



University of Eastern Piedmont Orientale “Amedeo Avogadro”

PhD in Molecular Medicine

Cycle XXVI

**BIODEGRADABLE POLYMERIC MICROPARTICLE FOR ANTIGEN AND PROTEIN
SUBUNIT DELIVERY**

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Annual report: 2013/2014

LIST OF ABSTRACT PAPERS

1. Characterization of PLGA (poly-D-L-lactic-co-glycolic acid) Microparticle Uptake and in vitro Inflammatory Responses (not submitted)

AIM: Microparticle generated from PLGA has been used for drug delivery due to their clinically proven compatibility and currently there is little knowledge and understanding, of the relevant length and time scales for microparticle-intracellular entry and localization within cells, and here we investigated the uptake of microparticle, and in vitro inflammatory response, and optimize a platform of vaccine delivery. METHODS: Microparticle using PLGA (poly D-L-lactide-co-glycolide) polymer was prepared by via the solvent evaporation method and morphological analysis, such as size and zeta potential was determined using Dynamic light scattering (DLS) and scanning electron microscope (SEM). The uptake amount by cells was determined by flow cytometry and fluorospectrophotometry and qualitative images was acquired by confocal microscopy and proinflammatory cytokines was determined by ELISA. RESULTS: The size of microparticle was ranging from 225-785nm with a smooth and spherical shape and possesses a -15 ± 2.12 mV zeta potential, which indicates the stability of the formulation. The internalization of microparticle across the Raw 264, U937 and MCF-7 cell for PLGA and PLGA-OVA microparticle formulation and the study demonstrated that U937, RAW264 and MCF-7 cellular uptake and transport of blank PLGA and PLGA with OVA is significantly affected by the dose, size, cell type and incubation time. The uptake of nanoparticle was rapid and confocal microscopy demonstrating their localization mostly in the cytoplasm as well as on the surface of the cells in the case of aggregated microparticles. There was a significant difference in the uptake of PLGA among cell types ($P < 0.05$) and the uptake reached a steady state after 4 hr. Macrophages exposed to PLGA displayed a dose dependent TNF- α induction and synergetic effect with LPS. The mitogenic of microparticles study demonstrated biocompatibility of microparticles at all range of the concentration used with the cells and could be used for localizing therapeutic agents into cell. Intracellular uptake micro/nanoparticles was observed to be the highest in RAW 264.7 and U-937 cells, which are a specialized phagocytic cell lines and the lowest in the MCF-7 cells. Results highlight the variability of uptake kinetics for the same material in different cell types. CONCLUSION: The study demonstrated that uptake of nanoparticles are dynamic and energy-dependent processes and a better understanding on the

mechanisms of cellular uptake, effect of nanoparticle formulation and composition as well as size and characterization of intracellular distribution of nanoparticles would be useful in exploring nanoparticles for intracellular delivery of therapeutic agents.

2. A novel formulation strategy, characterization, and in vitro evaluation of PLGA based microparticle for protein subunit delivery (not submitted)

AIM: Interleukin-10 (IL-10) is an anti-inflammatory molecule that has achieved interest as a therapeutic for infectious and autoimmune disease. Microparticle generated from PLGA has been investigated for drug or vaccine delivery due to their clinically proven compatibility. Here we explored a novel approach for making a single vaccine formulation and optimizes a platform in invitro condition for delivering protein subunits for therapeutics purpose. METHODS: Microparticle with IL-10, coumarin-6 were formulated from poly D-L- lactide-co-glycolide polymer by the solvent evaporation method. PLGA OVA was prepared by simply mixing OVA with PLGA. Morphological analysis, size and zeta potential was determined using Dynamic light scattering (DLS) and scanning electron microscope (SEM). The biocompatibility test, cell cytotoxicity, OVA adsorption, IL-10/OVA kinetic release, functional and stability of protein and uptake was performed using MTT, BCA assay, ELISA, flow cytometry, fluorospectrophotometry and confocal microscopy. RESULTS: The size of microparticle was ranging from 225-700nm with a smooth and spherical shape and possesses a -15 ± 2.12 mV zeta potential, which indicates the stability of the formulation. The microparticles displayed no cytotoxicity and, a direct correlation of MP dose and TNF- α induction was observed. The IL-10 encapsulating efficiency was about 23% and 82% adsorption of OVA. A two-phase release profile was observed for OVA and triphasic for IL-10. IL-10 release from PLGA complex accounts 10% of protein released during initial burst, followed by 30% release after 4 weeks and 63% release after 2months. There was an initial 30% release of OVA to the medium and loss stabilization to about 4% release. The IL-10 released was subjected to functional test and it inhibits the expression of the inflammatory cytokine, indicating that the biological activity of the IL-10 was preserved. The various range of OVA concentration tested for adsorption fitted with the Langmuir model. The degradation studies demonstrated that loss of 20% mass in the 2nd week followed by 53% loss during 7-9th week moreover, morphology of the PLGA was collapsed after the 2nd week. Cellular uptake of microparticle depends on cell type, the time of

incubation and the concentration of microparticle in the medium. The uptake of nanoparticle was rapid and their localization mostly in the cytoplasm and, few remains attached to the surface of the cells. CONCLUSIONS: The microparticles were biocompatible and can be used to make a single vaccine formulation and, slow release of drug can be overtime achieved. Uptake of nanoparticle is a dynamic and energy-dependent process, and better understanding on the mechanisms of cellular uptake would be useful for intracellular delivery of therapeutic agents.

3. Analysis of different biocompatible and biodegradable nanoparticle platforms for vaccination use (to be submitted.)

Nanoparticles are promising materials for various biomedical applications, gene/drug delivery, and autoimmunity/cancer treatment. To increase the effectiveness of NPs for delivery settings, high capture efficiency, controlled uptake of the particles by cells are required. Here, we evaluated the physicochemical properties, cellular toxicity, cellular internalization, and capacity of several types of new NPs to induce TNF- α secretion in comparison with PLGA polymer, approved by FDA for human use. Results showed the cytotoxicity of the nanoparticles was very limited which indicate that NPs were biocompatible. Analysis of cell uptake showed that the cellular uptake was proportional to the NP dose and time, and interestingly, SLN and BSSH-NS showed less uptake compared to a-b-g CD nanosponges and PLGA particles, and internalized particles were found mainly in the cytoplasm. Moreover, SLN and B-SH nanoparticles did not provoke an inflammatory response in human PBMC at any of the concentrations tested; however, PLGA and a-b-g CD nanoparticles elicited a pronounced inflammatory response above a threshold concentration of 600 μ g/mL, by demonstrating that inflammation in PBMC after exposure to nanoparticles depends on the concentration and composition of the particles. These data suggest that choice of the NP material can be crucial to modulate the vaccine capacity to trigger and direct the immune response and analysis of a wider pattern of cytokines might better

define these features. Moreover, NPs relatively inactive from an immunological point of view would be ideal to be loaded with mixtures of immunomodulatory selected to precisely direct the immune response triggered by the vaccine.

4. Camptothecin in nanosponges as therapeutic nanodelivery agent in prostate cancer: in vitro and in vivo evaluation (submitted and under review)

The use of the anti-tumor drug camptothecin (CPT) has been hampered by poor solubility and high degradation rate. We have previously shown that CPT encapsulated in β -cyclodextrin-nanosponges (CN-CPT) overcomes these disadvantages and ameliorates the CPT inhibitory activity on the growth of the prostate tumor cell lines DU145, PC-3, and LNCaP in vitro. This work extends those observations showing that CN-CPT also significantly inhibited adhesion and migration of these tumor cell lines and their STAT3 phosphorylation. The anti-adhesive effect was also exerted in human umbilical vein endothelial cells (HUVEC), in which CN-CPT also inhibited the angiogenic activity detected by the tubulogenesis and the sprouting assays. Finally, CN-CPT strikingly inhibited the PC-3 cell engraftment in SCID/beige mice in vivo without apparent toxic effects. Taken together, these results support the use of β -cyclodextrin nanosponge nanotechnology to deliver anticancer drugs for the treatment of prostate cancers.

5. PLGA nanoparticles for “inverse vaccination” in Experimental Autoimmune Encephalomyelitis (EAE) (Manuscript in Preparation)

Inverse or tolerogenic antigen-specific immunization induces regulatory/suppressive immune functions to inhibit autoimmune responses. It can trigger a suppressive loop spreading to epitopes, which is intriguing for MS where multiple autoantigens are involved. Initial therapeutics trials used administration of purified myelin antigens in experimental autoimmune encephalomyelitis (EAE), an animal model of Multiple Sclerosis (MS), however repeated injections needed due to their rapid clearance. Alternate approach to overcome this drawback is using DNA-based vaccines encoding for myelin autoantigens alone or in combination with “adjuvant” molecules, such as IL-4 or IL-10, to support development of regulatory immune cells. Recent phase I and II clinical trials with MBP-based DNA vaccines showed positive results in reducing MRI-measured disease activity and inducing tolerance to myelin antigens in MS patients. However, DNA vaccination has potential risks limiting its use in humans. An

alternative approach could be use of protein vaccines released from polymeric biodegradable lactic-glycolic acid (PLGA) particles (PLGA-NP), approved by FDA, to sustain release of antigens and regulatory adjuvants for extended periods. PLGA-NP maintain effective concentrations of the loaded protein for prolonged periods of times by trapping them in a hydrated polymer-network that enable slow-release, by modulating the polymer lactide-glycolide ratio, the molecular weight, and the crystal profile. Use of PLGA-NP per se can enhance tolerance induction in some delivery setting, such as nasal vaccination. Recent report suggested that intravenous infusion of either polystyrene or PLGA particles chemically coupled with an encephalitogenic peptides can prevent and cure EAE. This strategy stemmed from previous observations showing that autoantigenic peptides coupled to apoptotic leukocytes can induce antigen-specific tolerance in models of autoimmune diseases, allergy, and transplantation by inducing depletion and anergy of the antigen-specific lymphocytes.

Aim of this work was to develop protein-based tolerogenic PLGA-NP vaccines containing myelin autoantigen (MOG₃₅₋₅₅) and adjuvants to subcutaneously inverse vaccinate EAE (Experimental Autoimmune Encephalomyelitis) mice. Nanoparticles were formulated from poly D-L-lactide-co-glycolide polymer by solvent evaporation method. Morphological analysis such as size and zeta potential were determined using Dynamic light scattering (DLS) and scanning electron microscope (SEM). PLGA-NP shows a mean diameter of 591.7 nm with a smooth and spherical shape and possesses a -15 ± 2.12 mV zeta potential and did induce secretion of proinflammatory cytokines (TNF- α) on PBMC as dose dependent manner. IL-10 and MOG release from PLGA complex follows a triphasic and biphasic release profile respectively. The rIL-10 loaded-PLGA maintains its ability to inhibit TNF- α on PBMC, indicating the biological activity of the IL-10 was preserved. Cellular internalization of NP was evaluated on Raw-264 , U-937 and MCF-7 cells using confocal laser scanning microscope and flow cytometry .The study demonstrated that U937, RAW264 and MCF-7 cellular uptake and transport of PLGA is significantly affected by the dose, size ,cell type and incubation time. The uptake of nanoparticle was rapid and confocal microscopy demonstrating their localization mostly in the cytoplasm as well as on the surface of the cells. Cells exposed to PLGA displayed a dose dependent TNF- α induction and synergetic effect with LPS. The mitogenic of microparticles study demonstrated biocompatibility of microparticles at all range of the concentration used with the cells and could

be used for localizing therapeutic agents into. Preliminary therapeutic and prophylactic experiments showed that subcutaneous treatment with MOG₃₅₋₅₅- and rIL-10-loaded PLGA-NP significantly inhibited development of EAE in C57/B6 mice without detectable toxic effects. These data suggest that PLGA-NP-based inverse vaccination may be an effective tool to treat autoimmune diseases.

PUBLISHED PAPERS

- Differential induction of IL-17, IL-10, and IL-9 in human T helper cells by B7h and B7.1. Mesturini R, Gigliotti CL, Orilieri E, Cappellano G, Soluri MF, Boggio E, **Woldetsadik A**, Dianzani C, Sblattero D, Chiocchetti A, Yagi J, Rojo JM, Dianzani U. Cytokine. 2013 Oct; 64(1):322-30.
- Anti-cytokine autoantibodies in autoimmune diseases. Cappellano G, Orilieri E, **Woldetsadik AD**, Boggio E, Soluri MF, Comi C, Sblattero D, Chiocchetti A, Dianzani U. Am J Clin Exp Immunol. 2012 Nov 15; 1(2):136-46.

POSTER

- **PLGA nanoparticles for “inverse vaccination” in Experimental Autoimmune Encephalomyelitis (EAE). Oral presentation** on 36th National Congress of The Italian Society of Pharmacology, Turin, Italy, Oct 23-26, 2013.
- **Micro/nanoparticulate platforms for “inverse vaccination” in Experimental Autoimmune Encephalomyelitis (EAE)** .Poster presented on 15th International Congress on Immunology, Milan , Italy, Aug 22-27, 2013.

SEMINARS

2011

- **Abiy Woldetsadik** "Knock down of HIF-1 alpha in glioma cells reduces migration invitro and invasion in vivo and impairs their ability to form tumor spheres"
- **Dr Crescitelli ha** The Kruppel-like zinc finger protein ZNF224 recruits the arginine methyltransferase PRMT5 on the transcriptional repressor complex of the aldolase A gene.
- **Dr: Elisabetta BUGIANESI**, University of Turin "Metabolic syndrome: The gastroenterologist point of view"
- **PROF. LEONARD PETRUCCELLI** "Mechanisms and Models of TDP-43 Proteinopathies
- **Dr:Valerio NOBILI** *Liver fibrosis in children affected by NASH: how to investigate that*
- **Dr: Gilberto FILACI**, University of Genova Reverse vaccination in autoimmune
- **Dr Alessandro Di Nicola** "Ion Torrent technology for massive parallel sequencing"

- **Dr Leonard Petrucelli**, DEPARTMENT OF NEUROSCIENCE MAYO CLINIC, Florida USA "Mechanisms and Models of TDP-43 Proteinopathies"
- **Dr. StevenR. ELLIS**, University of Louisville, USA. "Iron Management in the Hepcidin Era"
- **Prof. Maurizio PAROLA**, Universita' Of Torino Hypoxia,angiogenesis and liver fibrogenesis"
- **Prof. Costantino Pitzalis MD PhD** "Importance of pathobiology in rheumatoid arthritis pathogenesis, disease evolution and response to treatment (The PEAC Project)"
- **Prof. Roberto BALDELLI** "Endocrine disturbances during Thyrosine Kinase Inhibitor treatment"
- **Dr. Sonia LEVI**, Vita-Salute San Raffaele University "Ferritins: ancient proteins with novel unexpected features"
- **Prof. Mauro Fasano**, University of Insubria "*Molecular pathogenesis and biomarkers of parkinson's disease*"

2012

- **Philip BEART**, Florey Neuroscience Institutes, University of Melbourne, Parkville, Australia, "Neuronal Death: Recruitment of programmed necrosis and autophagy"
- **Dr. Kim De Keersmaecker** VIB Center for the Biology of Disease, Center for Human Genetics, KU Leuven (Belgium), "Next generation sequencing in T-ALL"
- **Prof. Luigi Naldini** San Raffaele, Telethon Institute for Gene Therapy (HSR-TIGET), "Recent Advances in Hematopoietic Stem Cell Gene Therapy: from microRNA Regulation to Targeted Gene Transfer"
- **Dr .Molewaterplein Rotterdam, The Netherlands.** "Molecular control of human fetal globin expression: towards a potential cure for B-thalassemia and sickle cell anemia"
- **Prof. J. N. Bouwes BAVINCK** Department of Dermatology, Leiden University, Olanda. "High-throughput Biochemical Target Investigation Unveils a Novel Function of miR-21 as a Negative Modulator of Signal Transduction in T-lymphocytes"
- **Prof.Pino Macino** Dipartimento di Biotecnologie Cellulari ed Ematologia Università degli Studi di Roma "La Sapienza", "Linfomicutaneiprimitivi"
- **Prof. Andrea Danani** Chief of the Laboratory of Applied Mathematics and Physics-LAMFI Department of Innovative Technologies-DTI, University of Applied Science of Southern Switzerland-SUPSI, Lugano, CH , "Numerical simulations as virtual microscope at the nanoscale: some examples with dendritic molecules"
- **Prof.Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London

**London, UK, “Microparticles as noveffectors in Inflammation”
“Microparticlesasnoveffectors in Inflammation”**

- **Prof.Mauro PERRETTI, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK, “Resolvins and Omega-3 in Inflammation”**
- **Prof.ssa Isabel MERIDA Centro Nacional de Biotecnologia, Madrid, “Role of Diacylglycerol kinases in the control of T cell activation and differentiation programs**
- **”Dott.ssa Elena RAINERO Beatson Insitute for Cancer Research, Glasgow, UK, “Signalling pathways controlling integrin trafficking during invasion”**
- **Prof. Lötvall J., Department of Internal Medicine, Sahlgrenska Academy, University of Gothenburg, "Exosomes Shuttle RNA"**
- **Dr. Paolo Fortina, Department of Cancer Biology, Jefferson Genomics Laboratory, Kimmel Cancer Center, Thomas Jefferson University Jefferson Medical College, Philadelphia, PA, USA, “Next-generation DNA sequencing and target arrays in the clinics”**
- **Prof.Mauro PERRETTI, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK., “Alpha-MSH and the melanocortin system in inflammation”**

- **Prof.Mauro PERRETTI, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK., “Galectins-carbohydrate binding protein: sweet or sour?”**
- **Prof.Mauro PERRETTI, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK., “The resolution of inflammation: players and targets”**
- **Prof.Joseph A.Bellanti, Professor of Pediatrics and Microbiology-Immunology Director, International Center for Interdisciplinary Studies of Immunology Georgetown University School of Medicine , “New trends in allergy and immunology”**
- **Prof.SjaakPhilipsen Rotterdam, The Netherlands, “Molecular control of human fetal globin expression: towards a potential cure for B-thalassemia and sickle cell anemia”**
- **Prof. J. N. Bouwes BAVINCK Department of Dermatology, Leiden University, Olanda, “High-throughput Biochemical Target Investigation Unveils a Novel Function of miR-21 as a Negative Modulator of Signal Transduction in T-lymphocytes”**

2013

- Prof. Giuseppe Cornaglia, Dipartimento di patologia e Diagnostica, Università degli Studi di Verona. ***“Carbapenemases: a last frontier for beta-lactam antibiotics?”***
- Dr. Luca Pastorelli, Dipartimento di Scienze Biomediche per la Salute, IRCCS Policlinico San Donato. ***“Interleukin-33: a novel player in chronic intestinal inflammation?”***
- Dott. Girish Patel, Department of Dermatology and Wound Healing, School of Medicine, Cardiff University, United Kingdom. ***“Skin cancer in vivo models, what they have and can tell us”.***
- Dott. Giancarlo Ghiselli presidente, glyconova srl, startup , bioindustry park "silvano fumero" colleretto giacosa, torino" ***Heparan Sulfate: A versatile target for the development of new drugs.***
- Prof. Silvano Sozzani Dipartimento di Medicina Molecolare e Traslazionale, Università di Brescia. ***“Cytotoxic potential of plasmacytoid dendritic cells in autoimmune diseases”***
- Dott. Girish Patel Department of Dermatology and Wound Healing, School of Medicine, Cardiff University, United Kingdom. ***“Skin cancer in vivo models, what they have and can tell us”***
- ***PD Dr. Hans Bäuml*** *Head of the Research Department, Institute of Transfusion Medicine Berlin-Brandenburg Center for Regenerative Therapies Charité – Universitätsmedizin Berlin, Red blood cells as carriers for magnetically targeted delivery of drugs*
- DOTT. KYUNG-MIN NOH , PHD Rockefeller University New York, NY, USA ***Molecular interactions of histone modification and de novo DNA methylation***
- DOTT. FRANCO CAPOZZA Thomas Jefferson University Filadelfia, PA, USA, ***Cav1 protein in Skin Cancer Pathogenesis.***
- DOTT. ANTONELLA BANDIERA, PHD DIPARTIMENTO DI SCIENZE DELLA VITA UNIVERSITÀ DEGLI STUDI DI TRIESTE . ***“HELPS: biomimetic polypeptides for biomedical applications***
- Dott. Giuliana Pelicci, direttore di unità “biologia e trasduzione del segnale di cellule staminali neuronali normali e tumorali” dipartimento di oncologia sperimentale istituto

europeo di oncologia di milano . "*Glioblastoma stem cell biology and its implications in cancer therapy*

- Prof Steven Gravis, University of New Mexico, Center for Biomedical engineering, USA
Single cell and single particle analysis for biomedical diagnostics and medical discovery and biomimetic microparticles - construction, analysis, and applications. High throughput cell/particle screening for discovery.

TRAINING AND COURSES ATTENDED

- Summer School on Medical Technologies “Industrial Challenges and process of innovation in Medical technologies”, Grenoble, France, 18-23rd of Jul, 2011 Grenoble
- BD-flow cytometry training program, Buccinaso, Milan, 2-3 and 28 th of May, 2012
- Genetics and molecular medicine master class by Prof Steve Ellis, 21st May-1st Jun 2012
- Single cell and particle analysis master class by Prof Steven Gravis.
- **University of New Mexico, Center for Biomedical Engineering, USA, Sep 2013-Present** Training and research work focuses on Single cell and single particle analysis for biomedical diagnostics and medical discovery, Biomimetic microparticles construction, analysis, and applications. High throughput cell/particle screening for discovery

CONGRESS

- The German Society for Immunology (DGfI) and the Italian Society for Immunology, Clinical Immunology and Allergology (SIICA) annual meeting in Riccione, Italy (28th September - 1st October 2011).
- 8th International Congress on Autoimmunity, Granada, Spain, May 9-13, 2012
- 15th International congress of Immunology, Milan, Italy Aug 22-27, 2013.