University of Piemonte Orientale "Amedeo Avogadro"

PhD in Molecular Medicine

Cycle XXVI

BIODEGRADABLE POLYMERIC MICROPARTICLE FOR ANTIGEN AND PROTEIN DELIVERY

PhD student: **Abiy D Woldetsadik**

Tutor: **Prof. Dianzani Umberto**

Annual report: 2010/2011

A Novel in vitro strategy: encapsulating IL-10/OVA simultaneously in PLGA microparticle and morphological analysis

BACKGROUND

Interleukin-10 (IL-10) is produced by various cell types including T and B cells, monocytes, and macrophages *(Moore et al., 2001; Pestka et al., 2004).* This cytokine is highly pleiotropic in its biological activity that includes: inhibition of the synthesis of several cytokines, including IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, tumor necrosis factor (TNF-α), and interferon γ (IFN-γ) (de Waal Malefyt et al., 1991a,b); immunosuppressive effects on monocytes/macrophages *(Bogdan et al., 1991; Fiorentino et al., 1991; Gazzinelli et al., 1992)*; as well as immunostimulatory activity on a broad range of cells types, including T cells (MacNeil et al., 1990), B cells *(Defrance et al., 1992),* and mast cells. Furthermore, IL-10 down-regulates constitutive and IFN-γ or IL-4-induced class II major histocompatibility complex (MHC-II) molecules expression on monocytes, dendritic cells, and Langerhans *cells (de Waal Malefyt et al., 1991a,b; Groux et al., 1998)* as well as adhesion and co-stimulatory molecules on antigen-presenting cells (APCs) *(Willems et al., 1994; Creery et al., 1996)* and, suppresses the release of reactive oxygen intermediates *(Bogdan et al., 1991; Fiorentino et al., 1991).* IL-10 conditioned APC may also promote the differentiation of counter-inflammatory regulatory T cells *(Steinbrink et al., 1997; Steinbrink et al., 2002).* IL-10 has thus a strong anti-inflammatory activity and may act as a general suppressor factor of immune responses. Due to its immunoregulatory properties, this cytokine has been proposed to be used in several clinical applications *(Asadullah et al., 2003).* As already stated, cytokines, like IL-10, have attracted great attention due to their potential application in various medical fields, such as: vaccines *(Berzofsky et al., 2001),* allergies (Pullerits, 2002), infectious diseases *(Hubel et al., 2002),* acute inflammatory diseases *(Asadullah et al., 2003*), etc. Normally, proteins are expensive to produce on a large scale, are easily denatured loosing their bioactivity, and have a quite short half-life in vivo. So, it is essential to develop new delivery systems that allow efficient therapeutic effects at a minimum dosage. A promising method is the encapsulation using polymeric microparticles PLGA , which, by trapping the proteins in a hydrated polymer-network, minimizing denaturation, and enabling slow-release, while maintaining an effective concentration for the necessary period of time *(Murthy et al., 2002, 2003; Leonard et al., 2004; Kim et al., 2009).*

The polymers such as poly (L-lactide) (L.PLA) and co-polymers such as poly (D,L-lactide co-glycolide) (PLG) have been widely used for protein and drug delivery(Lewis,D.H.,Jalil,R., 1990). Their broad range of physico-chemical properties, degradation rates and biocompatibility gives wide scope for controlling protein and drug performance. The encapsulation of proteins and peptides in PLG matrices for vaccine formulation has generally been achieved using double emulsion/solvent evaporation techniques, originally developed by *Vranken and Claeys* and modified by *Ogawa et al*. These involve the formation of a primary emulsion consisting of droplets of polymer solution containing the antigen, which is subsequently mixed with a continuous aqueous phase containing a particle stabilizer/surfactant. This approach counteracts the partition of protein into the aqueous phase and subsequently results in efficient and reproducible encapsulation. One of the main attractions of using PLG microparticles to deliver antigens is the ability to vary the degradation rate from several days to over a year by selecting polymers with a particular lactide-glycolide ratio, molecular weight and crystallinity profile. The potential exists for producing pulse release of antigens by mixing two or more populations of microparticles in a single formulation which degrade at different rates and hence release entrapped antigen at predetermined times. This approach is of interest for replicating the conventional multiple dosing regimes which are generally required for many inactivated and sub-unit vaccines. Long-term, potent immune responses have already been measured after subcutaneous (s.c.) administration of antigen-loaded PLG

microparticles which are generally considered to result from degradation of the carrier in vivo permitting gradual release of entrapped antigens to stimulate immuno-competent cells.

The primary aim of the present study was to measure the capacity of fast and slow resorbing, OVA/IL-10-loaded PLG microparticles .*This work describes for the first time the strategy in simultaneously encapsulating OVA antigen and Il-10 as adjuvant in evaluating in vitro condition and in this study the following points will be addressed: (1) the expression in Hela cells and purification of murine IL-10 form (rIL-10).; (2) the evaluation of rIL-10 biological activity, using lymphocytes), and its comparison with that of a* ***commercially available IL-10 (cIL-10****)(if it is feasible); (3) the incorporation/release of IL-10 into/from the PLGA, and the stability and bioactivity of rIL-10 in the PLGA/rIL-10 complex.* (4) *Adsorption and release kinetics profile of OVA on the surface of il-10 loaded microparticles and blank PLGA microparticles will be addressed*

OBJECTIVES

The objectives of this research were to investigate protein loaded microparticle biodegradable polymer particulate system. The goals were to achieve desired release profiles for microparticles during pore diffusion process, and to improve the protein loading and release profiles with full preserved bioactivity of protein during microparticles preparation.

The specific hypotheses of this project are:

1) Due to the problems of protein release from biodegradable microparticles, we focused our research emphasis on the release profile of protein during pore diffusion stage. Considering the diverse properties of polymers, we would like to find the elemental relationship of microparticle morphology, protein distribution and release profiles. It was postulated that for relatively hydrophobic polymer, influence of morphology and drug distribution on release profiles during pore diffusion process is much pronounced on burst release; by contrast, for hydrophilic polymer this influence is significant at the slow release stage. Hence, to achieve desired release profiles different strategies of morphology and protein distribution modification are required.

2) To further improve the loading capacity and release profiles of protein loaded polymeric microparticles, we assumed that taking advantage of the microparticle surface charge, oppositely charged protein can be absorbed onto microparticle effectively through electrostatic interaction by adsorption process with full preserved bioactivity. Furthermore, with this variation of electrostatic interaction forces between protein and particles higher loading capacity of protein can be achieved on the microparticles with higher surface charge density. Also it is expected with this increase of electrostatic forces desired release profiles are possible to be achieved. For this purpose positively charged microparticles consisting of PLGA was prepared, and loading capacity of protein was investigated.

MATERIALS AND METHODS

Three types of PLGA copolymer (75:25,65:35 and 50:50 lactide:glycolide content) and Poly vinyl alcohol (PVA) were obtained from (S*igma*) and tested for their capacity in loading efficiency as well as in vitro characteristics .A 65% and 50% PLG copolymer containing ,65,50% D,L-lactide and 35%,50% glycolide respectively. (*Sigma*), was selected after evaluated based on specific parameters stated above and for our subsequent experiments we used the 65:35 PLG copolymer (S*igma)* since it degrades more slowly than the 50:50 PLG copolymer due to the increased lactide component. Polyvinyl alcohol (PVA) (Sigma), Ovalbumin , (SDS), muIL-10 ELISA were obtained from (*Sigma*) and Bicinchoninic acid reagent (BCA) (*Thermoscientific*). All other reagents obtained from sigma unless and otherwise stated.

Cloning and purification of hu IL-10 and mu IL-10

Aamplified fragment, IL-10 was gel purified using high pure PCR product purification kit (*Qiagen*), accordingn to manufacture protocol. The Product was then double digested with Nhe I and BssH I restriction enzymes (*NEB*) and then ligated into the NheI/BssH I doubke digested pHYGRO vector (provided from Prof.Sblattero.) using T4 DNA igase (*invitrogen*) . the ligation reaction was transformed in E.coli TOP10 competant cells and dispensed on LB (Luria-Bertani: LB, Agar(agar plate containing 50mg/ml Kanamycin. The colonies were screened by PCR and the colonies conaining the recombinat plasmid were selected and, then digested by Asc I and BsiW I restriction enzyme, the ligation mixture was transformed in E.coli TOP 10 competant cells and dispersed on LB containing ampicillin. The relased DNA band was purified and subcloned in AscI/BsiW I digested pUCOE expresstion vector. The reaction was transformed and bacterial colonies contanining the plasmid were screened, follwed by Hela cels transfection by Lipofactamine (*Invitrogen*) and controlled by fluorescence microscope and Harvesting of the transfected cells took place after 48 hours. 1 ml of supernatant has been collected after 24 and 48 hr, centrifuged at 1500rpm for 5 min and supernatant has been collected and SDS-PAGE, 100% acrylamide and western blot was performed for protein production analysis.

The postive clones were screened in a 6 well plate and stably transfcetd with pUCOE-Hu Il-10 and pUCOE-mu IL-10 using lipofatamine. 16 hr posttransfection, the cells were washed, trypsinized and split at a ratio of 1 in 10, into selective DMEM media containing 100µg/ml of Hygromycin B. the diluted cells were seeded onto a new 6 well plate and allowed to grow in selective media for 2-3 weeks. The selective media were refreshed every 3 days, until Hygromycin resistant can be identified. The selected clones were screened by exposing the cells to DMEM no FBS to facillitate selection process. The postive clones were transferred to a 24 well plate for maximum proliferation. 22 and 26 conolies for Mu and hu il-10 were selected and screened for by specific ELISA (according to manufacturer protocol) and three best clones for each were selected and vials of each clones was saved and kept under -80oC for future use.

Then, the collected supernatant was centrifuged 15000 rpm for 5 min and the bacteria cell pellet was removed and clear supernanat was collected and dissolved in a binding buffer (10 mM imidazole, 1.5mM NaCl, 1MNaH2PO4) (*Qiagen*). Recombinant IL- 10 (rIL-10) was purified from the cell-free supernatant by chromatography on a Ni2+-NTA agarose column. After washing the column with binding buffer, IL-10 was eluted with (250 mM imidazole, 50mMNaH2PO4, 150mM NaCl). Fraction containing rIL-10 was dialyzed against a PBS buffer pH 7.5 and was analyzed by SDS-PAGE electrophoresis and western blot.

Microparticle formulation

Il-10 loaded or blank microparticles prepared by a modified double emulsion solvent evaporation method. Briefly, 60mg of PLGA powder was dissolved in 1ml of dichloromethane (DCM) and emulsified in a homogenizer () at high speed for 1 min. the primary emulsion was added to 1ml of distilled water containing 50µl of polyvinyl alcohol (PVA 1%) and emulsion was stirred for 2 min. the formed water emulsion was stirred for overnight under fume hood, allowing DCM to evaporate. The emulsion solidified gradually as the diffusion of the solvent from the emulsion droplets into the external phase. The resulting microparticles were washed 5X in distilled water by centrifugation 13,000rpm and resuspending in PBS,pH ,7.4 or water depending on the experimental aim and stored at 4oC.

Blank microspheres (IL-10-free) were produced by emulsifying the respective polymer solution with a PVA surfactant solution and proceeding as described above.

PLGA microparticle/OVA mixtures*:* *PLGA/OVA* formulations were prepared by simple mixing of IL-10 loaded PLGA micro particles or blank PLGA microparticles (5-10mg/ml) and OVA solutions. Blank PLG microparticles (5-10 mg) were suspended in a 0.4% w/v solution of OVA in PBS to yield a 0.04-0.08mg/ml particle suspension and incubated at 37oC on a shaker for 24 h. Sample tubes were rotated end over- end with a mixer. An aliquot of the microparticle suspension was separated and the microparticles were isolated from the suspension medium by centrifugation (10,000 rpm, 1min). Samples of the supernatants were analyzed in duplicate by a bicinchoninic (BCA) protein assay (Thermoscientific) to provide an estimate of the unbound OVA fraction and the amount of OVA adsorbed to the microparticles, respectively.

Particle size measurement

Particle size measurements were performed using Malvern spectrometer (Malvern Instrument) on microparticle suspensions at 25oc±0.05 oC and each sample was analyzed a total of ten times to give an average value calculated for the particle diameter.

Scanning electron microscospy (SEM) ******

Measurements of microparticle size and information on shape and surface morphology will be obtained by Scanning electron Microscope (SEM). Cleaned microparticle suspensions will dropped onto aluminum stubs and allowed to air dry. Specimens will be sputter coated with gold prior to examination in the SEM (Hitachi Model).

PLGA OVA surface adsorption efficiency

OVA protein was diluted in PBS; pH7.4 at a concentration of 0.4mg/ml. PLGA was diluted at a concentration of 5mg/ml in PBS, pH 7.4 and 1volume of PLGA suspension was mixed with 1 volume of OVA solution. The mixture were briefly vortexed and reactions were left to occur at 37oC with moderate stirring end over end for 24 hr and to recover the unbound protein, aliquots were centrifuged 10min at 10000rpm. The supernatants were removed and centrifuged once more at 10,000 for 1min to get rid of all traces of microparticle. Unbound protein in each aliquot was quantified using BCA assay according to instruction of the manufacturer. Triplicate samples were analyzed in duplicate.

Analysis of surface OVA

The amount of OVA associated with the surface of IL-loaded microparticles or blank microparticles mixed with OVA solution was estimated by Treatment with 2% SDS. The left over samples were lyophilized and dried and 500 µl of 2% (w/v) SDS solution and agitated for 4 h using a shaker. The samples were centrifuged (10,000rpm, 1min) and the supernatant was analyzed for OVA by a BCA assay. At least three samples of microparticles were assayed for each formulation.

OVA adsorption to PLGA

For monitoring of adsorption kinetics, OVA protein was diluted in PBS, pH7.4 at a concentration of 0.4mg/ml. PLGA was diluted at a concentration of 5mg/ml in PBS, pH 7.4 and 500 µl was added to 500µl of protein solution. The mixture were briefly vortexed and 0.1 ml aliquots was removed immediately (t=0).

The adsorption reactions were left to occur at 37oC with moderate stirring end over end. At predetermined time intervals (30, 60, 90,120,150,180,210,240 and 270 min); 0.4 ml aliquots of microparticle suspension were taken. . To recover the unbound protein, aliquots were centrifuged 10min at 10000rpm. The supernatants were removed and centrifuged once more at 10,000 for 1min to get rid of all traces of microparticle. Unbound protein in each aliquot was quantified using BCA assay according to instruction of the manufacturer.

For adsorption isotherms, protein was diluted separately in PBS at different concentration (0.05-0.4mg/ml). 1volume of a 5mg/ml PLGA microparticle was added to 1vol of protein solution.

Reaction was left to occur at room temperature, with moderate stirring. Unbound protein collected and quantified as explained above.

Loading efficiency of the formulation (IL-10/PLGA) *******
The IL-10 content of microparticles will be determined according to the methods described by O'Hagan et *a1.(19)* Approximately 3.0 mg/ml microparticles solutions was dispersed in 0.5ml of 0.1 M NaOH containing 5% (w/v) SDS. The samples were agitated for 24 h on a shaker, centrifuged and the clear supernatant was collected and will be analyzed for IL-10 by muIL-10 ELISA. Each sample will be assayed in duplicate. Qualitative data will be obtained by SDS-PAGE and Commassie.

In vitro release rates of OVA/IL-10

The short-term, in vitro protein release rates from:

*(1) Il-10-loaded PLGA microparticles****;*****

(2) Microparticle/OVA mixtures; and

(3) OVA adsorbed to IL-10 loaded PLGA microparticles were measured to provide background data for analysis of the results of in vivo studies ****** will be or was determined.***

Il-10-loaded microparticles were incubated in release medium (PBS, pH 7.4, 37°C). Sample tubes contained a 3 mg of microparticles, accurately dispersed in 1 ml of release medium. Samples were retained in a water-bath or incubator and shaken intermittently to retain the microparticles in suspension. After 24 h, 48 hr and at intervals of 2 days up to 3 weeks or 1 month, the release medium was separated from the microparticles by centrifugation (2200g). Fresh medium was added to the microparticles and the release study was continued. Samples of release medium were stored at -20°C prior to testing in duplicate by a BCA assay. In *vitro* release studies were also performed with a mixture of blank PLG microparticles in OVA solution (5 mg microparticles, 0.4 mg OVA, 1.0 ml PBS) and separately a suspension of IL-10-loaded PLGA and OVA solution (5 mg PLGA/IL-10, 0.4 mg OVA in 1 ml PBS release medium) ,which will be carried out in order to see the release kinetics character and investigate whether the surface adsorbed protein has any effect on the release of the encapsulated protein.

Protein release profiles were or will be generated for:

(1) IL-10-loaded 65:35 PLGA;***
(2) OVA adsorbed to PLGA; and
(3) 65:35 PLGA microparticle mixtures in OVA solution.
Protein release profiles were generated for each microparticle system in terms of cumulative protein release (% w/w) vs. time.
Structural integrity of Il-10***
The integrity of IL-10 will be determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The released samples from the microspheres will be also lyophilized and reconstituted in PBS. The IL-10 samples, native IL-10, and a molecular weight reference marker (--) will be mixed with sample dilution buffer ( ), boiled for 2 min prior to electrophoresis, and then separated by SDS-PAGE in a 1.5 mm thick 4% stacking gel and 10% resolving gel. The electrophoresis will be performed at 200 V. The bands will be controlled by Coomassie blue staining.
Functional study ***
Bioassay of IL-10***

IL-10 bioactivity will be assayed by its ability to inhibit the production of TNF- in lipopolysacharide (LPS) and IFN-γ activated macrophages. Recombinant (rIL-10) in concentrations ranging from 0.1 ng/ml to 250.0 ng/ml will be added to the PBMC or macrophages, and the cells incubated at 37 oC in a 5% CO2 atmosphere for 1 h. Then, 0.1 ng/ml LPS and 1.0 ng/ml IFN-γ will be added to the cells to promote macrophage activation, and incubated for 24 h. As a positive control of macrophage activation, cells stimulated with LPS and IFN-γ, without IL-10, will be used; as a negative control, cells cultured in RPMI alone will be used. After incubation, culture supernatants will removed and stored at −20 oC until TNF-quantification.

- **Key ***: Ongoing investigation**

RESULTS

Characteristics of microparticles

PLGA Microparticle Characterization

The exhibition of the morphological characteristics of the microparticles, utilized in this work will be determined by Scanning Electron Microscope (SEM), this work will be completed in mid of Oct in Alessandria. The microparticles had a mean diameter of 591.7nm **(Fig -1),** varying in the range of 591-625nm; and the polydispersity index (PI) was 0.25, indicating a relatively homogenous size distribution and a zeta potential of −15±2.12 mV, which confirms the stability of the microparticles with protein.

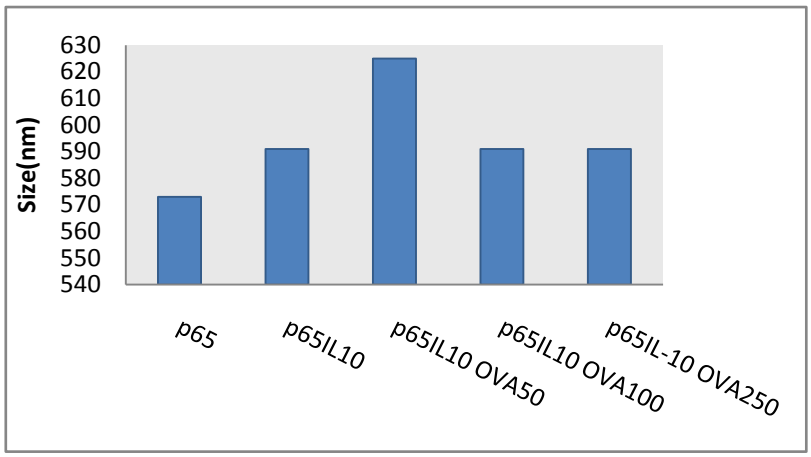

Fig-1 *Microparticle size by Malvern Instrument for blank PLGA 65:35, PLGA65:35/IL-10, PLGA65:35/IL-10/OVA (at different concentration 50-250µg/ml)*

OVA loading and surface analysis

The OVA/PLGA mixture resulted in high protein loading figures for both 50:50and 65:35 PLGA microparticles, lying in a fairly narrow range between 82.35% and 82.40% (w/w) respectively ***(Fig-2).*** Analysis of surface protein, however, revealed that a substantial part of the OVA content was associated with the surface of the microparticles. In contrast, analysis of the supernatant after separation of microparticles from the PBS release medium at 24 h, resulted in detection of only 17.64% and 17.59% OVA for 50:50 PLG and 65:25 PLG, respectively (***Fig-2).*** Thus, only loosely bound surface protein is removed into the PBS release medium and a large component remains strongly bound to the microparticle surface over a 24 hr period. The surface analysis finding showed that only up to 1-2% of OVA extracted from the beads and this further confirms that the rest of the OVA were still associated with PLG surface with a strong interaction.

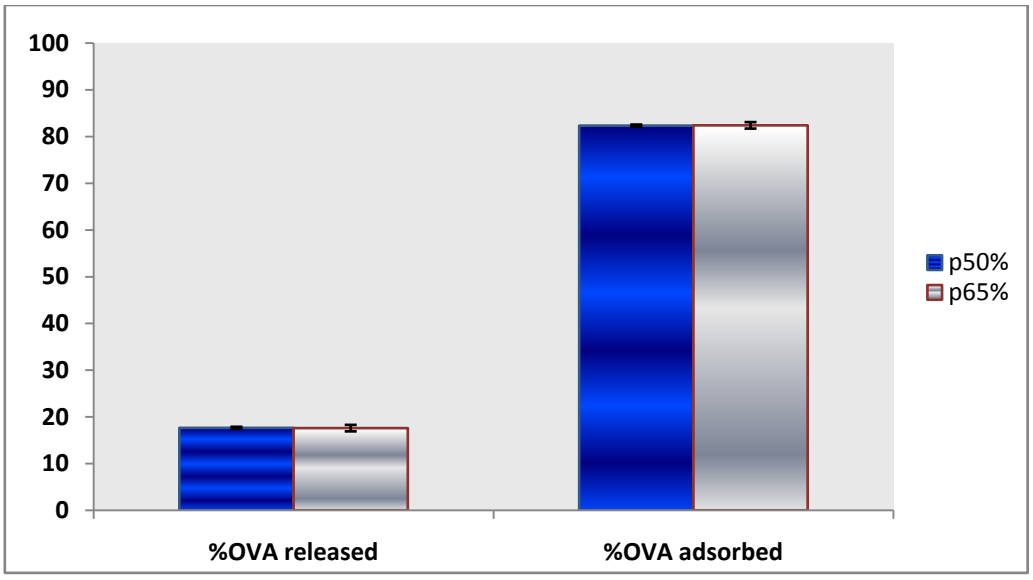

Fig-2: Surface OVA loading efficiency

OVA kinetics release

In vitro protein release of OVA for 0-16 hr was similar with the typical triphasic or biphasic release kinetics **(Fig-3).** A characteristic "burst release" of OVA occurs over the first 24 h, to a loss of about 30% of the OVA initially associated with the particles. This initial phase has been well-documented and is generally accepted as arising from release of surface protein. Following the burst phase, loss of protein stabilizes after 16 hr at a level around 4%, and is minimal from after a week. The same kind of study was conducted for extended period of time, 2 weeks and the result was consistent and further highlights or revealed that for a sustained release of OVA antigen for a few days, at least 300µg or more should be loaded (Fig-4).

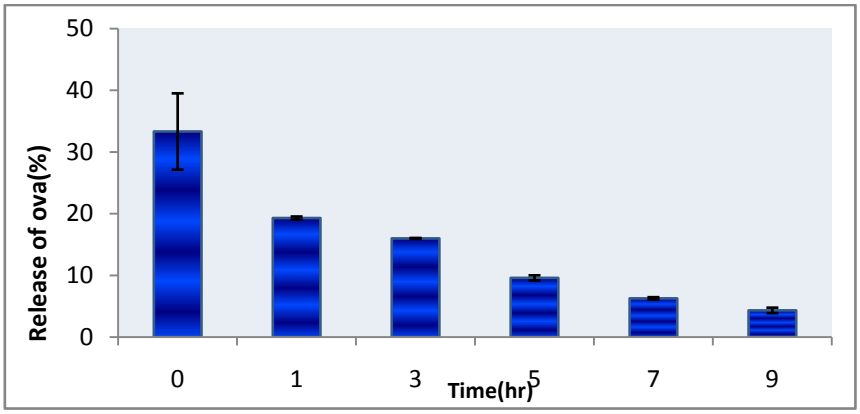

Fig-3: OVA (400µg/ml in 5mg/ml microparticle suspension) kinetic release from PLGA65:35 surface.

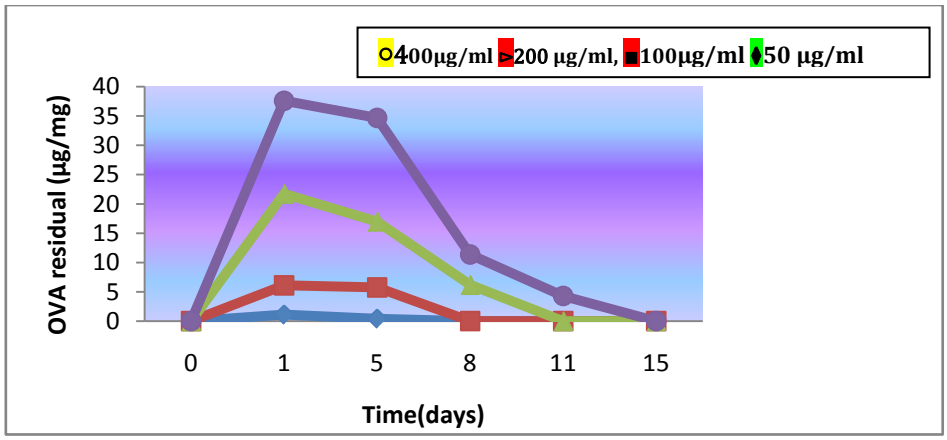

Fig-4: Time course of OVA kinetic release under various concentrations (0, 50,100,200,400µg/ml OVA).

Adsorption of OVA to Microspheres

The amount of OVA adsorbed on the PLGA substrate after incubation with OVA solution was significantly higher in using blank PLG microparticles. Complete adsorption of the available OVA had occurred giving rise to an association figure of 0.01-0.05% w/w. The cumulative protein release curve presented in Fig-5 gives an indication of the strength of association of OVA with the PLGA substrate. Gradual, almost linear release of adsorbed protein occurred over 60min in vitro. The release rate of OVA then tended to level off, resulting in just over 80% retention of the original protein load..

One of the main objectives of our study was to show that the OVA protein can be adsorbed on to PLGA micropartcile. Our result showed that the OVA adsorption was fast; at a protein to particle ratio of 0.08µg/mg, more than 80% of OVA was coated onto PLGA microparticle with in 60 min. afterwards a plateau was reached in both cases indicating the saturation of particle surface. Based on these observations, I fixed at 60min the duration of the adsorption reaction for subsequent experiments.

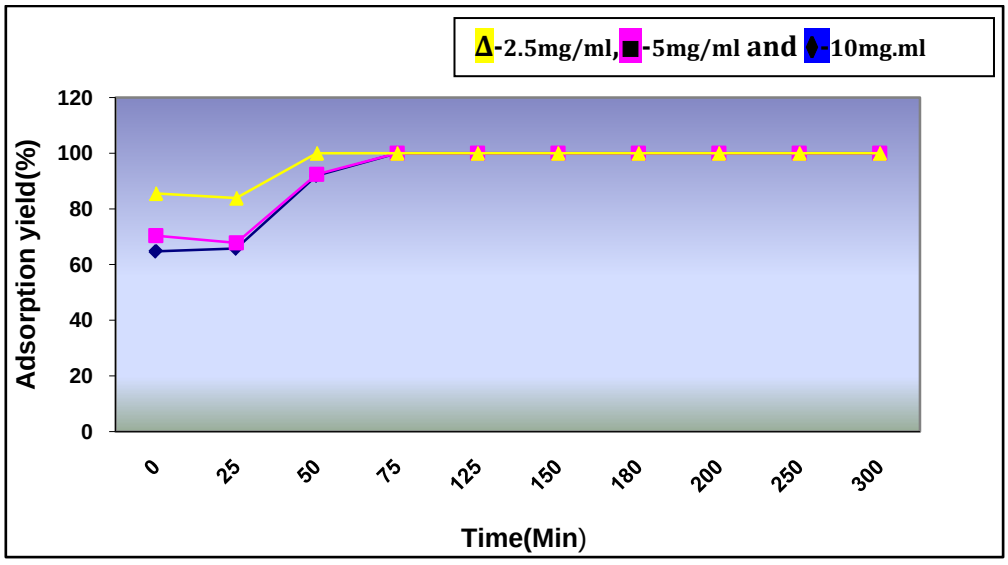

Fig-5: Time course of OVA adsorption to PLGA at various microparticle concentrations (10, 2.5 and 5mg/ml). Microparticles were suspended in a 0.4mg/ml OVA solution in 0.1M PBS buffer, pH 7.4).

OVA Adsorption Kinetics and Isotherms

Adsorption isotherm of OVA is reported on fig-6. For OVA protein, the initial slope of the curve was nearly vertical. This shows the affinities of the OVA protein to PLGA microparticles. In PBS buffer, complete saturation of PLGA particles with OVA correspond to coverage of 50-100 µg/ml. In contrast, no saturation of microparticle surface was observed with –protein, in the input concentration range tested here. Recently, *chesko et al*. has reported that protein binding data onto anionic PLGA/DDS microparticles could be fitted with the Langmuir model, supporting the description that a monolayer of adsorbed protein is formed. For our PLGA microparticle, the adsorption processes was of Langmuir type after evaluating the three consecutive time points and were averaged to obtain the data plotted. Given the initial high affinities of the protein for the microparticles, this result could be expected.

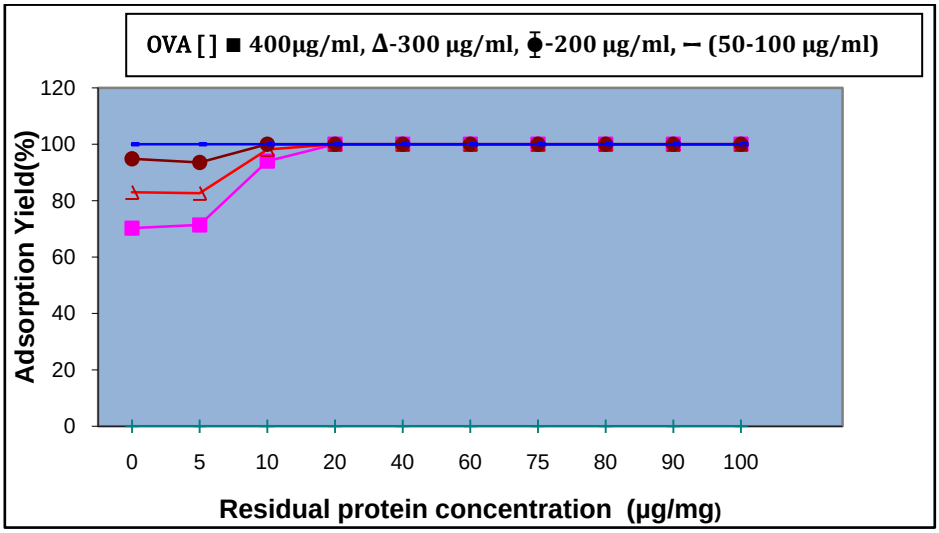

Fig-6: Adsorption kinetics of OVA onto 5mg PLGA microparticles (OVA range: 0-0.4mg/ml in PBS buffer, pH, 7.4.)

DISCUSSION

The adsorption of OVA to microspheres at various microsphere concentrations is presented in Figure -4. The kinetic profiles can be divided into three regions, the *pre-lag phase*, the *lag phase* and the *phase of rapid adsorption*. In these three regions, different phenomena may be occurring. In the pre-lag phase adsorption of about 30-70 µg/mg OVA occurred, presumably forming a monolayer. In the lag phase an alteration of either the polymer surface or the protein in bulk or the adsorbed protein occurred which resulted in the phase of rapid protein adsorption. The lag phase time was similar with among the samples and it appears to be independent on polymer surface.

Following the lag phase, protein adsorption was rapid and proceeded at a rate which appears to be independent of the polymer mass used for adsorption. The adsorption ceased only upon exhaustion of the protein in solution. The samples with 10 mg, 5mg and 2.5mg microspheres reached maximum adsorption in 60min in our case. Others reported that a lower concentration of microparticle might take a longer time to reach at equilibrium because of the surface area difference, but not always necessarily. More than 80% of the added OVA was depleted from the solutions at the end of maximum adsorption time irrespective of the microsphere concentration. This indicates that the continuous depletion of OVA by PLGA microsphere surface appears to be a

result of initial adsorption of the protein to the polymer surface with subsequent interaction of the peptide in solution with the already adsorbed protein. It is speculated that the integrity of the multilayer may be maintained by the hydrophobic interaction between protein molecules. Samples of microspheres with adsorbed OVA showed two distinct sedimentation layers after centrifugation; the lower layer was opaque and dense while the upper layer was translucent and loosely packed.

Figure 5 shows the removal of OVA from solution over a shorter time period using six OVA concentrations (0-0.4 mg/ml) in 0.1 M, pH 7.4, phosphate buffer. At all concentrations there was an initial uptake of 25- to 114 µg/ml OVA during the first 30 minutes. Adsorption continued after a short lag phase and equilibrium was attained between 60 and 90 minutes. At lower concentrations, 50 and 100 µg/ml, the equilibrium appeared to be a true equilibrium, however, at higher concentrations, the equilibrium was transient and adsorption continued after the equilibrium and shows incomplete release of the surface protein. The time at transient equilibrium decreased with increasing concentration of OVA. The second phase of adsorption at higher OVA concentrations can be attributed to formation of multiple layers of peptides on the surface of the polymeric microspheres. It appears that after the transient equilibrium changes occur in the bulk solution and at the polymer surface. In the solution protein-protein interaction may be occurring while at the polymer surface there is protein reorientation.

These processes may lead to further interaction at the surface. When this occurs there is no longer equilibrium but probably precipitation at the surface. The odsorption which results in a transient equilibrium during 60 to 90 minutes was used to construct the adsorption isotherms. The amount of OVA adsorbed from three consecutive time points in the equilibrium region were averaged to obtain the data plotted. It appears that the adsorption followed two different models depending on the concentration of the protein in solution. At lower concentrations, equilibrium was attained but at higher concentrations the adsorption continued beyond the monolayer formation. The first three concentration points in the isotherm were evaluated by the Langmuir equation using native and linearized model and the results were plotted. The data show a good correlation to the Langmuir model with a correlation coefficient 0.997 from the linear fit indicating formation of a monolayer. At higher protein concentrations the hydrophobic interaction between the peptide molecules may be stronger resulting in self-association of proteins. Adsorption may have occurred when self-association was complete. Then, the self-associated molecules adsorbed onto the polymeric surface forming mono layers.

The adsorption isotherms suggest these predictions: In dilute solutions, peptide-polymer interaction favored monolayer adsorption which fitted the Langmuir model as all the adsorption sites on the polymer surface are expected to be energetically equivalent. At higher peptide concentrations protein-protein interactions are favored ultimately resulting in monolayer adsorption which fitted the Langmuir model. The results suggest that adsorption occurred as a result of three phenomena: (1) protein-polymer interaction—adsorption of protein onto the polymeric surface. (2) protein-protein interaction on the polymer surface, in which adsorption of protein onto polymer was followed by adsorption onto previously adsorbed protein layer; this resulted in formation of monolayer as observed in the continuous depletion of OVA from the adsorption medium. (3) Adsorption onto polymer at high protein concentrations wherein self association of protein molecules (protein-protein interaction) preceded adsorption onto the polymer resulting in multilayer formation. This was evident from the delayed adsorption of OVA in the kinetic studies. Controlling the desorption over a favorable time should enhance the potential for applying the phenomenon of adsorption for controlled delivery of peptides.

Adjuvants may act in a number of ways to improve the immune response for example by protecting antigens, stimulating phagocytosis, activating lymphoid cells and by retaining the antigen at the site of ‘deposition *(Khan, M.Z.I,et.al,1994).* Antigen retention appears vital for repeated stimulation of the memory B-cell population and for maintaining antibody titers over long periods *(Gray.et.al.1988).* The adjuvant effect of water-in-oil emulsions (FCA/FIA), for example, is considered to arise from the creation of a short-term “depot effect”. In contrast the considerable research effort on microparticle-based vaccines has generated a number of strategies based on optimizing antigen release rates to produce single dose delivery systems. For example, pulse release of antigen from biodegradable microparticles is considered advantageous for simulating the conventional, multi-dose vaccine delivery regime. However, most microparticulate delivery systems are considered to function on the principles of efficient phagocytosis and transport to the lymph nodes and sustained antigen release over extended time periods which may present a continuous trickle of antigen to the immune system

Measurements of the amount of OVA associated with the surface of OVA-adsorbed to PLGA microparticles produced by mixing the two solutions together revealed high levels of surface protein amounting to ca 81% and 82% for the 50:50 PLG and 65:35 PLGA systems, respectively (Fig-2)**.** Thus, while an initial release of loosely bound surface protein occurs in vitro (Fig3-) a surface layer of strongly bound protein remains at the microparticle surface for at least 1 weeks depending on the amount of OVA loaded on the surface. *Verecchia et al* (23) reported similar phenomena for PLA nanoparticles (150 nm) prepared using an emulsion/microfluidization/solvent evaporation technique with human serum albumin as a stabilizer. Adsorption/desorption studies suggested a multilayer model of protein association and the presence of an irreversibly bound albumin fraction accounting for 35-45% of the originally associated protein. Verecchia et al.” considered that during nanoparticle production, the association of albumin with the particle surface probably occurred through an intermediate state involving accumulation then denaturation at the solvent/water interface.

The presence of strongly bound albumin at the surface of microparticles invites a reassessment of the current models of protein release from PLG microparticles and has major implications for vaccine design, formulation and performance. In the present case a change of concept is clearly called for from release of entrapped antigens alone to one which encompasses loss of surface antigen with greater emphasis placed on the role of substrate stability and degradation in controlling the presentation and depletion of surface antigen and consequently the duration of the immune response. While exposure and release of encapsulated antigen from PLG microparticles could contribute to long term immunity, the possibility of the loss of immunogenicity, due to the generation of acid products as the PLGA matrix degrades *(Park,T.G, et.al., 1995),* does render the general applicability of this mechanism for producing long-term immune responses highly questionable.

In the case of vaccine formulations prepared by simple mixing of blank pre-formed microparticles and OVA solutions, protein interaction with the substrate would be expected to differ from that existing in the solvent evaporation process. Protein adsorption and desorption from particulate substrates is governed by a complex interaction involving polar forces e.g. hydrogen bonding, hydrophobic interactions via the hydrophobic regions of protein molecules and weaker van der Waals forces and is influenced by the ionic strength and composition of the incubation medium *(Anick,S.T., et.al.,1983*). Maximum albumin adsorption is generally observed on hydrophobic surfaces. The binding of multiple charged proteins to terminal carboxylic acid end groups of PLG is considered to impede release of encapsulated protein from PLG microparticles *(Sah, H., 1994)* and is also applicable to the case of protein desorption from microparticle surfaces. Of particular relevance to the study reported here is the finding that OVA can be adsorbed to PLG microparticles

strongly and aim to investigate whether the strong adsorption of OVA has any effect or impede the release of the encapsulated IL-10 protein.

It has frequently been suggested that the higher immune response obtained when using OVA-loaded PLG microparticles could be attributed to an adjuvant (antigen retention/ presentation) effect rather than to slow release of encapsulated protein since antigens adsorbed onto microparticles have been shown to generate potent immune responses after s.c. *(, O'Hagen, D.T.et.al., 1993, Kreuter, J., et.al, 1988*) and nasal administration *(Alpar, S.T., 1994).* Conflicting reports do exist, however. In particular, *Eldridge et a1,* found that Staphylococcal enterotoxin B (SEB) mixed with blank PLG microparticles did not induce greater antibody responses than immunization with SEB alone. More recently, *Uchida et al* reported the absence of an immune response on administration of a mixture of OVA and blank PLG microparticles, although no details of dose or dosing regimen were provided. In contrast, *O'Hagan et al*,measured similar serum IgG antibody responses over 6 weeks following boosting, when 100 µg of OVA was either encapsulated in PLG microparticles or adsorbed to blank PLG microparticles.

A finding that PLG microparticles can confer greater immunogenicity on adsorbed labile antigens after a single dose would certainly be welcomed since harsh encapsulation procedures involving high shear rates and exposure to organic solvents (factors which may denature some antigens) would be avoided. Concern also exists over the acid degradation products generated by PLG microparticles which could denature some encapsulated proteins. Antigen adsorption would also provide a means of avoiding loss of antigenicity. Uncertainties exist, however, over protection and desorption of adsorbed antigens in vivo.

The presentation of strongly bound OVA (i.e. that remaining after burst release) at the surface of OVA-loaded microparticles can be expected to exert a major influence on the primary immune response. In the present study, 300-400µg of OVA, respectively, are estimated to lie at the surface of PLGA 65% microparticles produced by simply mixing the solutions together , can provide a sustained release of portion of the antigen for up to two weeks . Therefore, encapsulating antigen on the surface of PLGA microparticle provides as "depot –type" systems for sustained retention and presentation of antigen to the immune system is a very promising strategy.

REFERENCES

1. Aguado, M.T. and Lambert, P.H. Controlled release vaccinesbiodegradable polylactide/polyglycolide (PUPG) microspheres as antigen vehicles. lmmunobiology 1992, 184, 113-125.
2. Alonso, M.J., Cohen, S., Park, T.J. and Langer, R.S. Determinants of release rate of tetanus vaccine from polyester microspheres. Pharm. Res. 1993, 10, 945-953 .
3. Alpar, H.O. and Almeida, A.J. Identification of some of the physico-chemical characteristics of microspheres which influence the induction of the immune response following mucosal delivery. Eur. J. fharm. Biopharm. 1994, 40,198-202 .
4. Anick, S.T. and Hwang, J.Y. Adsorption of D. Nal (2)6, LHRH a decapeptide onto glass and other surfaces. Int J. Pharm. 1983, 16, 181-190
5. Asadullah, K., Sterry, W., Volk, H.D., 2003. Interleukin-10 therapy—review of a new approach. Pharmacol. Rev. 55, 241–269.
6. Ball, C., Vigues, S., Gee, C.K., Poole, S., Bristow, A.F., 2001. Rat interleukin-10: production and characterisation of biologically active protein in a recombinant bacterial expression system. Eur. Cytokine Netw. 12, 187–193.
7. Berzofsky, J.A., Ahlers, J.D., Belyakov, I.M., 2001. Strategies for designing and optimizing new generation vaccines. Nat. Rev. Immunol. 1, 209–219.

8. Bogdan, C., Vodovotz, Y., Nathan, C., 1991. Macrophage deactivation by interleukin -10. J. Exp. Med. 174, 1549–1555.
9. Bondoc Jr., L.L., Varnerin, J.P., Tang, J.C., 1997. Resolution of recombinant human interleukin 10 from variants by recycling free flow focusing. Anal. Biochem. 246, 234–238.
10. Challacombe, S.J., Rahman, D., Jeffery, H., Davis, S.S. and O'Hagan, D.T. Enhanced secretory IgA and systemic IgG antibody responses after oral immunisation with biodegradable microparticles containing antigen. fmmunotogy 1992, 76, 164-l 68.
11. Chichester, 1992, Chap. 7 O'Hagan, D.T., Jeffery, H. and Davis, S.S. The preparation and characterisation of poly(lactide co-glycolide) microparticles.
12. Cohen, S., Bernstein, C., Hewes, C., Chow, M. and Langer, R. The pharmacokinetics of and humoral response to antigen delivered by microencapsulated liposomes. Proc. Nat/. Acad, Sci. USA 1991, 68, 10440-10444.
13. Coombes, A.G.A. and Meikle, MC. Resorbable synthetic polymers as replacements for bone graft. C/in. Mater. 1994, 17, 35-67.
14. Creery, W.D., Diaz-Mitoma, F., Filion, L., Kumar, A., 1996. Differential modulation of B7-1 and B7-2 isoform expression on human monocytes by cytokines which influence the development of T helper cell phenotype. Eur. J. Immunol. 26, 1273–1277.
15. de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., de Vries, J.E., 1991a. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174, 1209–1220.
16. de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M.G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H., de Vries, J.E., 1991b. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J. Exp. Med. 174, 915–924.
17. Defrance, T., Vanbervliet, B., Briere, F., Durand, I., Rousset, F., Banchereau, J., 1992. Interleukin 10 and transforming growth factor beta cooperate to induce anti- CD40-activated naive human B cells to secrete immunoglobulin A. J. Exp. Med. 175, 671–682.
18. Eldridge, J.H., Staas, K., Meulbroek, J.A., McGhee, R., Tice, T.R. and Gilley, R.M. Biodegradable microspheres as a vaccine delivery system. Molec. Immun. 1991, 28,287-294.
19. Eldridge, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.R. and Gilley, R.M. Biodegradable and biocompatible poly(dl lactide co-glycolide) microspheres as an adjuvant for Staphylococcal Enterotoxin B toxoid which enhances the level of toxinneutralising antibodies. Infect. Immun. 1991, 59, 2978-2986.
20. Espartza, I. and Kissel, T. Parameters affecting the immunogenicity of microencapsulated tetanus toxoid. Vaccine 1992,10, 7 14-720..
21. Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., O'Garra, A., 1991. IL- 10 inhibits cytokine production by activated macrophages. J. Immunol. 147, 3815–3822.
22. Gazzinelli, R.T., Oswald, I.P., James, S.L., Sher, A., 1992. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. J. Immunol. 148, 1792–1796.
23. Gray, D. and Skarvall, H. B-cell memory is short lived in the absence of antigen. Nature 1988, 336, 70.
24. Groux, H., Bigler, M., de Vries, J.E., Roncarolo, M.G., 1998. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. J. Immunol. 160, 3188–3193.
25. Hoppen, H.J., Leenslag, J.W., Pennings, A.J., Van der Lei, B. and Robinson, P.H. Two-ply biodegradable nerve guide: basic aspects of design, construction and biological performance. Biomaferials 1990, 11, 286-290.
26. Hubel, K., Dale, D.C., Liles, W.C., 2002. Therapeutic use of cytokines to modulate phagocyte function for the treatment of infectious diseases: current status of granulocyte colony-stimulating factor, granulocyte-macrophage colonystimulating factor, macrophage colony-stimulating factor, and interferon. J. Infect. Dis. 185, 1490–1501.

27. Hunter, R., Olsen, M. and Buynitzky, S. Adjuvant activity of non-ionic block copolymers.lV Effect of molecular weight and formulation on titre and isotype of antibody. Vaccine 1991, 9, 250-256.
28. Jalil, R. and Nixon, J.R. Biodegradable poly(lactic acid) and poly(lactide co-glycolide) microcapsules: problems associated with preparative techniques and release prpperties. J.microencaps.1990,7,297-325.
29. Khan, M.Z.I., Optebeeck, J.P. and Tucker, I.G. Immunopotentiation and delivery systems for antigens for single-step immunisation. Recent Trends Proor. fharm. Res. 1994. 11. I 2-11.
30. Kreuter, J., Liehl, E., Berg, U., Soliva, M. and Speiser, P.P. Influence of hydrophobicity on the adjuvant effect of particulate polymeric adjuvants. Vaccine 1988, 6, 253-256.
31. Kim, S., Kim, J.H., Jeon, O., Kwon, I.C., Park, K., 2009. Engineered polymers for advanced drug delivery. Eur. J. Pharm. Biopharm. 71, 420–430.
32. Langer, R. Polymers for the sustained release of macromolecules. Their use in a single step method of immunisation. Meth. Enzym. 1981, 73, 57-75.
33. Leonard, M., De Boisseson, M.R., Hubert, P., Dalencon, F., Dellacherie, E., 2004. Hydrophobically modified alginate hydrogels as protein carriers with specific controlled release properties. J. Control Release 98, 395–405.
34. Lewis, D.H. Con&o//ed Release of Bioactive Agents from Lactide/g/yco/ide Polymers (Eds Chasin, H. and Langer R.). Marcel Dekker, New York, 1990, pp. 1-41.
35. MacNeil, I.A., Suda, T., Moore, K.W., Mosmann, T.R., Zlotnik, A., 1990. IL-10, a novel growth cofactor for mature and immature T cells. J. Immunol. 145, 4167–4173.
36. Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19, 683–765.
37. Murthy, N., Thng, Y.X., Schuck, S., Xu, M.C., Frechet, J.M., 2002. A novel strategy for encapsulation and release of proteins: hydrogels and microgels with acid-labile acetal cross-linkers. J. Am. Chem. Soc. 124, 12398–12399.
38. Murthy, N., Xu, M., Schuck, S., Kunisawa, J., Shastri, N., Frechet, J.M., 2003. A macromolecular delivery vehicle for protein-based vaccines: acid-degradable protein-loaded microgels. Proc. Natl. Acad. Sci. U.S.A. 100, 4995–5000.
39. Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T. And Shimamoto, T. A new technique to efficiently entrap leuprolide acetate into microcapsules of copolylactic/glycolic acid. Chem.
40. O'Hagan, D.T., Jeffery, l-l. and Davis, S.S. Long term antibody responses in mice following subcutaneous immunisation with ovalbumin entrapped in biodegradable microparticles. Vaccine 1993,11,965-969.
41. O'Hagan, D.T., Jeffery, H. and Davis, S.S. The preparation and characterisation of poly(lactide co-glycolide) microparticles. 3. Microparticlelpolymer degradation rates and the in vitro release of a model protein. Int. J. fharm. 1994, 103, 37-45.
42. Park, T.G., Lu, W.L. and Crotts, G. Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein encapsulated poly(DL lactic acid co-glycolic acid) microspheres. J. Confr. Rel. 1995, 33, 211-222.
43. . Pestka, S., Krause, C.D., Sarkar, D., Walter, M.R., Shi, Y., Fisher, P.B., 2004. Interleukin- 10 and related cytokines and receptors. Annu. Rev. Immunol. 22, 929–979.
44. Raghuvanshi, R.S., Singh, M. and Talwar, G.P. Biodegradable delivery system for single step immunisatuion with tetanus toxoid. Int. J. Pharm. 1993, 93, Rl-R5.
45. Reul, G.J. Use of vicryl (polyglactin 910) sutures in general surgical and cardiothoracic procedures. Am. J. Surg. 1977, 134, 297.
46. Russell-Jones, G.J. and Jeffery, H. In-vitro characterisation of biodegradable microparticles fdr oral antigen delivery. Proc. Int.Svmo. Contr. Rel. Bioact. Mater. 1994. 21. 873-874.
47. Steinbrink, K., Wolfl, M., Jonuleit, H., Knop, J., Enk, A.H., 1997. Induction of tolerance by IL-10-treated dendritic cells. J. Immunol. 159, 4772–4780.

48. Steinbrink, K., Graulich, E., Kubsch, S., Knop, J., Enk, A.H., 2002. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. Blood 99, 2468–2476.
49. Willems, F., Marchant, A., Delville, J.P., Gerard, C., Delvaux, A., Velu, T., de Boer, M., Goldman, M., 1994. Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. Eur. J. Immunol. 24, 1007–1009.
50. Sah, H., Toddywala, R. and Chien, Y.W. The influence of biodegradable microcapsule formulations on the controlled release of a protein. J. Conk Release 1994, 30, 201-211.
51. Singh, M., Singh, O., Singh, A. and Taiwar, G.P. lmmunogenicity studies on diptheria toxoid loaded biodegradable microspheres. Int. J. Pharm. 1992, 85, R5-R8.
52. Smith, P.K. et al. Measurement of protein using bicinchoninic acid. Analyf. Biochem. 1985, 150, 76.
53. Stieneker, F., Kersten, G., van Bloois, L. eta/. Comparison of 24 different adjuvants for inactivated HIV-2 split whole virus as antigen in mice. Induction of titres of binding antibodies and toxicity of the formulations. Vaccine 1995, 13, 45-53.
54. Tabata, Y. and Ikada, Y. Protein precoating of polylactide microspheres containing a lipophilic immunopotentiator for enhancement of macrophage phagocytosis and activation. Pharm. Res. 1989, 6, 296-301.
55. Uchida, T., Martin, S., Foster, T.P., Wardley, R.C. and Grimm, S. dose and load studies for subcutaneous and oral delivery of poly(lactide co-glycolide) microspheres containing ovalbumin.Pharm. Res. 1994, 11, 1009-1015.
56. Verecchia, T., Huve, P., Bazile, D., Veill&d,‘M., Spenlehauer, G. and Couvreur, P. Adsorption/desorption of human serum albumin at the surface of poly(lactic acid) nanoparticles prepared by a solvent evaporation process. J. Biomed. Mater. Res. 1993, 1019-l 028.
57. Vordermeier, M., Coombes, A.G.A., Jenkins, P. et al. Syntheticdelivery systems for tuberculosis vaccines: immunological evaluation of the M. tuberculosis 38 kDa protein entrapped in biodegradable PLG microparticles. Vaccine 1995, 13, 1576- 1582.
58. Vranken, M.N. and Claeys, D.A. Methods for encapsulating water and aqueous phase by extraction. U.S. Patent 3523907, 1970.
59. Washington, C. Particle Size Analysis in Pharmaceutics and Other Industries, Theory and Practice. Ellis Horwood, Chichester, 1992, Chap. 7.

PLAN OF WORK

- *Construction and purification of Plasmid/IL-10 protein production*
- *PLGA microparticle formulation*
- *Loading efficiency and In vitro release study of OVA/IL-10*
- *Microparticle morphological characterization*
- *Stability and functional study on encapsulated protein*
- *Microparticle Cellular uptake study in vitro and in vivo*
- *EAE induction and immunization of mice*
- *Immunological analysis and clinical evaluation of EAE*
- *Extending the study to SLE MRLlpr mice model after evaluating the results on EAE*

COURSES ATTENDED:

Summer School on Medical Technologies "Industrial Challenges and process of innovation in Medical technologies", Grenoble, France, 18-23rd of Jul, 2011 Grenoble

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).

SEMINARS

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"