Cellular interaction with Si- and Iron-based nanoparticles for bio-imaging.

A study of biocompatibility.

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Abstract **The overall objective of BONSAI project (FP6, EC) is the development of ultrasensitive bio-imaging techniques based on novel multifunctional nanoparticles (NPs) with tailored optical and magnetic properties for visualizing complex cellular structures (in tissues and organs), receptors, tumour cells and masses. An important aspect to take into consideration involves the cellular responses to the impact of exogenous structures represented by NPs themselves. As a preliminary sign of reaction, we start to investigate the basic cytotoxicity.**

1. INTRODUCTION

The field of bio-medical applications of NPs is still in its infancy as a consequence of the difficulties encountered in getting stable particles, easy dispersible in aqueous biological media and bio-compatible. True innovation in term of nanoparticles production rests on the capability to combine the preparation of "ad-hoc" NPs, having different properties and functions, with the development of advanced bio-imaging techniques.

The expected improvements of labelling cells and cellular structures with tailored NPs are sensitivity, speed and specificity in the visualization of biological systems. The goal of the BONSAI project is the development of stable solutions containing non-cytotoxic, Si- and iron-based NPs surface functionalized to improve and tune their optical properties and to increase their selectivity for bio-imaging of specific biological targets. These NPs will be most useful for the development of optical bio-imaging techniques aiming at: cell labelling for detection and tracking for lineage studies; optical imaging of in-vitro and in-vivo tumour cells for early cancer diagnostics.

Silicon is not a good light emitter, while nanostructured Si can emit photons in the visible- near IR range with a reasonable efficiency (1). It follows that silicon nanoparticles (Si-NPs) have the potential to overcome the inherent limitations in the biomedical use of Quantum Dots (QDs) because silicon is inert, non-toxic, abundant, economical and can be surface functionalized to allow stabilization and bioconjugation steps (2,3).

In this project we investigate weather these NPs will be biocompatible as compared to largely investigated, toxic CdSe QDs that require permanent biocompatible coatings.

Imaging can also be generated by exposure of cells or tissues to magnetic fields and by measuring dephasing times of water protons as in MRI (Magnetic Resonance Imaging). Actually, magnetic NPs are produced by complex multistep chemical procedures that unavoidably give rise to final particle sizes greater than 50 nm. The NP synthesis procedure proposed in this Project leads to the production of Fe-based magnetic NPs with size lower than 20 nm. The success in reaching this objective will lead to the production of new contrast agents for MRI with superior or compatible performances.

In this project we will also propose to use Solid Lipid Nanoparticles (SLNs) as a carrier of either Si- or iron-based NPs. SLNs offer the advantage of being biocompatibile and stable.

2. MATERIAL AND METHODS

NPs preparation: The NPs synthesys procedures employed in the BONSAI project is a one step process based on CO_2 laser induced pyrolysis of suitable gas-phase precursor.

Silicon nanopowders were produced by laser pyrolysis of silane. The as-prepared particles (diameter averaged 4 nm) were dispersed in ethanol at a typical concentration in the range of 8 g/l and were diluted in cell medium (1% FBS) before test.

Superparamagnetic iron oxide nanoparticles were produced by the fast laser heating of iron pentacarbonyl in an oxidant environment. Gases of high thermal conductivity (i.e. He, N_2) were used in order to favor the cooling of the nanoparticles as well as the decrease of the iron pentacarbonyl temperature during the synthesis. The result of this combined approach was the production of iron oxide nanoparticles with an almost spherical shape, TEM mean diameter of 3.0±1.2 nm and cell

parameters close to the magnetite (BONFEX4 sample). Complementary synthesis experiments were performed by use of SF_6 as reaction sensitizer and by increasing the laser power to obtain more crystalline nanoparticles, still keeping the average particle size below the superparamagnetic limit (SF39 samples, TEM mean diameter of 5.5 ±1.5 nm).

Technical platform is also developed to study the potential to encapsulate Fe-based NPs in Solid Lipid Nanoparticles (SLN) in order to obtain a stable dispersion suitable for administration in in-vitro studies. SLN are obtained by microemulsion at warm temperature of water, oil, surfactant and co-surfactant. After dispersion in cold water they spontaneously form droplets of the average diameter of 150 nm.

Cell culture: The cell lines used in this study are: A30 cells human lung epithelial cells (kindly provided by Prof P. Palestini, Università di Milano Bicocca), and L929 mouse fibroblast. All cells are maintained in their own complete medium supplemented with 10% FBS and grown in Petri dishes in an incubator at 37°C and 5% CO_2.

Trypan Blue exclusion Dye: The day before the incubation cells are plated on 35mm Petri dishes. For the incubation (6 or 24 hrs) with NPs at different concentration, we decreased the FBS in the medium to 1%. At the end of the exposure time cells are collected and resuspended in the medium. An aliquot is added to a mixture of Trypan Blue and is allowed to set for 5 minutes. Cells are counted on a hemocytometer; data are presented as live cells normalized to the total cells.

LDH assay: The day before the incubation cells are plated in a 96 wells plate at a density of 20000cells/well. On the day of the experiment the regular medium is substituted with complete medium with 1%FBS and selected concentration of NPs for 6 and 24 hours. At the end of the incubation the cells are treated following the protocol suggested by the kit producer (Clontech). In a separate set of experiments, NPs incubated at the maximal concentration tested were removed after 24 hours of exposure and after PBS washing, the complete culture medium is added to the cell monolayer. LDH assay is performed after 24 hours of recovery in regular medium.

3. Results

The aim of the BONSAI project is to develop nanoparticles (NPs) for bio-imaging. In these terms, a very important and preliminary task is to assay the biocompatibility of the newly synthesized NPs at the cellular level. The literature in this field reports controversial results. A major reason for such differences could in fact reside in the different methodologies used for the investigations. As an example, we compared two typical methodologies usually applied to discriminate whether a cell population is viable or not after exposure to a potentially toxic agent. Trypan blue exclusion test is a standard and well recognized procedure based on the uptake of the vital dye, while the LDH release assay is an enzymatic colorimetric method. Both of them depict a damage in the plasma-membrane: the former allowing the entrance of the blue dye (hence, the blue cells are damaged), the latter assaying the amount of a cytoplasmic enzyme (LDH, Lactate Dehydrogenase) released in the growing medium. Fig 1 shows the relationship between the two tests for Solid Lipid Nanoparticles (SLN) concentration progressively increased from 0.1 mg/ml up to 1mg/ml and for exposure time of 24 hours. One can note that an almost linear relationship holds between the two vitality assays although the toxicity index from LDH determination is four fold higher compared to Trypan blue exclusion test. One should also note that at a concentration of 0.4mg/ml, a 40% of dead cells detected by the Trypan blue test corresponds to about 90% of total LDH release, indicating that the conventional Trypan blue test highly underestimates the toxicity level of cells exposure to a selected substance.

Given the more objective and sensitive results, we decided to apply the LDH assay in order to investigate the biocompatibility of selected NPs.

A main problem encountered working with NPs in physiological medium is the tendency to the aggregation of the particles due to the interaction with proteins and/or salts present in the medium. For this reason we always measure the main particles diameter with the Dynamic Light Scattering (LDS) just before starting the incubation and at the concentration use for the experiment.

Si-based NPs were tested on human lung epithelial cells (A30) and on mouse fibroblasts (L929). The differences among the batches were mainly related to the NPs dimensions. As an example, fig 2 shows the level of toxicity in A30 cell, for 6 and 24hours exposure. Panel A refers to NPs with and average diameter of 200nm. Panel B shows higher toxicity level for the same NPs batch four month aged with a diameter of 367nm. Therefore, the increase in exposure time and in calibre affect the toxicity level.

Concerning the magnetic iron-based NPs, two products were studied: RSF39 and BONFEX4. To improve their stability in the cell medium the NPs were coated with biocompatible molecules such as citrate As shown in fig 3, both the cell lines tested exhibit low cytotoxicity for the magnetic NPs coated with citrate. Another possible alternative to the coating is the encapsulation of the iron-based NPs in Solid Lipid Nanoparticles (SLN-Fe). As fig. 4 shows the toxicity of the SLN-Fe was comparable to that citrate coated particles. Note that the SLN matrix shows no toxicity.

4. Discussion

The project BONSAI intends to develop multifunctional nanoparticles with tailored optical and magnetic properties for visualizing complex cellular structures (in tissue and organs), tumours and cell masses through ultra-sensitive bioimaging techniques. Recently, semiconductor nanocrystals (quantum dots) have been proposed for bio-imaging thanks to their high photostability, increased sensitivity through longer life time and excitability by a single wavelength. Unfortunately the most widely used quantum dots are highly cytotoxic (4,5,6).
Since the ambitious goal of the project is the production of NPs that may be employed in medical field, it is of primary interest

to investigate whether these particles are biocompatible with the organism. The first level of investigation was chosen to be the cell. We selected two cell lines with different properties. A lung epithelial and a fibroblast cell lines. Unlike the epithelial cells, fibroblasts do not form flat monolayers because their cell surface has not a polarized function as epithelial/endothelial cells. Therefore in living organism, they are assembled in three dimensional structures. We considered of interest to study the cellular response to NPs in cells either possessing or not polarized membrane function. The lung epithelium was also chosen as it represents the first barrier for air borne NPs.

An important task to consider for application in biology, medical diagnosis and therapy is stability of NPs in physiological solutions. This is particular hard to reach since the ionic strength in physiological fluids is high enough to decrease the electrostatic repulsion between NPs, which normally prevents aggregation. We make attempts to implement the homogeneous distribution of NPs in biological solution through biocompatible polymers coating or with SLN. We are facing a major problem of high variability in NPs diameters depending on the NPs concentration and on the medium used for the dispersion.

In all the cases presented the toxicity profiles of the different NPs tested may acutely develop by a marked increase in toxicity beyond a critical concentration threshold. From the data obtained we can conclude that fibroblasts are more sensitive to the NPs exposure compared to lung epithelial cells and reveal a higher toxicity for larger NPs. The direct relationship existing between the NPs radius and the biocompatibility may be an advantage since the luminescent properties are proportionally related with the radius of the particles.

Beside the raw data, the interesting question to be clarified is: what do we really intend with "toxicity"?. The assay we used identifies a plasma membrane alteration such as to cause the escape of a typical cytoplasmic enzyme (LDH, MW 35000 Da). An interesting step further may evaluate the possibility of reparative process once NPs are withdrawn from the medium. We will also evaluate how NPs exposure affect the integrity of the epithelial cell monolayer which is critically dependent upon the intercellular junctions.

The NPs developed inside the project are likely to have a significant impact on biomedical research based on improved resolution, sensitivity and versatility. However, a still poorly defined issue is the NPs-cell interaction that may impact over time and through different mechanisms on regular cell function. Therefore the studies concerning the use of NPs in nanomedicine imply a careful definition of the risks to benefits ratio.

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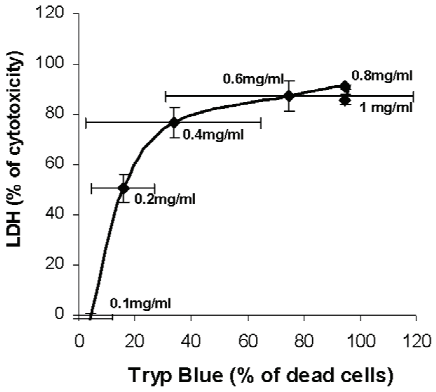


Fig.1 Relationship between Trypan Blue and LDH assay for NPs concentration 0.1mg/ml up to 1 mg/ml for 24 hours.

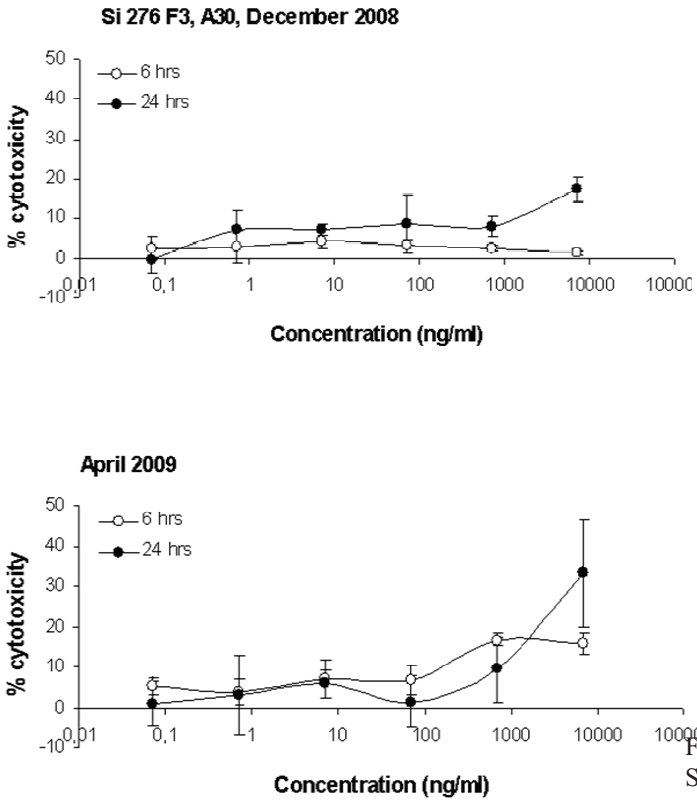


Fig.2 Cytotoxicity of a selected batch of SiNPs on A30 cells. Effect of NPs aging

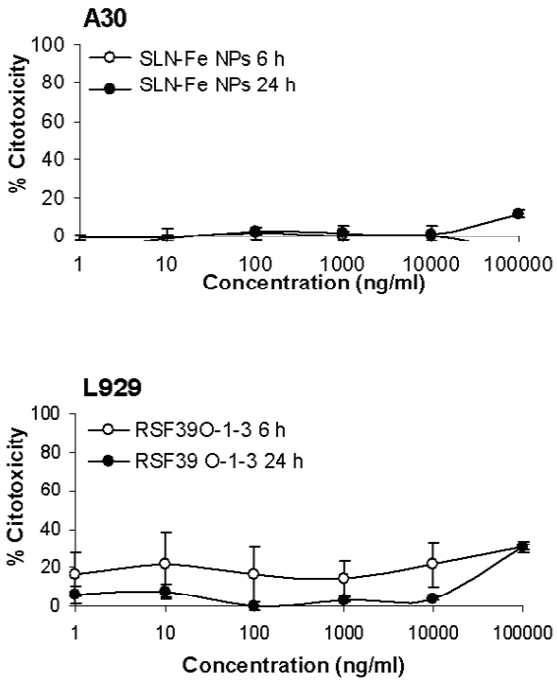


Fig.4 Cytotoxicity of Fe-base magnetic NPs encapsulated in SLN on A30 (upper panel) and L929 (lower panel) cells.

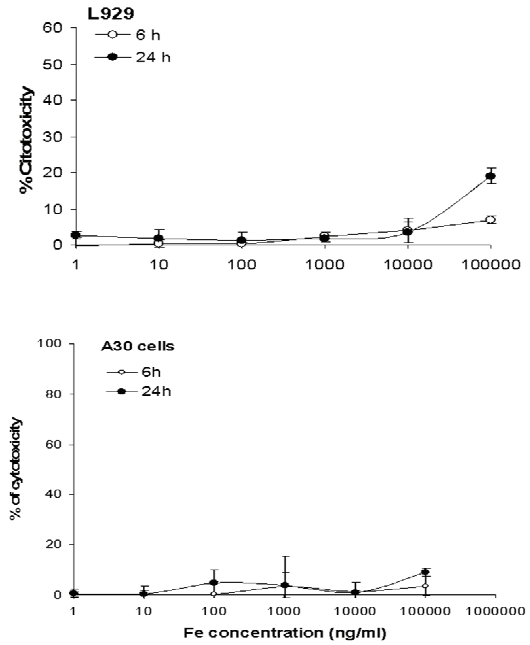


Fig.3 Cytotoxicity of a citrate coated Fe-base magnetic NPs on L929 (upper panel) and A30 (lower panel) cells.