

Advances in the preparation of novel functionalized nanoparticles for bioimaging

R. D'Amato¹, R. Alexandrescu², V. Bello³, V. Bouzas⁴, N. Carmona⁴, M. Chanana⁵, R. Costo⁶, F. Dumitrache², F. Fabbri¹, M. Falconieri¹, M.A. Garcia⁴, P. Gasco⁷, W. Gonzalez⁸, N. Herlin⁹, V. Maurice⁹, F. Huisken¹⁰, J.M. Idee⁸, V. Loschenov¹¹, G. Mattei³, G. Miserocchi¹², M.P. Morales⁶, I Morjan², Y. Nie⁵, M. Port⁸, V. Pustovoy¹¹, G. Riccio⁷, I. Rivolta¹², A. Ryabova¹¹, C. Robic⁸, G. Sancini¹², O. Sublemontier⁹, E. Trave³, S. Veintemillas-Verdaguer⁶, N. Vivenza⁷, D. Wang⁵, H. Xu⁵, E. Borsella¹

¹ENEA, Dept. FIM, Rome, Italy; ²Natl Inst Lasers Plasma & Radiat Phys, Bucharest, Romania; ³Univ. of Padova, Dept. of Physics, Padova, Italy; ⁴Univ. Complutense Madrid, Dept Fis Mat, Madrid, Spain; ⁵Max Planck Institute of Colloids and Interfaces, Dept. of Interfaces, Potsdam, Germany; ⁶ICMM-CSIC C/Cantoblanco, Madrid, Spain; ⁷Nanovector S.r.l., Torino, Italy; ⁸Guerbet Res, Roissy, France; ⁹Laboratoire Francis Perrin, SPAM, DSM, CEA-CNRS URA 2453, Saclay, France; ¹⁰Lab. Astrophysics and Cluster Physics Group, Inst. for Solid State Physics, F. Schiller-Universität Jena, Germany; ¹¹General Physics Institute- Natural Sciences Centre, Moscow, Russia; ¹²Dept. of Experimental, Environmental Medicine and Biotechnology, Univ. of Milano-Bicocca, Monza, Italy

Abstract—The EC BONSAI Project intends to develop multifunctional nanoparticles with tailored optical and/or magnetic properties for visualizing complex cellular structures (in tissues and organs), receptors, tumor cells and masses. In this framework, here we will report on recent advances on the preparation of luminescent silicon nanoparticles, magnetic iron oxide nanoparticles and Au nanorods for bio-imaging applications.

Keywords: Si nanoparticles, iron oxide nanoparticles, Au nanorods, bioimaging

I INTRODUCTION

The rapid development of bio-medical sciences demands new advanced techniques and instruments to investigate cells and cellular processes. In the last years, nanoparticles (NPs) have attracted steadily growing attention as a versatile and promising tool for bio-imaging. In this context, the EC BONSAI Project aims at the development of “smart” multifunctional NPs for visualizing complex cellular structures (in tissues and organs), receptors, tumor cells and masses.

In the following we will review the results obtained in the preparation and bio-testing of specific NPs with tailored optical and magnetic properties and will compare our achievements with the state-of-art.

Until now, cellular components and processes are mostly visualized through organic dye based fluorophores [1]. These dyes have several drawbacks such as rapid photo-oxidation, limited lifetime, need of different wavelengths to activate each dye, slow data acquisition for multi-parametric detection.

Recently a new class of fluorescent materials, the semiconductor quantum dots (QDs), raised increasing expectations for advanced biological research due to their peculiar optical properties [2]. QDs are generally II-VI semiconductor structures with all the physical dimensions smaller than the exciton Bohr radius (typically below 10 nm). In this size range, QDs can be exploited for multicolour experiments since nanocrystals with different sizes can be simultaneously excited by a single wavelength of light, at energies above the onset of their continuous excitation spectra,

resulting in multicolour, symmetrical spectral emission without red tails. Consequently, many colours can be distinguished without spectral overlap and different cellular compartments/processes can be labelled simultaneously, each with a different colour. The II-VI QDs have demonstrated useful for several applications ranging from cell labelling to tracking cell migration, from flow cytometry to genomic and proteomic detection, high throughput screening of biology, etc., however, their application in biology and medicine is hampered by their inherent chemical toxicity.

Unlike bulk Si that is not a good light emitter, nanostructured Si can emit photons in the visible- near IR range with a reasonable efficiency. It follows that silicon nanoparticles (Si-NPs) have the potential to overcome the inherent limitations in the biomedical use of QDs since silicon is inert, non-toxic, abundant and economical. Moreover, the silicon surface is apt to chemical functionalization, thus allowing for numerous stabilization and bioconjugation steps.

In order to exploit this potential for bio-medical applications, Si-NPs should remain highly luminescent and well dispersed in water and biological fluids over a wide range of pH and salt concentration. However, preparation of macroscopic quantities of single Si-NPs with stable and intense Photo-Luminescence (PL) emission and good dispersibility in water is still a challenging and difficult task that limits a widespread use of Si-NPs in bio-medicine. Our advances towards this objective are described in Paragraph II.

Imaging can also be generated by exposure of cells or tissues to magnetic fields and by measuring dephasing times of water protons as in Magnetic Resonance Imaging (MRI). Up to now, MRI shows excellent spatial resolution, but its sensitivity is relatively low, therefore MRI requires amplification mechanisms for its use in molecular imaging. To this purpose the help of contrast agents (CA) is required. Magnetic NPs actually used as CA have particle sizes ≥ 50 nm and consequently are cleared out by the RES system in very short times prior to any interaction with the biological target.

Superparamagnetic γ -Fe₂O₃ NPs are promising candidates as CA for MRI provided that powder samples are

disaggregated into colloiddally stable, well-dispersed, and biocompatible NPs with hydrodynamic sizes below 100 nm. A number of approaches are possible to get surface coatings of the magnetic NPs and will be reported in the Paragraph III.

Another class of materials of great interest for bio-imaging are the metallic NPs. In fact, when compared with molecular species such as organic chromophores, the absorption and scattering cross section of Au and Ag NPs are several order of magnitude higher. As a result, these NPs have recently been explored as CA for optical imaging of tumors. In addition, it is possible to tailor the spectral dependence of absorption and scattering coefficients of Au and Ag spherical NPs by engineering their geometrical parameters. The optical resonant behavior is due to the collective electronic or plasmonic resonance which depends not only from the metal but also from the particle shape. Tuning the plasmon resonance, combined with the easy bioconjugation of Au nanostructures, results in a combination of features which are ideal for biomedicine. Achievement in development of Au nanorods for detection in blood are reported in paragraph IV.

A major concern that might restrict the application of NPs in medicine, arises from the potential harm that could derive from their reduced size. To this respect, our studies dealing with cytotoxicity response of cells exposed to Si-based and Fe-oxide NPs are here summarised. More details can be found elsewhere [3].

II Si NANOPARTICLES

The EC funded BONSAI Project aims at replacing toxic QD such as CdSe with light emitting Si and Si-based NPs having lower toxicity, broader excitation band, size dependent optical emission and a reduced tendency to photo bleaching.

Sizeable quantities of Si-NPs with size ≤ 7 nm are currently produced by laser pyrolysis. This technique is based on the use of a laser beam to initiate and sustain the chemical reactions that generate the NPs in the gas phase [4]. Different, complementary strategies were followed to reduce the NPs size, by keeping a good productivity (300-500 mg/hr). By using continuous wave laser, NPs with size of 4-5 nm are produced by dilution of the precursor (silane) in a sensitizer and by collection of the NPs very near to the laser beam to limit the particle growth [5]. Alternatively, decrease of the size down to 3-4 nm is obtained by 10-20 μ s high power pulsed laser irradiation (see Fig. 1) in a silane/He flow.

As prepared Si-NPs, however, exhibit weak or even no PL. The emission intensity increases as a result of (non-controllable) exposure to air [4] or passivation in liquids of pristine NPs with size ≤ 5 nm or after ad-hoc optimised (soft) wet-oxidation processing of NPs with size $\leq 7-8$ nm [6].

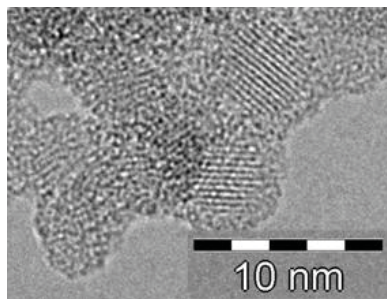


Figure 1. SiNP obtained by 10 μ s pulsed laser irradiation

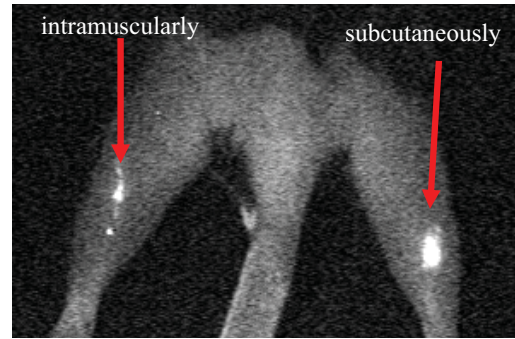


Figure 2. Si NPs (20 μ l of 0.1 g/l) were injected subcutaneously and intramuscularly into the right and left flank, respectively, of a nude mouse, and imaged immediately.

The PL emission of Si-NPs falls in the range 600-1000 nm and typical radiative lifetimes are in the range 0.05-0.3 ms. Furthermore, using two-photon excitation we were able to excite Si-NPs in the IR (at about 900 nm) where the human skin transmittivity is high. [7]

Preliminary in vivo experiments were performed by detection of the PL signal in continuous mode after intravenous injection of a colloidal solution of softly oxidised pyrolytic Si-NPs in tail vein of a mouse, as shown in Fig. 2. No acute toxicity was observed, however the signal intensity was not yet sufficient to evaluate the specific distribution of luminescent Si-NPs inside the organs. Moreover, applications in biology, medical diagnosis and therapy require that NPs are well dispersed and stable in physiological media. This could be a particularly hard task since the ionic strength in physiological fluids is high enough to decrease the electrostatic repulsion between NPs, which normally prevents aggregation. To this aim, a great effort was devoted to the preparation of stable colloidal solutions of Si-NPs in aqueous media. Both acid etching and alkali etching procedures result problematic, but successful results were achieved in the disaggregation of dried powders to well-dispersed Si-NPs in water by a combined alkali-etching procedure terminated by addition of H_2O_2 . It was also found that the use of HF/ HNO_3 mixture as etching agent can make the Si-NPs photoluminescent with various emission colors depending on the etching time (see Fig. 3).

For in vitro and in vivo applications, NPs should be coated with a biocompatible polymer to prevent the formation of large aggregates in order to improve biodistribution. Consequently, the second step was the fabrication of colloiddally stable and biocompatible Si-NPs by grafting hydrophilic polymer chains, such as poly(ethylene glycol), on the Si-NPs. First, disaggregated Si-NPs were coated with functional silanes terminated with amine or epoxy groups, and then were conjugated with poly(ethyleneglycol). It was found that the PEGylated Si-NPs remained stable in water for weeks.

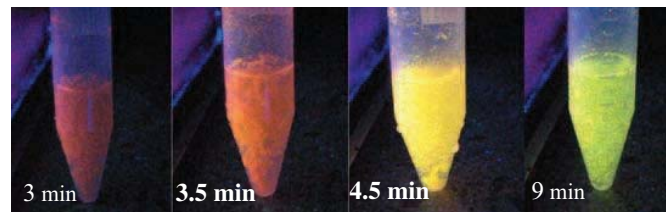


Figure 3. HF/ HNO_3 mixtures are used to disaggregate Si powders to Si NPs with size and wavelength emission decreasing with increasing etching time.

Negligible cytotoxicity of PEGylated Si-NPs was observed by vitality tests on epithelial cell lines known to be fairly sensible to noxious agents, i.e. A30 cells (located at the air/blood barrier).

III IRON-OXIDE NANOPARTICLES

Magnetic iron oxide NPs can be used as negative MRI CA [8]. They have marked T2 relaxivity due to their high magnetic moment, which generates microscopic field inhomogeneities. Consequently they produce a strong decrease in signal intensity in the organs in which they accumulate. There are currently two distinct classes in this family of products depending on the size of the particles: SuperParamagnetic Iron Oxide (SPIO), with mean particle diameter greater than 50nm, and Ultrasmall SPIO particles (USPIO) smaller than 50nm. By modifying the size and the nature of NPs coating, it is possible to modify the organ distribution and the pharmacokinetics and consequently the clinical application.

Commercial SPIO-CA are cleared from the blood flow and accumulated in the liver and spleen in short times. This issue impairs the clinical application of CA-enhanced MRI to the early detection of cancer or inflammatory lesions in regions other than the liver.

On this premise, the BONSAI Project aims at the development of stable colloids of non-toxic, non-immunogenic NPs with: (i) high magnetization (ii) particle size small enough and adapted coating to remain longer in circulation after injection (“stealth NPs”).

The NPs synthesis procedure employed by the BONSAI partners is a one-step process based on the laser pyrolysis of suitable gas-phase precursors [9,10]. Using this method in continuous operation mode, the BONSAI partners succeeded in the production of gram quantities of iron oxide powders, with small particle size (1.9-3.6 nm), narrow size distribution and good magnetic properties. (Fig. 4)

The intensity of the Magnetic Resonance signal depends on the magnetization of the iron oxide NPs, thus, it is crucial to obtain NPs with a magnetization strong enough and ensure that this magnetization can be reached at moderated fields.

It was found that magnetization values of the Project Iron Oxide magnetic NPs are similar to those of commercial NPs. Consequently, the strength of the magnetic signal reached in the BONSAI samples is good enough for MR applications. It was proved that the samples can be saturated at low magnetic fields when they are highly crystalline (Fig. 5).

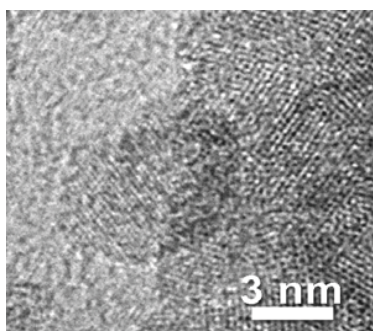


Figure 4. HR-TEM image of iron oxide samples produced by laser pyrolysis of Fe(CO)₅ using ethylene as sensitizer. Oxidation is made *in-situ* by introduction of air in the system

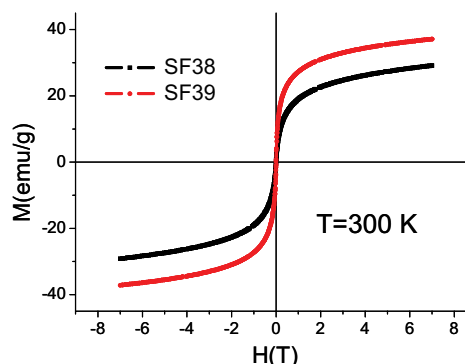


Figure 5. Magnetization curves for iron oxide NPs (at 300 K).

The use of γ -Fe₂O₃ NPs as CA for MRI requires colloiddally stable, well-dispersed, and biocompatible NPs with hydrodynamic sizes of 20-80 nm. The particles are dispersible in H₂O, but are not stable over long term. To overcome this limitation, the γ -Fe₂O₃ powder were disaggregated into individual NPs (i) stable in water and (ii) stable in organic solvents such as hexane and toluene. To this purpose, citrate acid (in the first case) and oleic acid/oleylamine (in the second case) were used as both etching agent and stabiliser of pyrolytic iron oxide NPs with no alteration of their magnetic properties. The water-citrate samples reached the USPIO level (D<40 nm) after the removal of aggregates, and presented good stability at high concentrations (>5mg Fe/ml). The organic-oleate approach produced colloids with D<200 nm. Both types of coated NPs are able to be redispersed after drying with little change in the aggregate size distribution.

Alternative further coatings have been developed in order to make the colloids stable and biocompatible in the body. In the BONSAI project a number of catechol ligands have been developed to disaggregate magnetic NPs powder. By these ligands, we succeeded in stabilizing the resulting NPs at physiological salt concentration of 150mM NaCl, and in culture medium (see Fig. 6).

Catechol ligand was chosen for its affinity to iron oxide and offers a number of advantages: high water dispersibility and high colloidal stability due to the presence of COOH group, together with immense flexibility for conjugation of dyes and peptides due to NH₂ group. By modification of the terminal groups, such as amine and carboxylic acid group of the catechol-ligands, we also succeeded in labelling magnetic NPs with dyes, which allows *in vitro* fluorescent bioimaging.



Figure 6. Coated Fe₃O₄ in PBS in the presence of a magnet

The Nanovector proprietary technical platform to prepare Solid Lipid Nanoparticles was applied to coat or to load BONSAI γ -Fe₂O₃ NPs, in order to obtain stable dispersions suitable for *in vitro/in vivo* studies [11]. By these coatings the colloidal stability was indeed improved without alteration of the particle size distribution and with final hydrodynamic sizes <80 nm - Intensity mean.

It was checked that there is not any degradation of the magnetic properties after lipid coating/loading processes. First stable freeze-dried formulations show iron concentration in the range usually administered in animal model and can be sterilised by filtration.

Preliminary Nuclear Magnetic Resonance Dispersion experiments, based on recording the relaxation enhancement (longitudinal *a/o* transverse) versus a magnetic field of variable intensity (i.e., a range of Proton Larmor Frequencies), have been performed as well (Fast Field Cycling NMR - FFC NMR). It was confirmed that the relaxometric behaviour of project NPs is substantially kept after lipid coating.

Bio-testing is a major issue in the BONSAI project. The study of the uptake of the MNP in epithelial and PMA-activated macrophage-like THP-1 cells and other cell lines was performed and is reported elsewhere [3].

IV AU-NANORODS

Au NPs exhibit a strong absorption in the green part of the spectrum (at about 540 nm) due to the surface plasmon resonance (Fig. 7). The absorption is so intense that it is possible to detect optically small quantities of Au NPs. This property, in addition to the high biocompatibility and easy functionalization, renders Au NPs optimal candidates for biomedical applications. Thus nowadays, their use for cell labeling, DNS recognition, and others are well established and commercial devices are available. While *in vitro* applications are fairly developed this is not the case for *in vivo* applications. The optical absorption of Au NPs is almost coincident to that of the blood as Fig. 7 illustrates. Thus it is not possible to detect optically small concentrations of Au NPs in the bloodstream. A possibility to overcome this problem is to prepare non-spherical Au NPs, as the increase of the aspect ratio shifts the absorption band towards the infrared [12].

Au nanorods were prepared by seed-mediated growth technique controlling the aspect ratio to shift the absorption band over 700nm where blood does not absorb strongly, so that the nanorods can be detected optically (Fig. 7). However this synthesis procedure provides Au nanorods capped with CTAB and BDAC surfactants which are toxic. Therefore, capping exchange is carried out to render the nanorods biocompatible.

At this stage, Au nanorods detection with a spectrophotometer is well achieved. We address now, in the framework of the BONSAI project, the design and fabrication of miniaturized devices to detect Au nanorods in blood. To this purpose we are developing small devices specifically designed to work at fixed wavelengths, allowing the use LEDs, bandpass filters and photodiodes, as optical elements. Last prototypes sizes are of the order of cm, and detection limits are below micrograms/liter.

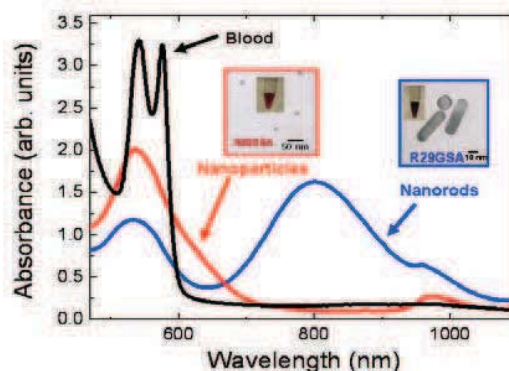


Figure 7. Optical absorption of blood (reference), spherical Au NPs and Au nanorods. Inset shows the morphology of the Au nanostructures.

REFERENCES

- [1] F. Wang, W. B. Tan, Y. Zhang, X. Fan and M. Wang, "Luminescent nanomaterials for biological labelling", *Nanotechnology*, vol. 17, pp. R1-R13, 2006.
- [2] K. Klostranec and W. C. Chen, "Quantum Dots in biological and biomedical research: Recent progress and present challenges", *Adv. Mater.*, vol. 18, pp. 1953-1964, 2006.
- [3] I. Rivolta, R. D'Amato, R. Alexandrescu, M. Falconieri, I. Morjan, M. Chanana, V. Bouzas, R. Costo, F. Fabbri, C. Fleacé, M.A. Garcia, P. Gasco, W. Gonzalez, M.P. Morales, Y. Nie, G. Riccio, C. Robic, G. Sancini, N. Vivenza, H. Xu, V. Bello, V. Maurice, O. Sublemontier, G. Mattei, N. Herlin, D. Wang, J.M. Idee, E. Trave, M. Port, S. Veintemillas-Verdaguer, E. Borsella and G. Miserocchi, "Cellular interaction with Si- and Iron-based nanoparticles for bio-imaging", *IEEE NANO 2009 Proceedings*, in press.
- [4] F. Huiskens, G. Ledoux, O. Guillois and C. Reynaud, "Light emitting silicon nanocrystals from laser pyrolysis", *Adv. Mater.*, vol. 14, pp.1861-1864, 2002.
- [5] R. D'Amato M. Falconieri, F. Fabbri, M. Carpanese, F. Dumittrache and E. Borsella, "Synthesis of Si nanoparticles with controlled size, morphology and crystallinity in a CO₂ laser pyrolysis reactor", *Proceedings of the 2nd International Congress on Ceramics*, 6P-020, 2008.
- [6] E. Trave, V. Bello, G. Mattei, M. Mattiazzi, E. Borsella, M. Carpanese, F. Fabbri, M. Falconieri, R. D'Amato and N. Herlin-Boime, "Surface control of optical properties in silicon nanocrystals produced by laser pyrolysis", *Appl. Surf. Sci.*, vol. 252, pp. 4467-4471, 2006.
- [7] M. Falconieri, R. D'Amato, F. Fabbri, M. Carpanese and E. Borsella "Two-photon excitation of luminescence in pyrolytic silicon nanocrystals", *Physica E* (2008) doi: 10.1016/j.physe.2008.08.055
- [8] S. Laurent, D. Forge, M. Port, A. Roch, C. Robic, L. Vander Elst and R.N. Muller, "Magnetic iron oxide nanoparticles: Synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications", *Chem. Rev.*, Vol. 108, pp. 2064-2110, 2008.
- [9] Y. Leconte, S. Veintemillas-Verdaguer, M.P. Morales, R. Costo, I. Rodriguez, P. Bonville, B. Bouchet-Fabre and N. Herlin-Boime, "Continuous production of water dispersible carbon-iron nanocomposites by laser pyrolysis: Application as MRI contrasts", *Journal of Magnetism and Magnetic Materials*, Vol. 311, pp.120-124, 2007.
- [10] I. Morjan, F. Dumittrache, R. Alexandrescu, R. Birjega, C. Fleaca, I. Voicu, L. Gavrila, I. Soare, G. Filoti, V. Kuncser, G. Prodan, V. Ciupina and L. Vekas, "Nanoscale Maghemite Iron Oxide Powders Prepared by Laser Pyrolysis", *Nanotech 2007 (Technical Proceedings of the 2007 NSTI Nanotechnology Conference and Trade Show)*, Vol. 4, pp. 234-237, 2007.
- [11] M. R. Gasco "Lipid nanoparticles: perspectives and challenges", *Advanced Drug Delivery Reviews*, Vol.59, pp 377-378, 2007.
- [12] Yu-Ying, Ser-Sing Chang, Chien-Liang Lee and C.R. Chris Wang "Gold Nanorods: Electrochemical Synthesis and Optical Properties", *J. Phys. Chem. B*, vol. 101, pp. 6661-6664, 1997.