show overlay of blood (A) and liver (B) protein spots of treated samples (Cy3 and Cy5) and pooled internal standard (Cy2). The three samples show overlap based on distinct color of protein spots. Sample proteins were separated using a 24 cm, non-linear, pH 3-11 strips and 12.5 % polyacrylamide gel.

24 hr of exposure, suggesting an early acute response and a recovery of the induced effect after longer time of exposure.

2 RESULT AND DISCUSSIONS

We investigated protein expression profiles of whole blood and liver proteome of rats treated with different NPs (SiO_2 NPs and QDs) by intravenous injection. We used 6 nm CdSe/ZnS quantum dots synthetized according to Galeone et al.[6] and positively and negatively charged SiO_2 NPs (posSiO_2 NPs and negSiO_2NPs) of 25 nm in size [11]. NPs were extensively characterized by TEM, DLS and Z-pot before their use (not shown).

We used Sprague Dawley (SD) rats weighing 250 g that were treated and cared for the proteomics experiments in accordance with the principles guideline of animal handling. We selected single intravenous injection (IV) into caudal vein of rats at a dose of 1mg/kg (nanoparticles/rats); for the control groups physiological saline solution was administered. More in details, for both types of SiO_2 NPs, 24 female rats were divided randomly into 6 different groups (n = 4), according to the charge of the administered SiO_2 NPs, the 2 scheduled necropsy dates (24 hr and 2 weeks), and the respective control groups (24 hr and 2 weeks). Similarly for QDs, 16 female rats were randomly split into 4 groups (n = 4) according to the same temporal intervals, and the respective control groups (24 hr and 2 weeks). Rats were then sacrificed, and the whole blood and liver samples were collected for further processing by 2D-DIGE analysis. 2D-DIGE (two-dimensional fluorescence difference gel electrophoresis) enables the focalization of several thousand proteins based on their charge in the first dimension, and size in the second allowing for an accurate and reproducible quantification of proteins expression for control and treated sample. Moreover enabling the co-detection and quantification of differences between two samples in the same gel, significantly reduce the pitfalls of inter-gel variability associated with conventional 2D-PAGE. Representative multiplexed 2D spot maps of whole proteome of plasma (Figure 1A) and liver (Figure 1B) exposed to NP treatment are reported in Figure 1. Protein patterns are reported as distinctive color images or as a multiplexed image of NP treated (Cy3 and Cy5) and the pooled internal standard (Cy2). Images revealed a good separation and resolution of proteins in both liver and blood samples. The changes in protein pattern among our samples (Ctrl, posSiO_2NPs and negSiO_2NPs, QDs) were quantitatively analyzed by using the Decyder software and by applying Principal Component Analysis (PCA) and Extended Data Analysis (EDA) (Figures 2 and 3). Figure 2 reports on SiO_2 NP treatment. In the case of blood sample, we initially found about 2000 protein spots. This first set of data was further filtered finding that 61 plasma proteins were differently expressed in the different groups ($p < 0.001$ by Two-way ANOVA). For liver, the total number of proteins was about 3300. By further filtering the protein set (including proteins present in at least 75% of the spot maps) we obtained a subset containing 1787 proteins of which 141 were found to be up- or down-regulated with statistical significance ($p < 0.001$ by two-way ANOVA). We found that the first primary parameter impacting on proteomics expression profile of rat blood was the time, as it is possible to observe from PCA analysis. This trend can be explained

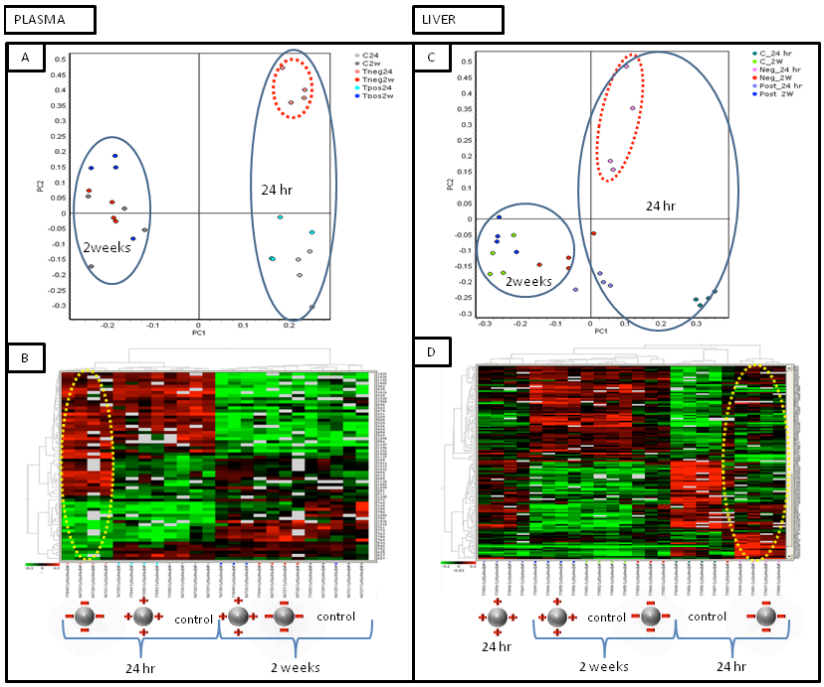

Figure 2: Protein clustering analysis by PCA (A, C) and EDA (B, D) of blood (A, B) and liver (C, D) proteome of rats treated with differently charged (positive or negative) SiO_2NPs.

as an early biological response of the animal to the NP treatment followed by a completely recovery of the animal functions (all experimental groups present protein spots whose variance is not statistically relevant after 2 weeks). Particularly, it is also possible observing that negSiO_2NPs are the type of NPs that mostly produce such effect (red and yellow circles in Figure 2). We found that posSiO_2NPs did not affect the blood proteome being very similar to the control. A similar protein clusterization is reported by EDA module (Figure 2B, D) as well. After applying statistics ($p < 0.001$ by Two-way ANOVA), protein spots were grouped into families by hierarchical clustering analysis. Note that this analysis properly discriminates the experimental groups (Ctrl, posSiO_2NPs and negSiO_2NPs at the two different time of exposure). The three like structures dendograms show the similarities and distances among the proteins in the experimental groups confirming that, unlikely posSiO_2NPs and Ctrl (belonging to the same family), treatment by negSiO_2NPs was the solely to induce an early acute response in rats as reflected by the statistically significant change of the protein expression profile in the blood. Interestingly, the same specific time- and charge-dependent effect is observed in liver proteome (Figure 2C, D), suggesting a correlation about these two important biological samples. The same experimental approach was used for plasma proteome analysis in the case of QDs. In particular, a comparison between the two types of NPs has been performed by spots map normalization procedure carried out by Decyder software in the EDA module. PCA and EDA analyses (Figure 3A, B) showed a number of protein clusters related to the different NP treatments. Importantly, these clusters discriminate all the experimental groups. In particular, it is evident that, also in the case of QDs, plasma proteome profile is altered at 24 hours. Similarly to SiO_2 NPs induced effect, a total recovery of this alteration after 2 weeks (yellow and red dots in the blue circle) was noticed. Moreover, within the first 24 hours, proteins, which are differentiated by silica and QDs nanoparticles are different, suggesting that the two types of nanoparticles trigger a different biological animal response by activating and/or inhibiting diverse biochemical pathways.

Identification of the differently expressed protein in both samples by MALDI-TOF/TOF is currently ongoing in our laboratory. Knowledge of biological functionalities related to the identified proteins (which are up- or down-regulated in both liver and blood upon NP treatment) could allow us to understand the molecular mechanisms – and the specific biochemical pathways possibly related to toxicity effects – activated in animals by the negatively charged NPs and QDs. Moreover, it may allow understanding whether the differently-expressed proteins found in the blood, maybe related to liver molecular damage. This latter point is quite intriguing, since it may opens up the possibility to consider the blood a peripheral treasure source of unknown biomarkers that, in principle, could reflect the ongoing physiologic state of other central tissues (especially liver).

Furthermore, by this approach the structure-activity relationship (effect of surface charge or chemical composition and relative animal response) may be highlighted, providing novel opportunities for the production of safer nanoparticles by design.

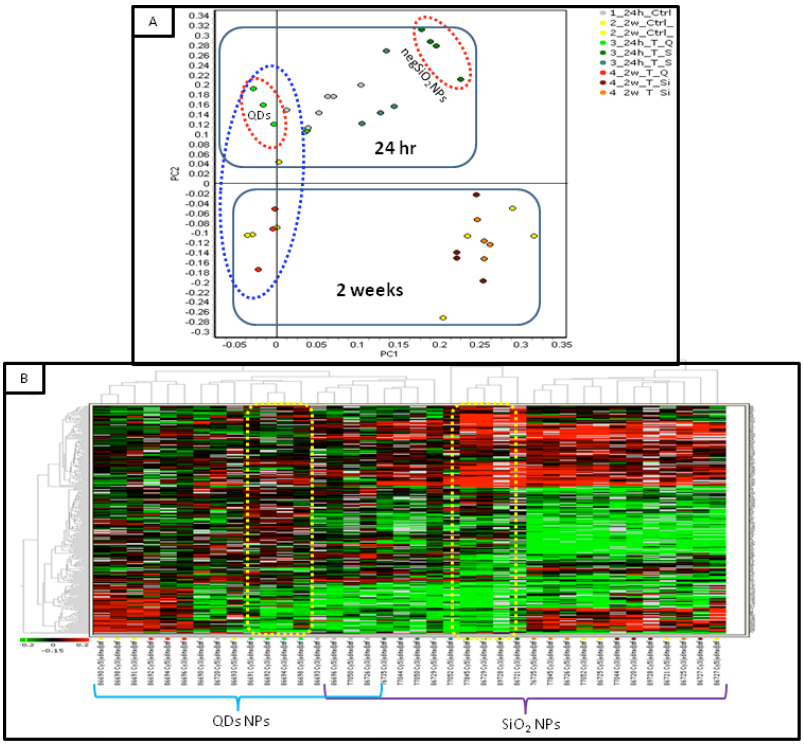

Figure 3: Protein clustering analysis by PCA (A) and EDA (B) of blood proteome of rats treated with differently charged (positive or negative) SiO_2 NPs and QDs after protein spot maps normalization against controls at 24 hours. Normalization was performed by EDA module of Decyder software.

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