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Protein Delivery from Polymeric Nanoparticles

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***Abstract*—**Aim of this work was to compare the efficacy of two loading methods of proteins onto polymeric nanocarriers: adsorption and encapsulation methods. Preliminary studies of protein loading were done using Bovine Serum Albumin (BSA) as model protein. Nanocarriers were prepared starting from polylactic co-glycolic acid (PLGA) polymer; production methods used are two different variants of emulsion evaporation method. Nanoparticles obtained were analyzed in terms of dimensions by Dynamic Light Scattering and Loading Efficiency of BSA by Bradford Assay. Loaded nanoparticles were then submitted to in-vitro protein dissolution test in order to study the effect of the delivery system on the release rate of the protein.

***Keywords*—**Drug delivery, nanoparticles, PLGA, protein adsorption, protein encapsulation.

I. Introduction

SEVERAL studies demonstrated that numerous kinds of nanoparticles can penetrate into human cells by methods not already well known. Their uptake is indirectly proportional to their small dimensions: in fact particles having mean diameter of about 100 nm are phagocyted by the cells 2.5 times more than particles having 1 μm diameter [1]. Regarding their dimensions, nanoparticles are able to easily interact with mucosal portions and pass through mucus to reach submucosal zones more than microparticles that normally stay on the mucosal surface [1]. All data obtained by several studies showed that nanoparticles can be usefully applied both to mucosal and intravenous administration of peptides and proteins [2, 3]. Many papers already discussed about the possibility to use PLGA and its derivatives to encapsulate proteins or polypeptides; the emulsion evaporation method was validated as an appropriate process to encapsulate proteins into PLGA based micro and nanoparticles [4, 5, 6]. However, this method could be affected by several factors such as the risk of protein degradation due to interaction with solvents/surfactants and homogenization/drying processes [4]. With the intention to overcome these issues, some recent studies were based on the investigation of the adsorption process of the protein on the particles surface [7]. This technique is based on a complex mechanism of interaction between polymeric surface and protein which is settled by many variables including pH, ionic strength, temperature, the properties of protein molecule and polymer, and by the nature of the solvent and other components present in the medium [8, 9, 10, 11]. Starting from this introduction, aim of this work was to set the formulative and manufacturing parameters for preparing nanoparticles loaded with BSA by *adsorption or encapsulation* methods; experimental comparison of two processes was performed in order to verify which one appears as more suitable for protein loading.

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II. Materials and Methods

A. Materials

Polylactic co-glycolic acid (molecular weight 34kDa, inherent viscosity: 0.32-0.44 dL/g) was purchased from Boehringer Ingelheim Pharma GmbH & Co. KG (Germany); Bovine serum albumin (BSA) was obtained from VWR DBH Prolabo (UK) Chitosan Glutamate G213 (C) (molecular weight: 470kDa, acetylation grade: 70-90%, apparent viscosity 20 mPas) was purchased from Protasan Ultra-pure (Norway); Bradford reagent was obtained from AppliChem (Germany); other reagents used are all of analytical grade.

B. Nanoparticles preparation and characterization

First batch of loaded nanoparticles, named PA, was obtained by double emulsion evaporation method. The internal water phase was composed by 1 mg of BSA dissolved into 0.5 ml of water; the oily phase was obtained by dissolution of PLGA (1% w/v) into 10 ml of Dichloromethane; the external phase was composed by a water solution of PVP (5% w/v). The W/O/W emulsion was obtained by homogenization with Ultraturrax T25 (IKA, Switzerland) of the internal phase with the polymeric oily phase; this W/O emulsion was then mixed with the external water phase and emulsified for 10 minutes at 24,000 rpm in order to obtain a stable emulsion.

Unloaded batches coded as PB and PC and designed for adsorption of BSA on their surface, were prepared by simple emulsion evaporation method, dryed and successively submitted to BSA loading by adsorption technique. They differs each other for the presence of Chitosan Glutammate on the matrix of PC. This variation was studied in order to obtain two matrixes chemically different: PB was mainly composed by lactic and glycolic molecules characterized by the presence of several carboxylic groups that confer a net negative charge to the matrix. Addition of Chitosan Glutammate in PC batch was done in order to neutralize the carboxylic groups with chitosan aminogroups and consequently modify the effective superficial charge of the polymeric matrix.

PB was obtained by simple oil in water emulsion evaporation process. 10 ml of DCM, in which were dissolved 1.25% w/v of PLGA, were added drop-wise into 20 ml of aqueous solution of PVP kept under constant magnetic agitation. The mixture was then sonicated for 2 minutes using a probe sonicator.

PC, was obtained varying the simple emulsion evaporation method previously described by addition of 0.7% w/v of chitosan glutammate in the water phase. Nanoparticles suspensions were exsiccated in ventilated oven at 27°C for 24 hours in order to permit the complete evaporation of the water and the obtainment of dried material (Table I).

TABLE I
COMPOSITION OF PRODUCED NANOPARTICLES

Batch	PLGA (w/v%)	Chitosan Glutammate (w/v%)	BSA (w/v%)
PA	1	---	0.2
PB	1.25	---	---
PC	1.25	0.7	---

All batches produced were analyzed by Dinamic Light Scattering (Nanosizer N5, Beckman Coulter, USA) in order to determine the mean particle size. Dimensional distribution of the nanoparticles is reported as Polydispersity Index (PI) that indicates the dimensional range of particles. Few drops of suspension, diluted in distilled water to 5 ml, were analyzed after evaporation process. About 1 mg of dried nanoparticles, previously dispersed in 5 ml of distilled water, vortexed for 30 seconds and sonicated for 5 minutes, was analyzed for evaluating the effect of evaporation process on the nanoparticle formation. Formulations characterized by nanometric size, uniform size range and low Polidispersity Index, were submitted to adsorption process of BSA on their surface.

C. Adsorption process of BSA on Nanoparticles Surface

Batches of nanoparticles PB and PC, not previously loaded with BSA were submitted to adsorption test. Superficial adsorption of protein was carried out by mixing 1 ml of BSA water solution (1mg/ml) with 5 mg of nanoparticles gently mixed and vortexed for 1 minute for obtaining an homogeneous suspension. This it was then immediately transferred to a rotating platform at room temperature (speed 50 rpm) to facilitate the adsorption process. After 1 hour of incubation, suspensions were centrifuged at 15000 rpm at room temperature for 15 minutes and supernatant was withdrawn and analyzed by Bradford's assay in order to quantify the amount of non-adsorbed BSA.

D. Evaluation of Loading Efficiency

Loading efficiency of BSA into PA nanoparticles was evaluated by direct analysis: an exactly weighted amount of nanoparticles was suspended into 1 ml of 2M NaOH and stirred for 2 hours at 40 rpm in order to degrade the polymeric matrix and favorite the complete protein release from nanoparticles. Suspension was then centrifuged for 15 minutes at 15,000 RPM in order to guarantee the obtainment of a clear solution of NaOH.

10 µl of obtained solution were then mixed with the dye reagent and analyzed by UV-Vis spectrophotometer at 595 nm. Any possible interference between polymers and protein was checked prior to analysis.

The adsorption efficiency of BSA on PB and PC nanoparticles was calculated by the indirect method able to quantify the protein found in the supernatant after incubation process. An aliquot of the particle suspension previously incubated was centrifuged at 15,000 rpm for 15 minutes at room temperature in order to obtain clear supernatant containing exclusively the not adsorbed BSA; protein quantification was done by Bradford assay at 595 nm. Possible influence due to polymers or surfactant was evaluated. The adsorption efficiency (AE) was calculated from the following equation:

AE (%) = (total amount of protein-non-adsorbed protein)/ total amount of protein x 100

E. In-vitro Release test of Protein

Test was performed using a thermostatic rotational apparatus able to avoid the sedimentation of the nanoparticles; speed was set at 50 rpm and temperature at 37°C. An amount of nanoparticles corresponding to 10 mg of protein was tested by disperding the sample into 50 ml of phosphate buffer pH 7.4, test was carried out for 1 day; samples (100 µl) were withdrawn at selected time intervals and measured spectrophotometrically at 595 nm by Bradford assay in order to determine the amount of protein released which was calculated referring to the calibration curve prepared in phosphate buffer pH 7.4 (R^2: 0.999). An equal volume of fresh medium was added after each sampling to maintain sink conditions.

III. RESULTS

A. Nanoparticles Characterization

Dried particles were analyzed in terms of particle size in order to establish the influence of preparation method on their dimensions. Measured mean diameters are strongly affected by the emulsion process, in fact PB batch, obtained by sonication, is composed by nanoparticles with mean diameter of about 50 nm, the addition of chitosan glutammate increases dimensions up to 76 nm (PC); this method guarantees a PI lower than 0.2 due to a monodimensional range of nanoparticles. On the contrary, homogenization process

TABLE II
DIMENSIONAL CHARACTERIZATION OF NANOPARTICLES

Batch	Mean dimensions ± SD	PI±SD
PA	318±28	0.213±0.08
PB	49±6.1	0.189±0.05
PC	75±2.3	0.056±0.01

Mean dimensions of nanoparticles are expressed as nanometers ± Standard Deviation. Each analysis was performed in triplicate.

carried out by Ultraturrax produces nanoparticles with 318 nm mean diameter and PI of about 0.2.

Results show that both methods tested are suitable for nanoparticles production; nevertheless, preformulation studies have demonstrated that homogenization technique by Ultraturrax does not permit to obtain particles lower than 300 nm (Table II).

Nanoparticles exsiccation in oven was preferred to others processes because of this method avoid drastic conditions of work as low temperatures and pressures that can damage not only the physical structure of the nanoparticles but also the quaternary structure of BSA. More over this method avoids the addition of excipients as cryoprotectant often necessary for lyophilization process.

B. Loading efficiency evaluation

Formulations PB and PC were submitted to adsorption process in distilled water as previously described. Data obtained show that formulation PB, is able to adsorb 49% of protein dissolved into the reaction medium; as expected the result is highly influenced by the development of repulsion forces between nanoparticles, negatively charged due to the presence of carboxylic groups and BSA and characterized by an isoelectric point of 4.7. The adsorption of a low percentage of BSA on the surface of PLGA nanoparticles characterized by negative superficial charge, it is mainly due to hydrophobic interactions between the hydrophobic polymers and the hydrophobic parts of the proteins [12], steric interactions [13] and a very low quantity of electrostatic forces.

PC, dispersed in distilled water, is able to load 98% of BSA; this behavior is due principally to attraction forces between aminogroups of chitosan with BSA negatively charged at neutral pH. As previously described, the high ratio between Chitosan Glutammate and PLGA in PC formulation confers a very high positive charge to nanoparticles that determines the total adsorption of negatively charged BSA on the polymeric surface.

Encapsulation efficiency of BSA into PA nanoparticles appears not affected by electrostatic interactions, in fact 90% of protein is found in the medium after degradation of the polymeric matrix (Table III).

TABLE III
EVALUATION OF LOADING EFFICIENCY PERCENTAGE

Batch	Encapsulation Efficiency±SD	Adsorption Efficiency±SD
PA	91.5±12.3	---
PB	---	49.4±2.5
PC	---	98.6±5.3

Values are experessed in as percentage of protein loaded ± Standard Deviation; Every test was performed in triplicate

C. Dissolution test

Dissolution test was performed into phosphate buffer pH 7.4 in order to simulate the effective capability of loaded nanoparticles to release the protein. As it is possible to observe from Figure 1, release rate profile of nanocarriers is strictly influenced by their dimensions, by BSA loading method and by composition of the matrix. Encapsulation of BSA into the matrix appears as a method applicable to control the release rate of the protein; BSA in fact is slowly released from the matrix because of the slow degradation of the PLGA. Degradation of polymer varies dimensions of nanoparticles and permits the dissolution of the protein into the acceptor medium. It is reasonable to expect that lower dimensionate nanoparticles and different degradation time of the polymer would influence the release rate of the protein.

Adsorption process of BSA on the surface of nanoparticles appears as a good method able to overcome the slow release obtained through encapsulation method. In fact, as it is possible to observe from the showed curve related to PB in-vitro behavior, this method of release is mainly affected by the affinity of BSA to the acceptor medium, to the polymeric matrix and its degradation time: formulation PB is able to release almost 35% of adsorbed BSA after 1 hour of test thanks to a relatively rapid desorption process and only after 6 hours a further small amount of protein is released probably because of the slow polymer degradation. Formulation PC shows an improved behavior respect to PB batch; after 1 hour of test about the 40% of protein is released from the surface of the nanoparticles and after further 6 hours the amount of BSA found in the acceptor fluid increases to about 50%. The presence of C, not only significantly increases the quantity of adsorbed protein but also ameliorates its release rate during the test.

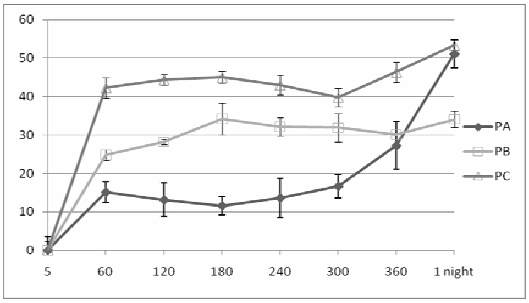

Fig. 1 Dissolution test of BSA loaded nanoparticles in phosphate buffer pH7.4

IV. DISCUSSION

Adsorption process appears as an appropriate method for loading proteins into nanoparticle systems. This method in fact avoids the direct contact of the proteins with agents such as solvents and surfactants that might cause degradation of therapeutically active ingredients. The superficial charge of proteins and nanoparticles and the chemical affinity between polymers and proteins are the main factors influencing the adsorption efficacy. Results obtained highlight the importance of electrostatic interactions and ionic strength between reactive groups of polymers and proteins as they could act as the driving forces for the adsorption process. The repulsive and attractive forces are influenced not only by the

concentration of BSA but also by the ionic characteristics of the polymers: positively charged C interacts with the negatively charged BSA by its cationic groups increasing the adsorption capability. Other important factor is the low interaction between the hydrophobic groups of PLGA and the hydrophilic protein BSA. Although data obtained demonstrate the weak possibility of hydrophobic or steric interactions due to flexible protein structure in which hydrophobic zones are present and determine the formation of a stable protein corona. On the contrary encapsulation appears as a very rapid and efficient method to entrap proteins into a polymeric matrix overcoming chemical interaction and affinities not affected by ionic interactions; however, the possible degradation of protein structure should be accurately evaluated before carrying on nanoparticles formation and direct protein encapsulation. Differences on release rate due to entrapment method should be positively used for preparation of carriers able to release proteins within two different steps: a rapid first step due to the adsorption process and a second slower step due to the degradation of the polymer that permits the release of the encapsulated protein.

V. Conclusions

This study belong to the first part of a bigger project that aims to develop an innovative system of protein delivery. Preformulation studies are important in order to individuate the best carriers and methods for protein or peptides administration. Polymeric nanoparticles have been chosen as protein carriers because of their several possibilities in drug delivery, however each kind of polymer and protein show a specific behavior due to several factors. Loading studies showed in this paper were performed in order to completely understand the effective interaction between carriers and proteins before to proceed with more complex studies necessary to develop a nanoparticulate systems for therapeutic uses. Further studies will be carried out in order to ameliorate the matrix composition of designed protein carriers; new polymers and new ratio between them will be evaluated. Starting from this point different tests of adsorption process will be carried out to find the main important parameters that influence this reaction and to discover the effective affinity between proteins and polymeric matrix. Moreover, basic point is also to study the desorption process of the proteins and their release rate. Linkages between polymers and proteins due to chemical characteristics will be also evaluated in order to define the best time of protein release. In-vitro studies will be followed by ex-vivo and in–vivo tests able to demonstrate the efficacy of the developed protein delivery system.

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