BANDO "PROMUOVERE LA RICERCA D'ECCELLENZA"
Anno 2009

Project

Nanomedicine in ageing-associated prototypic diseases: activation of a scientific and technological platform challenging seminal aspects of pathogenesis, diagnosis and therapy.

Principal Investigator: Prof. Piercarlo Mustarelli, Dept. of Chemistry

Introduction

The project aims are: i) to contribute to the elucidation of key pathogenic aspects of two prototypic diseases associated to physiologic ageing; ii) to offer new sensitive diagnostic and effective therapeutic tools tailored on the pathogenic basis of the diseases.
We have identified 4 biomedical activities: A) clinical and pathological research on patients affected by the "prototypic diseases" in which the ageing contributes to the pathogenesis; B) investigation of structure-function relation on genes and proteins involved (directly or indirectly) in the pathogenesis; C) biological studies on cells directly responsible for the diseases and on cells eligible for substitutive therapies; D) establishment of models of the diseases presenting different grade of complexity from simple cellular model, to pluricellular organisms, to mammalian (mouse) models of the diseases. These 4 bio-medical activities are supported by the "technological platform" acting in three different activities. α) preparation and characterization of nanomaterials, and applications of micro-electronic devices in preparing new diagnostic tools for proteomic and genomic studies. β) Spectroscopy and microscopy of organic and biologic nanomaterials. γ) Exploitation of new nanomaterials and bio-engineering in developing bio-reactors aimed to prepare artificial tissues applicable to the reparative and regenerative medicine. δ) Pharmacological and toxicological studies on products of potential clinical use supporting all the activities that are transferrable into a clinical practice.
The project joins 20 research units of the University of Pavia, and it has been co-financed by Regione Lombardia (project SAL-45, P.I.: prof. Vittorio Bellotti). The P.I.s are supported by a steering committee collecting the WPs responsibles. This project has been financed with 100000 Euro destined to buy instrumentation of general interest. In the following the main results obtained by the research units will be reported.

Results

WP 1.1 Molecular base of *misfolding*-induced diseases and studies of new therapeutic approaches

R.U. Prof. Bellotti
The project is providing seminal new data on the molecular mechanism of inhibition of TTR amyloidogenesis through the interaction with new palindromic molecule MDS84. This molecule simultaneously binds the two binding sites of TTR, thus escaping the negative cooperativity of all the other mono-valent ligands. Such a property makes this molecule the most potent inhibitor of TTR amyloidogenesis so far designed. The first paper supporting the undergoing activity has been released last November in PNAS (1). At this stage we are comparatively characterizing by spectroscopic techniques, in stopped flow, the binding kinetics of wild type and pathologic variants of TTR. Meanwhile our research of new molecules interfering with the protein fibrillogenesis through the stabilization of the folded state has successfully provided new nanobodies the specifically recognize the dimeric form of DN6 b2-microglobulin (2) and we are now dissecting the

mechanism of stabilization of the dimer that in the absence of the antibody is, in vivo, highly amyloidogenic.

- Kolstoe SE, Mangione PP, Bellotti V, Taylor GW, Tennent GA, Deroo S, Morrison AJ, Cobb AJ, Coyne A, McCammon MG, Warner TD, Mitchell J, Gill R, Smith MD, Ley SV, Robinson CV, Wood SP, Pepys MB. Trapping of palindromic ligands within native transthyretin prevents amyloid formation. Proc Natl Acad Sci U S A. 2010 Nov 23;107(47):20483-8
- Domanska K, Vanderhaegen S, Srinivasan V, Pardon E, Dupeux F, Marquez JA, Giorgetti S, Stoppini M, Wyns L, Bellotti V, Steyaert J. Atomic structure of a nanobody-trapped domain-swapped dimer of an amyloidogenic beta2-microglobulin variant. Proc Natl Acad Sci U S A. 2011 Jan 25;108(4):1314-9

WP 1.4 Pharmacological targets for neurological and age-related diseases

R.U. Prof. Mattevi

Imidazolines are compounds that were originally discovered as anti-hypertensive agents and three distinct pharmacological targets have been identified as I_1, I_2, I_3 binding sites. It was proposed that the outer mitochondrial membrane monoamine oxidases (MAO A and MAO B) possess a I_2 binding site and our structural studies demonstrate this hypothesis. We have solved the crystal structure of human MAO B in complex with 2-(2-benzofuranyl)-2-imidazoline (2-BFI) at 1.6 Å resolution, which clearly shows that the inhibitor binds in a site distinct from the substrate-binding cavity. Interestingly, the 2-BFI affinity for the MAO B active site significantly increases when the enzyme is inhibited with tranylcypromine, an inhibitor with anti-depressant action which forms a covalent linkage with the enzyme flavin cofactor. Crystallographic analysis showed 2-BFI and tranylcypromine both bound to human MAO B with the former occupying the same site as that in the MAO B-2BFI complex and the latter being located in the substrate-binding cavity and interacting covalently with the flavin cofactor.

These studies have been published as follows:

- Bonivento, D., Milczek, E.M., McDonald, G.R., Binda, C., Holt, A., Edmondson, D.E., Mattevi, A. (2010) Potentiation of Ligand Binding through Cooperative Effects in Monoamine Oxidase B. *J. Biol. Chem.* 285, 36849-36856.

W.P. 2.2 Pluripotent stem cells

R.U. Prof. Garagna

In the first year of research, a protocol to differentiate megakaryocytes (MKs) from mouse embryonic stem cells (mESCs) has been developed. The protocol we have developed is very efficient, higher than previous published protocols, as 75% of differentiated cells are positive to CD61, a specific marker of megakaryopoietic cells. ES-derived MKs are rich of proplatelets from which platelets are released, evidencing a correct differentiation.

W.P. 2.3 Identification of drugs active fo*r* piastrinopoiesis

R.U. Prof. C. Balduini

The work to date performed has given the following results:

a) Identification by means of in vitro models of megacariocytopoiesis of a molecule able to stimulate platelets production in people with hereditary thrombocytopenia from gene MYH9 mutations (MYH9 correlated disease), and its effectiveness test by clinical trials.

This translational research allowed for the first time to demonstrate that a molecule mimicking trombopoietine, the hatural hormoon stimulating platelets production, is able to eliminate or at least reduce the platelets deficiency and the subsequent haemorrhagic diatesis of one of the most common types of hereditary piastrinopenia. This drug is therefore a good alternative to platelets transfusion, which at present is the only available treatment.[1]

b) Identification of age-related modifications of platelets production.

The variability of the platelets production parameters vs. ageing and sex has been demostrated for the first time by investigating Sardinian isolated sites with more than 10000 people. The platelets counting is inversely proportional to the age, and significantly higher for females. Moreover, the platelets volume is inversely proportional to their number. Both these parameters are gene-determined.[2]

c) Identification of a new gene responsible for hereditary piastrinopenia.

The analysis of a wide population of patients with hereditary piastrinopenia of unknown origin allowed to understand *ANKRD26* gene mutation as one of the most common reasons of domestic piastrinopenia.[3] The role of this gene has been to date unknown, and the discovery of the new disease will allow to understand its role in platelets production.

- Pecci A, et al., Eltrombopag for the treatment of the inherited thrombocytopenia deriving from MYH9 mutations. Blood. 2010 Dec 23;116(26):5832-7.
- Biino et al., M. Analysis of 12517 inhabitants of a Sardinian geographic isolate reveals that propensity to develop mild thrombocytopenia during ageing and to present mild, transient thrombocytosis in youth are new genetic traits. Haematologica. 2011 Jan;96(1):10-3.
- T. Pippucci, et al., Mutations in the 5'UTR of the ankirin repeat domain 26 gene (ANKRD26) cause an autosomal dominant form of inherited thrombocytopenia (THC2). Am J Hum Genet. 2011 Jan 7;88(1):115-20.

WP 2.5 – identification of biological markers in Alzhaimer disease

R.U. Prof. Lanni

An altered protein conformational state of p53, independent from point mutations, has been reported in tissues from patients with Alzheimer's disease (AD) and has been proposed as possible biomarker **[**1-3].

Our group recently described a blood-based cytofluorimetric method for conformationally altered p53 protein detection that allows to distinguish AD patients from control subjects and patients affected by other dementias [2]. Recent research suggested that onset of AD is commonly preceded by a phase known as mild cognitive impairment (MCI), a transitional state between prodromal and full-blown AD. The purpose of our research was to further investigate whether conformationally altered p53 expression may be applied to those patients falling in the ill defined category of MCI and in particular to predict which subjects among MCI patients will progress to AD. We found that unfolded blood p53 protein predicted progression to AD in preclinical patients with MCI two years before clinical diagnosis of AD was made [4].

Other purpose of our research was to examine the molecular mechanisms underlying the impairment of p53 activity, starting from recent findings showing that p53 conformation may be regulated by HIPK2, **one of the major activators and controllers of p53** [5]. We **observed that** soluble beta-amyloid (Aβ) induces degradation of HIPK2 causing loss of its transcriptional repressor activity and inducing the altered conformational state of p53 observed in cells of AD patients. The consequence of this conformational change is the loss of p53 transcriptional activity and failure to activate the proper apoptotic program when cells are exposed to noxae [6]. These results support the existence of a novel amyloid-based pathogenetic mechanism in AD potentially leading to the survival of injured dysfunctional cells.

- Uberti et al. Neurobiol Aging. 2006; 27(9):1193-201
- Lanni et al. Mol Psychiatry 2008; 13(6):641-73
- Zhou and Jia. Neurosci Lett. 2010 468(3):320-5
- Lanni et al. J Alzheimers Dis 2010; 20(1): 97-104
- Puca et al. Exp Cell Res. 2009;315(1):67-75
- Lanni et al. PLoS One. 2010 14;5(4):e10171

WP3.2 Animal Models of skeletal dysplasias

R.U. Proff. Rossi and Forlino

During this first period of research activity our group (Forlino-Rossi) achieved the following objectives:
-we completed the collection of long bone samples (tibia) from 21 days old knock in murine model of Diastrophic Dysplasia. We extracted the RNA extraction from the microdissections of the growth plate. We are now performing the molecular analysis to quantify by Real Time PCR the expression level of about 50 genes involved in cell cycle, differentiation and proliferation (Wnt, Ihh, etc). We are also planning to investigate the expression of some extracellular matrix genes;
- we set up a bone fracture protocol that allowed us to obtain compound fractures in the tibia of the knock in murine model for Osteogenesis Imperfecta, BrtlIV. By immunohystochemistry we demonstrated that after in utero bone marrow transplantation the donor cells are recruited at the fracture site of BrtlIV and WT mice and that these cells differentiated into osteoblasts. pQCT analysis are ongoing to evaluate the effect of such donor normal cells into the process of fracture repair.

- Gualeni B, Facchini M, De Leonardis F, Tenni R, Cetta G, Viola M, Passi A, Superti-Furga A, Forlino A, Rossi A. Defective proteoglycan sulfation of the growth plate zones causes reduced chondrocyte proliferation via an altered Indian hedgehog signalling. Matrix Biol. 2010 Jul;29:453-60.

WP 3.3 Biology of telomeres during ageing
R.U. Prof. Giulotto
After discovering that human telomeres are actively transcribed into RNA molecules called TERRA (Azzalin et al, Science 318: 798, 2007), we identified specific promoters, responsible for the transcription of TERRA, which are located in the subtelomeric region of several chromosomes (Nergadze et al., RNA 15: 2186, 2009). We recently showed that the methylation status of these CpG rich promoters regulates TERRA levels (1) supporting the idea that these non-coding RNA molecules play fundamental roles in telomere biology. We also carried out a preliminary genome-wide study of the DNA sequences bound by telomeric proteins and showed that the TTAGGG DNA repeat proteins 1 and 2 (TRF1 and TRF2) bind not only to telomeric DNA but also to several interstitial telomeric sequences (2). Interestingly, the TRF-binding sites are often located in the proximity of genes or within introns suggesting that the functional state of telomeric DNA may contribute to gene regulation.

- FARNUNG BO, GIULOTTO E, AZZALIN CM.(2010) Promoting transcription of chromosome ends. Transcription, 1: 140-143
- SIMONET T, ZARAGOSI L-E, PHILIPPE C, LEBRIGAND K, SCHOUTEDEN C, AUGEREAU A, BAUWENS S, YE J, SANTAGOSTINO M, GIULOTTO E, MAGDINIER F, HORARD B, BARBRY P, WALDMANN R, GILSON E. (2011) The human TTAGGG Repeat Factors 1 and 2 bind to a subset of interstitial telomeric sequences and satellite repeats. Cell Research, In Press

WP 3.4 –Tissue engineering
R.U. Prof. Conti
We investigated biodegradable block copolymers made of polyethylenglycol and polylactide, with the final goal to use them in the cartilage and/or tendon regeneration. Polyethylenglycol (PEG) is known as biodegradable, biocompatible polymer, already used in drug delivery applications; it can be successfully employed to modulate poly-alfa-hydroxyacids characteristics in terms of hydrophilicity and elasticity. The exposure to ionizing-radiation is the most effective method for the terminal sterilization of moisture- and heat- sensitive polymer devices. The purpose of these first months work was to investigate the long term effect of the gamma irradiation treatment on the functional properties of PEG-d,lPLA and PEG-PLGA films and to evaluate the biocompatibility of sterilized samples.

Chemical, thermal properties and biocompatibility of sterilized films were detected for samples at time zero and after storage at 4°C for 60 days. An *in vitro* degradation study was carried out on polymer samples to examine the effect of sterilization on the degradation performances of copolymers films. Incubated samples were characterized in terms of film surface structure (SEM), chemical (GPC) and thermal properties (DSC).
The study performed on films upon gamma sterilization showed no remarkable changes of the PEG-d,lPLA and PEG-PLGA film structure, while GPC analysis highlighted that the effect of gamma irradiation was dependent to the Mw and composition of polymers. DSC traces suggested more pronounced gamma-ray effects on the PEG-PLGA multiblock copolymer. During the stability study important changes in terms of structure surface, thermal properties and biocompatibility were observed and investigated. Data collected during the *in vitro* degradation study emphasized the need to know and investigate the degradation performances and behaviour of polymer or polymer systems (e.g. scaffolds) treated by gramma-ray.

- R. Dorati, C. Colonna, C. Tomasi, G. Bruni, I. Genta, T. Modena, B. Conti *" Gamma irradiation long-term effect on functional properties and cytotoxicity of multiblock copolymers films"* International Journal of Pharmaceutics submitted for publication January 2011.

R.U. Prof. Visai
In the first period of the project, Dr. Visai group and her collaborators focused her research on the study of *in vitro* interaction and differentiation of human SAOS-2 cells (hSAOS-2) (1) and human adipose stem cells (hASC) (2) to osteoblasts using different type of scaffolds.
The first type of study was performed using bioglasses scaffolds. Bioactive glasses were synthesized by the sol-gel technique in the laboratory of Prof. Mustarelli. In view of the potential clinical applications, we performed a detailed *in vitro* study of the biological reactivity of synthesized 58S bioactive glass containing-zinc, in terms of osteoblast morphology, proliferation, and deposition of a mineralized extracellular matrix (ECM). Human Sarcoma Osteoblast (SAOS-2) cells were used. In comparison with pure silica and 58S, the 58S-Zn0.4 bioglass showed a significant increase in cellular proliferation and deposition of ECM components such as decorin, fibronectin, osteocalcin, osteonectin, osteopontin, type-I and –III collagens. Calcium deposition was significantly higher than on pure silica and 58S samples. Also Alkaline phosphatase (ALP) activity and its protein content was higher with respect to pure silica and 58S. qRT-PCR analysis revealed the up-regulation of type-I collagen, bone sialoprotein and osteopontin genes. All together these results demonstrate the cytocompatibility of 58S-Zn0.4 bioglass and its capability to promote osteoblast differentiation.
The second type of study was performed using a different type of scaffold and pluripotent adipose tissue-derived stem cells (hASCs) that can differentiate into various mesodermal cell types such as osteoblasts, chondroblasts, and myoblasts. Isolated hASCs were induced to the osteogenic differentiation for 28 days on three different synthetic scaffolds such as polylactide-co-glycolide (PLGA), polylactide-coglycolide/hydroxyapatite (PLGA/HA), and trabecular titanium scaffolds (Ti6Al4V). PLGA and PLGA/HA were prepared in the laboratory of Prof. Conti. The aim of this study was to investigate the performance of PLGA and PLGA/HA scaffolds with a higher porosity, ranging between 75% and 84%, with respect to Ti scaffolds but with smaller pore size, seeded with hASCs to develop a model that could be used in the treatment of bone defects and fractures. Osteogenesis was assessed by ELISA quantization of extracellular matrix protein expression, von Kossa staining, X-ray microanalysis, and scanning electron microscopy. The higher amount of protein matrix on the Ti scaffold with respect to PLGA and PLGA/HA leads to the conclusion that not only the type of material but the structure significantly affects cell proliferation.

- *In vitro* calcified matrix deposition by human osteoblasts onto a zinc-containing bioactive glass. Saino E, Grandi S, Quartarone E, Maliardi V, Galli D, Bloise N, Fassina L, De Angelis MG, Mustarelli P, Imbriani M, Visai L. *Eur Cell Mater. 2011 Jan 14;21:59-72*;

- Stem Cells Grown in Osteogenic Medium on PLGA, PLGA/HA, and Titanium Scaffolds for Surgical Applications. Asti A, Gastaldi G, Dorati R, Saino E, Conti B, Visai L, Benazzo F. *Bioinorg Chem Appl. 2010:831031. Epub 2010 Dec 23.*

WP 4.1 Toxicity studies

R.U. Prof. Manzo

The Internal Medicine Toxicology Division Unit has completed the first set of studies included in the experimental plan aimed at developing and validating new testing methods that should improve the predictive value of safety and biocompatibility studies of nanomaterials (NMs). The adopted models and testing protocols are among the methodologies proposed by the National Cancer Institute (NCI, Bethesda, USA) for assessing toxicity and biocompatibility of NMs intended for clinical and diagnostic applications.

Studies are addressed to different types of "model" nanomaterials obtained from various sources. The parameters examined are those considered by regulatory guidelines and by recent directives on safety assessment of NMs and include general and target organ toxicity as well as systemic biocompatibility. An integrated set of molecular endpoints are investigated in order to correlate physicochemical characteristics, biokinetics and toxicological profile of nanomaterials and thus interfacing all toxicological results obtained with different methods (e.g. morphological, biochemical and analitycal).

Cytotoxicity of model NMs has been assessed in cell cultures, representative of target organs, by evaluating (i) membrane integrity and cell morphology (*Dye exclusion assay*: *Trypan blue, Calcein AM/Propidium Iodide*), (ii) cell function alterations (mitochondrial dysfunctions, energetic process, protein synthesis (*MTT, Neutral Red Uptake, ATP*), and (iii) cytolysis (*LDH*). A clonogenic assay has been developed and used in preliminary experiments evaluating the impact of NMs on cellular growth and proliferation.

Some of the results obtained during the first step of the project have been published in international journals (1-3). Preliminary data have also been presented in International meetings (4-9).

- COCCINI T, RODA E, SARIGIANNIS DA, MUSTARELLI P, QUARTARONE E, PROFUMO A, MANZO L. Effects of water-soluble functionalized multi-walled carbon nanotubes examined by different cytotoxicity methods in human astrocyte D384 and lung A549 cells. *Toxicology 269: 41–53, 2010.*
- RODA E, COCCINI T, ACERBI D, CASTOLDI AF, MANZO L. Comparative *in vitro* and *ex-vivo* myelotoxicity of aflatoxins B1 and M1 on haematopoietic progenitors (BFU-E, CFU-E, and CFU-GM): species-related susceptibility. *Toxicology in Vitro 24 217–223: 2010.*
- RODA E, COCCINI T, ACERBI D, BARNI S, VACCARONE R, MANZO L. Comparative pulmonary toxicity assessment of *pristine* and functionalized Multi-Walled Carbon Nanotubes intratracheally instilled in rats: morphohistochemical evaluations. *Histology and Histopathology, 26: 357-367 (2011).*
- MANZO L, RODA E, COCCINI T. Nanovectors. New Opportunities for Boron Neutron Capture Therapy. *Invited lecture, BIT's 3rd Annual World Cancer Congress, Singapore June 22-25, 2010, p. 437.*
- RODA E, COCCINI T, BARNI S. MANZO L. Comparative pulmonary toxicity of pristine and functionalized multi-walled carbon nanotubes intratracheally instilled in rats. *Congress of the International Union of Toxicological Societies, IUTOX Barcelona 19-23 July 2010. Toxicol Letters* 196S: 277 (2010).
- COCCINI T, RODA E. SIGNORINI C, GOLDONI M, GIARDINI A, MUTTI A, MANZO L. Kinetics and oxidative stress evaluation of silica nanoparticles doped with cadmium after intratracheal instillation in rats. *Congress of the International Union of Toxicological Societies, IUTOX Barcelona 19-23 July 2010. Toxicol Letters* 196S: 277-278 (2010).
- SABBIONI E, OLIVATO I, BONARDI ML, GROPPI F, MANENTI S, MANZO L. Multifunctional radionanomedicine: a new theranostic approach. *Nuclear Med Biol* 37: 719

(2010).

- COCCINI T, RODA E, MANZO L. Safety Evaluation of Engineered Nanomaterials. Tiered Testing Strategy and Examples of Application. *Invited lecture, Int. Conf. NanotechItaly, Venice 20-22 October 2010, p. 37-38.*
- COCCINI T, RODA E, FABBRI M, SACCO MG, MANZO L, GRIBALDO L. Gene expression analysis in rat lungs after intratracheal exposure to nanoparticles doped with cadmium. *International Conference on Safe Production and Use of Nanomaterials (NanoSafe2010), Grenoble, France 16-18 November 2010. J. of Physics – Conference Series, in press*

WP. 5.1 Development of micro-nanostructured materials and their interactions with biological structures

R.U. Prof. Conti

The research carried out in the first months work involves a preliminary study on preparation and characterization hydroxyapatite-Alendronate (HA:ALN) composite materials. Biphosphonate (BPs) such as Alendronate have been in widespread use for the treatment of several bone diseases characterized by osteoclast-mediated bone resorption such as Paget's disease, tumour-induced hypercalcemia, metastatic bone diseases and osteoporosis. One drawback of this drug class is the low bioavailability either through oral or intravenous administration. The alternative route able to achieve high drug concentration at the intended situ is a local treatment by drug delivery systems (DDS), such as microspheres and scaffolds.

The purpose of this preliminary work was to synthesize HA-ALN nano-composites as carriers for ALN improving the drug specific activity on bone as its target site.

HA-ALN composites were prepared by the co-precipitation method. Process parameters such as HA:ALN w/w ratio (1:1, 5:1, 10:1) and HA incubation time were evaluated as critical process parameters. Morphological, physico-chemical characterization and biocompatibility tests were performed. TEM and SEM confirmed the ALN precipitation as fine network onto HA. Composite size ranged between 200 – 800 nm as determined by granulometric analysis. FTIR, DSC, NMR confirmed ALN interaction with HA. The best value of ALN content in the composites was obtained between 60 and 80% for the HA:ALN 5:1 and 10:1 ratios. The HA:ALN 10:1 composition allowed suitable ALN release up to 80% in 4 days. Biological test revealed that complexation with HA increased in a three times order ALN biocompatibility. This preliminary results demonstrate that HA-ALN composites can be considered suitable carriers to control ALN release. Next step of the work involves loading of the HA:ALN nanocomposites into polymeric scaffold for bone regeneration.

- Priscilla Capra, Rossella Dorati, Claudia Colonna, Giovanna Bruni, Franca Pavanetto, Ida Genta, Bice Conti **"** *A preliminary study on the morphological and release properties of hydroxyapatite-Alendronate composite materials",* Journal of Microencapsulation. accepted for publication. January 2011.

R.U. Prof. Pallavicini

Silver nanoparticles (Ag NP) have been synthesized and characterized both coated with biocompatible molecules (cysteine, glutathione) and grafted on bulk surfaces. The separation, purification, stability and persistence in biomimetic solutions of the coated NP have been studied and improved. The mechanism of interaction with *E.Coli* and *S. Aureus* bacterial strains has been examined both on coated (solution dispersed) and on surface-grafted Ag NP. The optical characterization of the nanostructures has been carried out by means of FTIR, ATR, and spectrosopic ellipsometry in the visible, in order to determinate their optical response, their composition and thickness, particularly on self-assembled monolayers (SAM) grafted on silica and silicon surfaces. The optical data have been integrated with topographic and morphological measurements with Atomic Force Microscopy (AFM) and electron microscopy (SEM and TEM). The coating with SAM of pharmaceutically interesting molecules of monolayers of Ag NP is currently under study, to obtain surfaces capable of exerting a double action against resistant strains of bacteria. Innovative synthetic

approaches have also been developed to obtain asymmetric gold nanoparticles (Au NanoStars), with tunable dimensions and aspect ratio. These syntheses are also capable of positioning the absorbance wavelength (LSPR) at will in the 700-1500 nm range, and to produce nanoobjects that are perfectly stable in in-vivo conditions. The Au NanoStars interaction with bacterial strains is under study, together with their use for imaging and the exploitment of their huge phothermal effect. Moreover, fluorescent sensors for pH windows have been prepared thanks to the self-assembly of molecular components inside polymeric biocompatible micelles, based on amphiphilic co-polymers of polyhydroxy aspartamide (PHEA) functionalized with PEG e n-alkyl chains, that are stable under biomimetic conditions. The regulation of the position on the pH axis of the fluorescence "ON" interval was obtained by comicellization of charged species (surfactants, polyelectrolytes). Interactions with cells and the use for in-vivo imaging is under study.

R.U. Prof. P. Mustarelli
Magnetic core nanoparticles (Fe_2O_3, Fe_3O_4) have been synthesized be wet chemistry methods. We have been able to prepare particles with diameters ranging from 6 to 20 nm. The dispersion of the particles can be controlled by emulsifiers like oleic acid. Preliminary exchange procedures among oleic acid and polyethylene glycols with different molecular weight have been set up. This way we have obtained core/shell NPs with diameters ranging between 20 and 40 nm. The NPs have been characterized by means of thermal analysis, DLS, Raman spectroscopy and TEM. In the following some drugs will be added to the NPs and cytotoxicity and release tests will be performed.

R.U. Proff. Fagnoni and Profumo
We focused on the preparation of carbon nanotube protein conjugates that have been recently considered both for the delivery of therapeutics and for their application as electrochemical biosensors.
We then develop a facile strategy to obtain multiwalled carbon nanotubes (MWCNTs) functionalized with covalently bonded lysozyme. Lysozyme is a good model system to investigate the advantages related to the CNTs covalent functionalization. In fact, lysozyme is well known to kill Gram-positive bacteria, by hydrolyzing β 1,4 *O*-glycosidic subunit among *N*-acetylmuramic acid and *N*-acetylglucosamine subunits of peptidoglycane, which is an essential component of bacterial.
Accordingly, the aim of our work was to demonstrate the feasibility of a facile procedure for the covalent immobilization of lysozyme onto MWCNTs, and to test the biological activity of the resulting conjugate in comparison with that of the free enzyme. The basic idea was to perform an acidic etching of CNTs in order to form accessible carboxyl groups at nanotube tips. Lysozyme was then bound to the oxidized nanotube by using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) in the presence of *N*-hydroxysuccinimide (NSH) as the coupling agent.
The actual functionalization has been proved by means of several techniques, including thermogravimetry, Raman spectroscopy, transmission electron microscopy, and cyclic voltammetry. A functionalization of about 1 lysozyme molecule every 4000 carbon atoms was obtained.
The modified lysozyme-CNTs nanocomposite shows a significant increase of the antibacterial activity towards the Gram-positive *S. aureus* strain if compared with lysozyme in solution so demonstrating that the covalent functionalization does not alter its native functional properties but, in contrast, does enhance the enzyme biological activity.

- *Merli, D.; Ugonino, M.; Profumo, A.; Fagnoni, M.; Quartarone, E.; Mustarelli, P.; Visai, L.; Grandi, M. S.; Galinetto, P.; Canton, P.* "Increasing the antibacterial effect of lysozyme by immobilization on multi-walled carbon nanotubes"J. Nanosci. Nanotechnol. ***2011****, in press*.

WP. 5.2 Micro- and nano-structured platforms for advanced diagnostics
R.U. Prof. Patrini
The activity has focused on the design, realization and optical characterization of photonic micro- and nano-structured devices to be proposed as high sensitivity biosensors for proteins and complex

molecules. These devices will be exploited for the detection of organic monolayers covalently bonded or chemically/physically adsorbed on functionalized surfaces through specific recognition events. All-dielectric devices have been proposed, where e.m. field confinement effect is achieved by means of an interference effect in periodic multilayers that support Bloch surface modes (BSW). These optical resonances can be tuned to specific target wavelengths in the entire visible-NIR range through proper design of the dielectric structure. Then, the optical sensing signal is measured via micro-reflectance, diffraction and fluorescence spectroscopies. The surface functionalization with standard peptide solutions is under study in order to calibrate the sensing ability and specificity of the substrates. In the meantime, we are assembling an optical setup dedicated to fluorescence and Raman scattering/SERS microscopy of photonic and plasmonic platforms exploiting the strong signal enhancement due to surface-bounded modes, e.g. surface plasmon resonances and Bloch surface waves (SPR and BSW).

R.U. Prof. Cristiani

We developed an innovative microfluidic chip for the measurement of the mechanical properties of single cells in the frame of a collaboration with Istituto di Fotonica e Nanotecnologie (IFN-CNR, Milano). The chip is fabricated by the femtosecond laser micromachining technique in a silica substrate. Both a microfluidic channel and properly alligned optical waveguides are fabricated on the chip. The cells under test flowing into the microchannel are trapped and then elongated thanks to the effect of radiation pressure exerted by the optical beams emitted by the channel waveguides. The chip is used in combination with a phase contrast or a fluorescence microscope. The analysis of the cell elongation as a function of the optical forces is obtained through a Matlab software that is able to automatically process the images of the elongated cells captured by a high resolution CCD camera. In order to increase the quality of the cell images a second version of the device has been developed that is obtained fabricating the optical waveguides into a commercial microfluidic chip in which the inner surfaces of the channel have a negligible roughness. Indeed in the first realization the channel was obtained by selectively etching the silica through HF. In this case the residual roughness has an average value of 200 nm; such a value can alter the image quality thus limiting the measurement reliability.

The chip has been tested by measuring the elasting properties of human red blood cells in physiological e hypotonic solution.

Budget

As already stressed, the grant received by AMT Foundation has been devoted to the acquisition (in case within a co-funding scheme) of instrumentation of general interest of the project. To date, four instruments have been individuated, and the details are given in the following table:

Instrument	**Total cost (IVA included)**	**AMT contribution**	**Scientific responsible**	**Purchase order state**
Laica microscope M165FC	24.000	24.000	V. Bellotti	Complete
Solid state NMR 400 MHz Bruker Avance III	342.000	24.000	P. Mustarelli	To be emitted
Sorgente "Laser Quantum" Torus CW	24.000	24.000	M. Patrini	To be emitted
Proteins purification apparatus	38.000	24.000	A. Mattevi	Money to be transferred to the Department of Genetics.
Total		96.000		

The first instrument has been already purchased. For the second and the third ones the P.O.s must be emitted. Concerning the last one, the AMT contribution will be transferred to the Dept. of Genetics and Microbiology where the P.O. has been emitted.