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Research paper

Ex vivo evaluation of prolidase loaded chitosan nanoparticles for the enzyme replacement therapy

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Abstract

Prolidase loaded chitosan nanoparticles were set up in order to suggest an innovative therapeutic approach for Prolidase Deficiency (PD), a rare autosomal inherited disorder of the connective tissue. The satisfactory drug loading efficiency (42.6 ± 2.1%) as well as the suitable physical characteristics (mean diameter of 365.5 ± 35.1 nm and a positive zeta-potential of 17.94 ± 0.12 mV) was achieved. In order to verify the compatibility of the chitosan nanoparticles with cells, the influence of the nanoparticles on the growth and the viability (MTT assay) of cultured skin fibroblasts were determined: the nanoparticles showed a good biocompatibility up to 5 μg of chitosan/10,000 fibroblasts. Uptake of chitosan nanoparticles by fibroblasts was verified by confocal microscopy using FITC-labelled chitosan nanoparticles. The *ex vivo* experiments were performed by incubating different amounts of prolidase loaded chitosan nanoparticles with skin human fibroblasts from PD patients for scheduled times. The restored prolidase activity was quantitatively monitored by a capillary electrophoretic method and confirmed by cells morphological observations. Standing from the nanoparticles internalization, the enzymatic activity was progressively restored reaching the best value (about 66%) after 5 days of co-incubation. Moreover, prolidase loaded chitosan nanoparticles permitted to restore prolidase activity in PD fibroblasts for a prolonged period of time (8 days).

Keywords: Chitosan nanoparticles; Cytotoxicity; Cellular uptake studies; Enzyme replacement therapy; Prolidase Deficiency

1. Introduction

Chitosan is a cationic polyelectrolyte and one of the more abundant polysaccharides present in nature. It has shown advantageous biological properties, such as biodegradability, biocompatibility, low immunogenicity and non-toxicity as well as a favourable mucoadhesiveness and the ability to increase membrane permeability. Chitosan's unique characteristics make it potentially useful in a variety of pharmaceutical applications, such as wound healing, implantation and drug carrier [1–4]. Among the drug delivery strategies, a great deal of attention has been directed to chitosan nanoparticles as promising systems which are able to improve drug bioavailability, modify pharmacokinetics and/or protect the encapsulated drug [5]. In particular, chitosan nanoparticles have been extensively studied as delivery systems of complex molecules such as peptides, proteins, oligonucleotides and plasmids [6–8]. These molecules are very unstable with a reduced bioavailability and need to be protected from degradation in the biological environment; their efficacy is often limited by the ability to cross biological barriers and to reach the target site, especially when an intracellular or intranuclear site of action must be reached. At this purpose, chitosan nanoparticles represent a meaningful challenge for macromolecules delivery. In fact, chitosan nanoparticles improve transmucosal permeability enhancing transport through

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the paracellular pathway thanks to the good bio- and mucoadhesive properties of the nanoparticles and to an induced structural reorganization of *tight junction* – associated proteins [9,10]. Moreover, the formulation of chitosan into nanoparticles significantly promotes the cellular uptake. Various endocytic processes, which have not been fully identified, may be responsible for the uptake of chitosan nanoparticles by different types of cells: this uptake appears to occur predominantly by adsorptive endocytosis and in part by a clathrin-mediated process [11,12].

Prolidase Deficiency (PD) is a rare autosomal recessive disorder of the connective tissue, chronic in nature, progressive and debilitating due to the lack of prolidase. Prolidase is a manganese-requiring homodimeric iminopeptidase, which releases carboxy-terminal proline or hydroxyproline from oligopeptides that participates in collagen metabolism and in the terminal degradation of endogenous and dietary proteins. PD typically begins in childhood and common symptoms include chronic intractable skin ulceration and mental retardation; the conditions generally progresses in adulthood to include splenomegalia and recurrent respiratory infections [13,14]. The only "treatment" currently applied for PD is a topical conservative management of the severe skin ulcerations diffused on patients' lower limbs. Prolidase replacement therapy has been attempted for PD patients by blood transfusions: this therapeutical approach has given only transitory effects caused by prolidase *in vivo* instability and its lacking cellular uptake. To overcome these limitations, trials of prolidase encapsulation have been proposed by our research group: namely biodegradable PLGA microspheres that showed a good loading efficacy with a reduced efficacy due to their sizes unsuitable for endocytosis, and liposomes, characterized by the appropriate and narrow size distribution and excellent properties in delivering prolidase into cells [15–17].

In the attempt of suitably combining the colloidal size to the polymeric carrier, the aim of the current study was to investigate the feasibility of using chitosan nanoparticles for successful prolidase replacement therapy: the choice of a hydrophilic cationic polymer is intended to have the chance of working in mild conditions, useful for improving the loading of the enzyme in its active form, and to promote the cellular uptake, thanks to the well-known bioadhesive properties of chitosans.

The preliminary evaluation of nanoparticles toxicity and the study of cellular uptake were assessed, respectively, by the growth and the viability of human fibroblasts and by confocal microscopy using FITC-labelled nanoparticles. Then, in the *ex vivo* studies fibroblasts from PD patients were incubated with prolidase loaded nanoparticles and the enzymatic restored activity was determined by a capillary electrophoretic (CE) method. Furthermore, we established correlations between dose and cytotoxicity, and between dose and restored enzymatic activity in relation to time. In particular this correlation could be useful for the further development of such particles for *in vivo* applications.

2. Materials and methods

2.1. Materials

Chitosan glutamate (Protasan G213, M_w 300,000 Da, degree of acetylation 15%, glutamic acid content 30–50%) was obtained from Pronova Biomedical (Norway). Prolidase (M_w 54,000–56,000 Da), tripolyphosphate pentasodium salt (TPP, M_w 367.9 Da), Cibacron Brilliant Red 3B-A (M_w 995.23 Da, dye content 50%), glycine-proline (Gly-Pro, M_w 172.18 Da), 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium (MTT, M_w 335.43 Da), fluorescein 5(6)-isothiocyanate (FITC, M_w 398.38), 4′,6-diamidino-2-phenylindole (DAPI, M_w 277.32 Da) were purchased from Sigma–Aldrich Chemical Company (UK).

2.2. General working conditions

Nanoparticles preparation was carried out under aseptic conditions in a laminar flow hood. Vials and flasks were heat sterilized and all solutions were sterile-filtered before use. All experiments including prolonged incubation steps were carried out under conditions avoiding microbial contaminations.

2.3. Preparation of chitosan nanoparticles

The preparation of chitosan nanoparticles was performed by a method previously set up in our laboratory [18]. Placebo nanoparticles were prepared by combining ionotropic gelation and ultrasonication treatment. Briefly, 1 ml of TPP solution, at a constant rate of 0.5 ml/min, was added to 2.5 ml of 1 mg/ml solution of chitosan glutamate under magnetic stirring (300 rpm). Then, the nanosuspension was ultrasonicated once under discontinuous mode for 4 min, using an ultrasonic probe with diameter of 3 mm and a 70 W high-intensity ultrasonic processor (GM2070 Bandelin Sonopuls, Germany) operating at 20 kHz. Nanoparticles were recovered by centrifugation at 4 °C at 12,000 rpm for 15 min (Centrifuge 5417R, Eppendorf, Germany) and resuspended in 500 μl of distilled water. Prolidase loaded chitosan nanoparticles were prepared by dropping 250 μl of enzyme solution (200 I.U./ml) to the chitosan solution before adding cross-linking agent.

2.4. Characterization of placebo and prolidase loaded chitosan nanoparticles

Nanoparticles were analyzed with transmission electron microscopy (TEM 208 S, Philips, The Netherlands). Nanoparticle suspensions were diluted tenfold with distilled water, an aliquot of 15 μl was then deposited on a Formvar coated grid (300 mesg, AGAR Scientific, United Kingdom) and stained with 1 M uranyl acetate solution as the negative staining material. The excess of reagent was removed by means of filter paper.

Mean diameter, polydispersity and *Z*-potential of chitosan nanoparticles were measured using a NICOMP 380 ZLS apparatus (Particle Sizing Systems, USA).

The amount of chitosan employed in the nanoparticles preparation was calculated from the difference between the total amount of chitosan used for nanoparticles preparation and the amount of free chitosan remaining in the clear supernatant after the centrifugation performed for nanoparticles recovery. The concentration of chitosan in the supernatant was measured by the colorimetric reaction between Cibacron Brilliant Red 3B-A and the free chitosan, using UV spectrophotometer (Beckmann DU7500, USA) at 575 nm [19].

The evaluation of encapsulation efficiency and prolidase loading level was assessed by a spectrophotometric method [18]. The amount of prolidase encapsulated in the nanoparticles calculated by measuring the difference between the total amount of the enzyme added in the nanoparticle preparation solution and the amount of non-entrapped prolidase remaining in the clear supernatant after the centrifugation. The supernatant was analyzed for prolidase concentration, which accounted for the non-entrapped prolidase, by UV spectrophotometry (Beckmann DU7500, USA) at 280 nm.

Prolidase loading level in nanoparticles was calculated based on the amount of prolidase and chitosan in the nanoparticles.

2.5. Evaluation of chitosan nanoparticles toxicity

2.5.1. Ex vivo studies on the growth of cultured skin fibroblasts

Different amounts of placebo nanoparticles were incubated with fibroblasts to determine their toxicity. Cells were grown in Petri plates (3.5 cm diameter) containing 5×10^4 cells in Dulbecco modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS). At confluence, the media were removed and replaced with 2 ml of DMEM containing 1% Insulin-transferrin-sodium selenite (DMEM+ITS+3), serum substitute, and different amounts of placebo nanoparticles in the concentration range from 2.5 to 50 μg of chitosan/10,000 fibroblasts. After 1, 2, 5 and 12 days of incubation at 37 °C, the cells were trypsinized, centrifuged and resuspended in a small amount of medium (about 2 ml). Aliquots (10 μl) of these suspensions were used to assess the cells number by means of the Neubauer chamber.

2.5.2. Ex vivo studies on the metabolism of cultured skin fibroblasts

The effects of chitosan nanoparticles on cell viability were assessed with the 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium (MTT) assay [20], using 96 Well Cell Culture Cluster with 10,000 fibroblasts plated in contact to different amounts of placebo nanoparticles corresponding to chitosan amounts from 0.625 to 5 μg.

Briefly, fibroblasts were cultured in wells in DMEM supplemented with FCS for 6 h at 37 °C, then media were removed and fresh DMEM with the different amounts of nanoparticles was restored. After 40 h, 25 μl of MTT working solution (5 mg/ml in DMEM) were added into wells. Cells were incubated for 2 h at 37 °C to allow MTT oxidation by mitochondrial dehydrogenase in viable cells. After 2 h, a suitable detergent was added to dissolve the resulting blue formazane crystals. The results can be read on a multiwell scanning spectrophotometer (Microplate Reader Model 680, Bio-Rad Laboratories, USA). The absorbance was measured at 595 nm with 655 nm as reference wavelength. Cell viability was calculated as the percentage of untreated cells (control).

2.6. Synthesis of FITC-labelled chitosan, preparation of fluorescent nanoparticles and uptake studies

FITC-labelled chitosan was synthesized by adding 5 ml of methanol followed by 2.5 ml of FITC in methanol (2 mg/ml) to 5 ml of chitosan (1% w/v in 0.1 N CH_3COOH). The reaction was run for 3 h in the dark at room temperature. Then the labelled polymer was precipitated in 0.5 M NaOH (till to pH 10). The precipitate was recovered by centrifugation at 25,000 rcf (10 min) and washed in methanol/water (70:30 v/v). The washing and the pelletization were repeated until no fluorescence was detected in the supernatant (Perkin-Elmer Luminescence Spectrophotometer LS 55, USA). The labelled chitosan was then dissolved in 0.1 N CH_3COOH and dialyzed in the dark against water for 3 days. Finally, the FITC-labelled chitosan was freeze-dried. The effective grafting of the polymer with FITC was assessed by ^1H NMR analysis (Avance™ Ultra Shield 400 working in FT, Bruker, Germany), and the labelling efficiency (% FITC/FITC-labelled chitosan, w/w) was determined by measuring the fluorescence intensity of the FITC-labelled chitosan solution against standard solution of FITC.

Fluorescent nanoparticles were prepared with FITC-labelled chitosan (1 mg/ml in 0.1 N CH_3COOH) and characterized following the procedure described in Sections 2.3 and 2.4, respectively.

For the confocal microscopy studies, 40,000 fibroblast cells were seeded onto Will Co-Dish and cultured in 2 ml of DMEM supplemented with 10% FCS at 37 °C in the incubator. After 24 h of culture, 8 μl of FITC-labelled nanoparticles (corresponding to 2.5 μg of chitosan/10,000 fibroblasts) were added to the medium. After different time of incubation (4, 6, 8, 24 h), the culture medium was removed. The cell monolayers were washed with PBS and fixed with 70% ethanol for 10 min and stained with 300 μl of DAPI (1 μg/ml) in order to highlight the cells nucleus. The specimens were examined under an inverted confocal laser scanning microscope (Leica TCS SP2, Leica Instruments, Germany). Each uptake experiment was performed in triplicate and six images of each time point were analyzed.

2.7. Ex vivo evaluation of prolidase activity on cultured skin fibroblasts from PD patients incubated with prolidase loaded nanoparticles

Fibroblasts (1×10^6) from two PD patients were plated in T75 flasks containing DMEM supplemented with 10% FCS. After 24 h, the initial medium was changed with DMEM+ITS+3 supplemented with different amounts of prolidase loaded nanoparticles suspension from 25 to 200 μl: the corresponding amounts of prolidase incubated with PD cells are expressed as the amount (μmol) of substrate Gly-Pro hydrolyzed/min at 37 °C (I.U.) and reported in Table 1.

At the beginning in order to set up the best incubation time, fibroblasts were incubated with 25 or 100 μl of prolidase loaded nanoparticles for 1, 2, 5, 6, 8 and 10 days. Once assessed the best time of incubation, different amounts of prolidase loaded nanoparticles suspension (Table 1, NP25–NP200) were added to the flasks and let in co-incubation with cells. After the period of incubation, the medium was removed and stored, and the cell layers were washed three times with phosphate saline buffer (PBS, 7.4 pH), resuspended with PBS containing protease inhibitor (4 mM sodium ethylendiaminetetraacetic acid bihydrate, EDTA; 10 mM benzamidine; 2 mM *N*-ethylenmaleimide, NEM; 1 mM phenylmethylsulfonilfluoride, PMSF) and Triton X-100, denaturated at 80 °C for 15 min and then centrifuged at 8000 rpm for 30 min at 4 °C. The supernatant was filtered using a Microcon concentrator (Millipore, USA) for 5 min at 6000 rpm.

The enzyme activity on cellular extracts was determined by a capillary electrophoretic method developed in our laboratory [15] and using a Beckman P/ACE 2100 system (Fullerton, CA, USA) equipped with an UV detector. Samples were injected by pressure (10 s, 0.07 MPa) onto an uncoated fused silica capillary of 50 cm effective length × 50 μm I.D. operating at 25 °C and applying a voltage of 25 kV. Separations were performed using 50 mM sodium tetraborate, pH 9.3, containing 30 mM cyclodextrin as the background electrolyte and analytes were monitored at 214 nm. By this set up CE method, it was possible to evidence the typical Gly-Pro peak at its migration time (about 12 min) in cellular extracts of PD fibroblasts.

The restored prolidase activity was determined as the reduction of the Gly-Pro peak of the PD cellular extracts and it is expressed as μmol of Gly-Pro hydrolized/mg of protein and calculated as difference between the Gly-Pro amount (μmol) in untreated PD cells and the Gly-Pro amount (μmol) in treated PD cells (μmol Gly-Pro hydrolized); this value is normalized by the amount (mg) of endocellular protein determined by a spectrophotometric method at 660 nm with Lowry Assay [21]. In order to highlight better the efficacy of the treatment, the restored activity is expressed as specific activity (%) in terms of percentage (%) of the total Gly-Pro amount in untreated PD cells.

Morphological characteristics of PD fibroblasts were studied by an optical microscope (Laborlux 11 Pol, Leitz, Germany). The analyses were performed on samples of plated fibroblasts from PD patients and controls, and on fibroblasts cultured after different days of incubation with prolidase loaded chitosan nanoparticles.

2.8. Statistical analysis

Data were expressed as means ± standard deviation (SD). Comparison of mean values was performed using one-way analysis of variance (ANOVA). A statistically significant difference was considered when $P < 0.05$.

3. Results and discussion

3.1. Nanoparticles characterization

Prolidase loaded chitosan nanoparticles were prepared by TPP ionotropic gelation combined with one cycle of ultrasonication in controlled conditions. This preparation method permitted to obtain nanoparticles of regular and spherical shape (Fig. 1).

Results of the granulometric analysis revealed nanoparticles with mean diameter of about 360 nm (365.5 ± 35.1 nm) with a positive *Z*-potential of 17.94 ± 0.12 mV ($n = 10$). Chitosan analysis indicated that the yield of process was quite satisfactory ($54.2 \pm 5.7\%$) and the results concerning the actual enzyme content showed a loading efficiency of about 43% ($42.6 \pm 2.1\%$): the composition of the nanoparticles was calculated as $19.3 \pm 0.9\%$ and $80.7 \pm 1.1\%$ by weight for prolidase and chitosan, respectively. Table 2 reports the main features of placebo and prolidase loaded nanoparticles.

Table 1
Samples of chitosan nanoparticles incubated with PD fibroblasts for the *ex vivo* evaluation of prolidase activity

Sample	μl of nanoparticles suspension incubated	μg of chitosan/10,000 fibroblasts	Total amount of chitosan in the sample (μg)	Total activity of prolidase in the sample[a] (I.U.)
NP25	25	0.625	62.5	0.75
NP50	50	1.25	125	1.5
NP100	100	2.5	250	3
NP200	200	5	500	6

[a] The corresponding amounts of prolidase incubated with PD cells are expressed as the amount (μmol) of Gly-Pro hydrolyzed/min at 37 °C (I.U.).

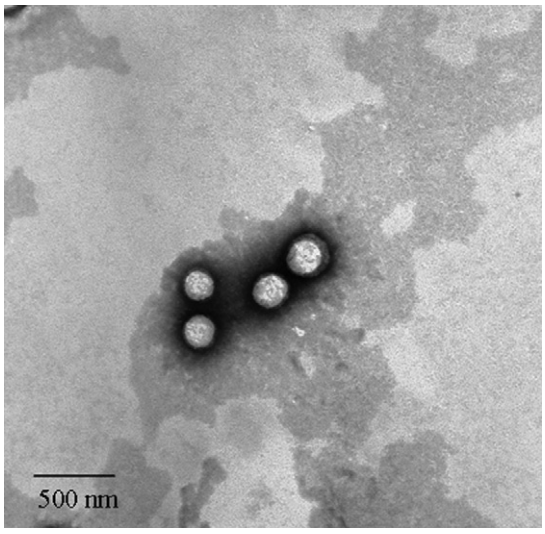

Fig. 1. TEM photomicrograph of prolidase loaded chitosan nanoparticles. TEM photomicrograph (magnification 85,000×) shows the image of few distinctive single particles, each possessing a typical spherical shape and similar nanometric dimension.

3.2. Evaluation of chitosan nanoparticles toxicity

As a preliminary step of this study, different amounts of placebo nanoparticles were incubated with cultured healthy fibroblasts to verify their toxicity towards these cells. Figs. 2 and 3 and show the results obtained by the cellular growth test and the MTT assay, respectively.

The highest nanoparticles amounts corresponding to 50 and 10 μg of chitosan incubated with cells seemed to deeply affect the fibroblasts growth (Fig. 2); after 2 days of incubation the collected cells number was of 71,500 ± 1000 and 83,000 ± 750: these values represent about 63% and 55% of the growth values of the controls. The reduction of proliferation rate was better highlighted after 5 days of incubation: at this time, the growth values obtained resulted to be about 30% and 40% of control values. By these results, it is possible to highlight the cellular toxicity of these amounts of nanoparticles. For the nanoparticles amounts corresponding to 5 and 2.5 μg of chitosan/10,000 cells, fibroblasts kept the growth profiles superimposable to controls and the collected values can be considered comparable to control values, even if higher standard deviations are evident (Fig. 2). For this reason, the chitosan concentration of 5 μg/10,000 fibroblasts represented the highest concentration employed for the MTT test.

The viability assay (MTT test) revealed that the amounts of nanoparticles considered in this study are not

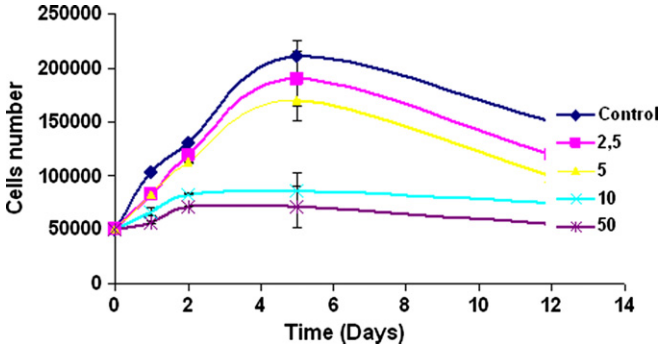

Fig. 2. Curves of cellular growth of cultured skin fibroblasts after incubation with placebo nanoparticles. The legend is referring to untreated cells as control and to different amounts of nanoparticles as μg of chitosan/10,000 cells. Data shown are means ± SD ($n = 6$).

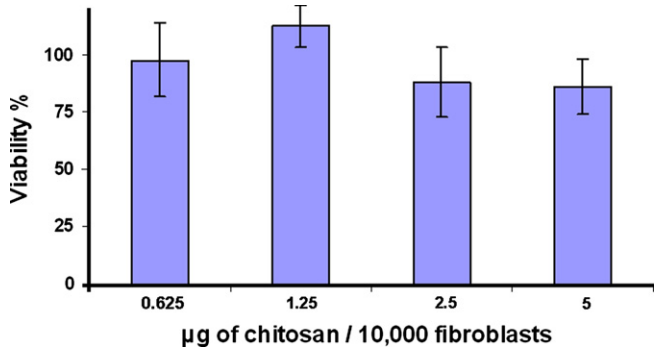

Fig. 3. The influence of different amounts of placebo chitosan nanoparticles on fibroblasts viability (MTT test). Data shown are means ± SD ($n = 6$).

toxic for the fibroblasts metabolism (Fig. 3). A very narrow reduction in the viability percentages was obtained only for samples made of 5 and 2.5 μg of chitosan/10,000 fibroblasts, that obtained 85.6 ± 10.8% and 87.7 ± 12.1%, respectively.

In any case, all the studies on nanoparticles cellular toxicity confirmed the good nanoparticles biocompatibility up to 5 μg chitosan/10,000 cells, corresponding to sample NP200 (Table 1) considered for the *ex vivo* studies of prolidase activity.

3.3. Synthesis of FITC-labelled chitosan, preparation of fluorescent nanoparticles and uptake studies

In order to highlight the cellular uptake of the chitosan nanoparticles by confocal microscopy, the chitosan glutamate was labelled with FITC. The successful labelling

Table 2
Main features of placebo and prolidase loaded nanoparticles

Batch	Mean diameter (nm)	*Z*-Potential (mV)	Yield of the preparation process (%)	Prolidase loading efficiency (%)
Placebo nanoparticles	388.6 ± 6.8	16.45 ± 0.75	55.4 ± 2.4	–
Prolidase loaded nanoparticles	365 ± 35.1	17.94 ± 0.12	54.4 ± 5.7	42.6 ± 2.1

Data are reported as mean values ± standard deviation (SD).

was shown by ^{1}H NMR spectra with the peak of the fluorescein aromatic groups evident at about 7.00 ppm (unreported data). The weight fraction of FITC per weight unit of chitosan is 2.7% (w/w). The FITC-chitosan conjugate was successfully transformed by ionotropic gelation with TPP into nanoparticles with a spherical shape, mean diameter of 273.9 ± 30.6 nm and *Z*-potential of 10.51 ± 1.2 mV. Such characteristics suitably allowed the use of FITC-labelled particles as a model of chitosan nanoparticles for the uptake studies.

Confocal images of fibroblasts after uptake experiments showed as few minutes lead to the internalization of the FITC-labelled chitosan nanoparticles and as the interaction between nanoparticles and cellular membrane is kept close up to 4 h of co-incubation (Fig. 4a). Fig. 4b shows the FITC-labelled nanoparticles still accumulated into the perinuclear zone after 6 h of co-incubation. A three-dimensional analysis of the confocal data, in particular the reconstruction of *z*-axis, confirmed that fluorescent signals are located within the cells incubated with nanoparticles (Fig. 4c). When the incubation of fibroblasts with nanoparticles was prolonged up to 8 or 24 h (unreported data), the collected confocal images did not reveal any significant difference with the shorter times investigated, so the uptake of the chitosan nanoparticles seemed to be a saturable event which is involving an a-specific adsorptive endocytosis [11,12].

3.4. Ex vivo evaluation of prolidase activity on cultured skin fibroblasts from PD patients incubated with prolidase loaded nanoparticles

The *ex vivo* experiments on cultured skin fibroblasts from PD patients were performed in order to verify whether the nanoparticulate drug delivery systems suggested in this study could be effective in restoring the prolidase activity in the cells. For this reason, based on the toxicity data evaluated on placebo nanoparticles, 25 and 100 μl of prolidase loaded nanoparticles suspension, corresponding to samples NP25 and NP100, respectively (Table 1), were added to fibroblasts flasks and at fixed times cellular extracts were collected. As stated in a previous work [15], the cellular extracts from PD fibroblasts evidenced the typical Gly-Pro peak by CE at its migration time of 12.0 ± 0.12 min. The presence of this peak was due to the intracellular accumulation of Gly-Pro caused by the inefficient endogenous prolidase in PD patients [22]. At the

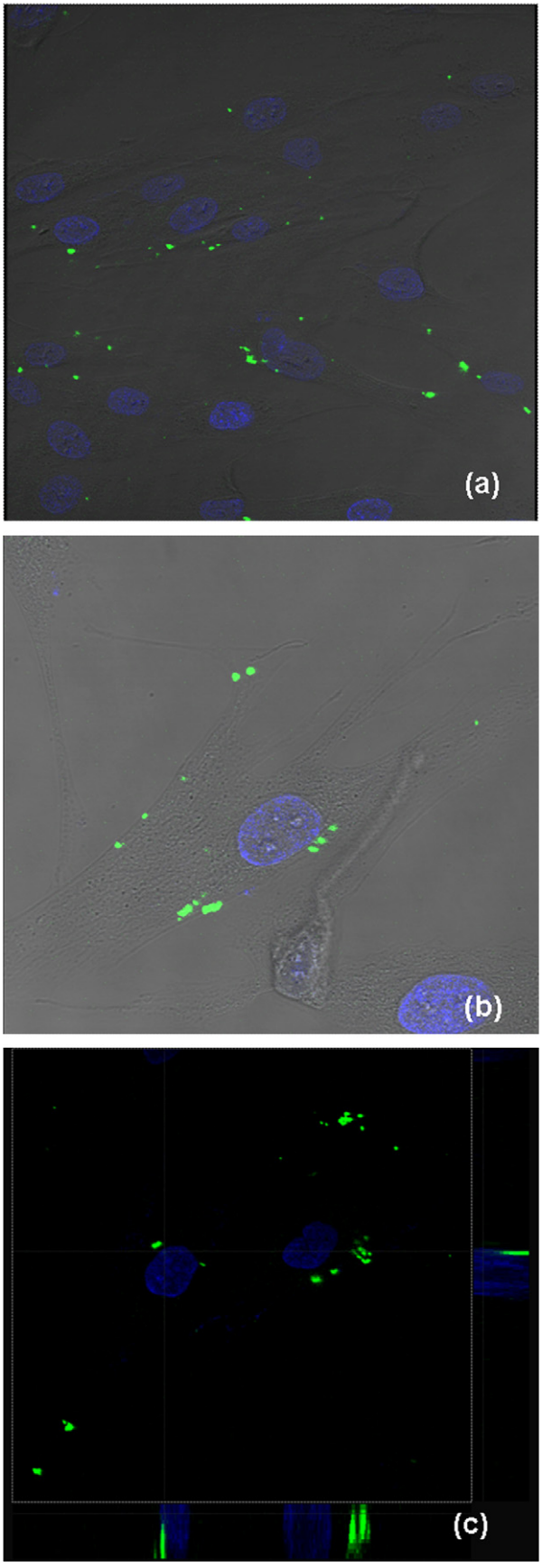

Fig. 4. Confocal images of fibroblasts incubated for 4 h (a) and 6 h (b) with 8 μl of FITC-labelled nanoparticles, corresponding to 2.5 μg of chitosan/10,000 fibroblasts.(a) Chitosan nanoparticles are visualized as fluorescent spots closely adhered to cellular membrane. (b) Chitosan nanoparticles are internalized into the fibroblasts. (c) Optical section (*x*, *y*-axis), with respective projections of *x*, *z*- and *y*, *z*-axis of fibroblasts incubated for 6 h at 37 °C with FITC-labelled nanoparticles. The cross points represent internalized FITC-chitosan nanoparticles.

beginning, free prolidase solutions were added to the culture media, but no area shifts in the electrophoretic peaks were detected: this result was not surprising since prolidase has no specific membrane carriers, so it could not be uptaken by the cells in free form. Once the PD fibroblasts were incubated with prolidase loaded chitosan nanoparticles, the area of the recorded peaks reduced: this fact confirmed that the delivery of prolidase across the cellular membrane was successful and effective in restoring the hydrolytic function of the enzyme. Given this positive standing point, fibroblasts from PD patients were incubated with encapsulated prolidase (samples NP25 and NP100, Table 1) for different time intervals in order to evaluate the best incubation time. As shown in Fig. 5, the prolidase activity detected in cellular extracts evidenced the best restored activity after 5 days of incubation for both samples. NP25 and NP100 (Table 1) showed very similar profile of restored activity: prolidase was transferred to fibroblasts since the 1st day of incubation (about 30% expressed as μmol Gly-Pro hydrolized/mg protein), but the activity values gradually increased and culminated after 5 days of incubation when NP25 and NP100 reached about 56% and 66% of restored enzymatic activity, respectively. Longer incubation times did not induce any better enzyme replacement and the collected data showed a sensible reduction in hydrolytic activity percentages: at 6th day of incubation this value (about 35%) was superimposable to data collected at the 1st day of incubation for both samples and after 12 days the prolidase activity was quite hardly detectable ($\leqslant$10%).

Stated 5 days as the more suitable time of incubation, experiments were carried out adding different amounts of prolidase loaded nanoparticles to the culture media in order to understand whether the enzymatic replacement is a dose-depending phenomenon. As shown in Fig. 6, the replaced enzymatic activity did not seem to be affected by nanoparticles concentration up to NP50 ($52.31 \pm 4.95\%$), then it was possible to improve these results by NP100 ($65.85 \pm 4.51\%$, $P < 0.05$) and surprisingly the activity fell to lower values when PD fibroblasts were incubated with the higher amount of nanoparticles ($17.69 \pm 8.64\%$). For all the samples evaluated, nanoparticles uptake is confirmed to be a saturable event driven by electrostatic interaction between the chitosan nanoparticulate systems and the cellular membrane [23]. The decrease in binding affinity and uptake capacity for NP200 could be explained by an unsuitable particle concentration gradient.

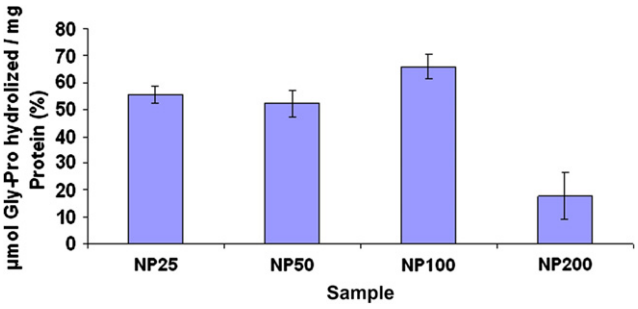

Fig. 6. Prolidase activity detected in cellular extracts of fibroblasts after 5 days of incubation with different amount of prolidase loaded chitosan nanoparticles. The legend is referring to different volumes (μl) of nanoparticles suspension as specified in Table 1. Data shown are means ± SD ($n = 6$).

Fig. 7 shows the morphological improvement of PD fibroblasts after incubation with prolidase loaded nanopar-

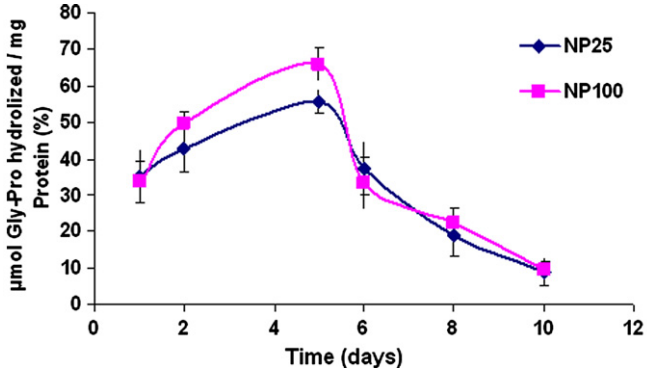

Fig. 5. Prolidase activity (expressed as μmol Gly-Pro hydrolized/mg protein %) detected in the cellular extracts after different time of incubation of PD fibroblasts with prolidase loaded chitosan nanoparticles. The legend is referring to 25 or 100 μl of nanoparticles suspension as NP25 and NP100, respectively. Data shown are means ± SD ($n = 6$).

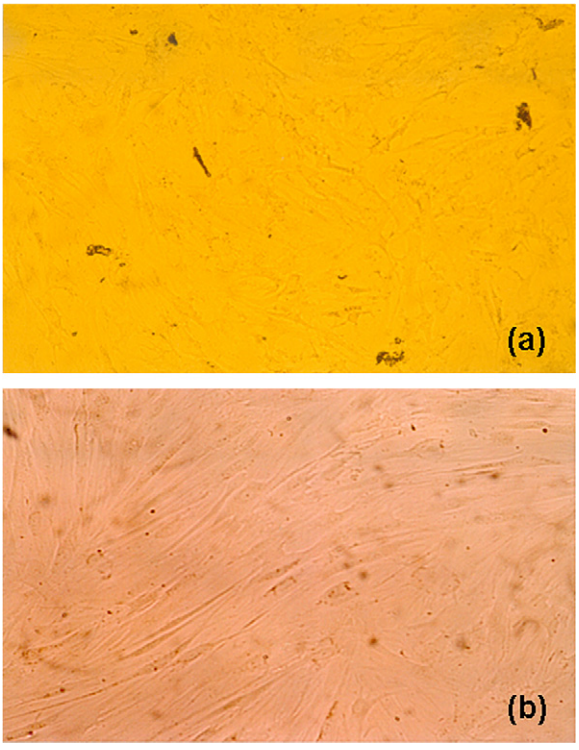

Fig. 7. Photomicrographs (magnification 10×) of PD patients fibroblasts before (a) and after 5 days of incubation (b) with 100 μl of suspension of prolidase loaded chitosan nanoparticles. (a) PD fibroblasts are swollen and disorganized. (b) PD fibroblasts have recovered a more regular shape and morphology.

ticles. As shown in Fig. 7(a), fibroblasts from PD patients are swollen and disorganized. The incubation with prolidase loaded nanoparticles deeply affected the cellular morphology: progressively fibroblasts appeared more regular shaped and get normal nuclei, achieving the best morphological aspect after five days of incubation (Fig. 7b): this further experimental evidence indicated that the prolidase was delivered in its active form across the cellular membrane by chitosan nanoparticles.

4. Conclusions

The formulation of prolidase into chitosan nanoparticles significantly protected the enzyme against the degradation and created the chance of a suitably effective enzyme replacement therapy for PD. In fact, because of the nanoparticles small size, the positive Z-potential and the good biocompatibility, the internalization of the nanoparticles by cells seemed to occur by absorptive endocytosis through non-specific interactions between nanoparticles and cell membranes. This phenomenon brought to the release of the enzyme in the active form into the cytoplasm and let to restore the prolidase activity into fibroblasts obtained from PD patients for a prolonged period of time. Thanks to these results, we assessed a solid base for the future potential application of prolidase loaded nanoparticles in topical management of the severe skin ulcerations of PD patients.

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References

[1] M.N.V. Ravi Kumar, A review of chitin and chitosan applications, React. Funct. Polym. 46 (2000) 1–27.
[2] T. Minagawa, Y. Okamura, Y. Shigemasa, S. Minami, Y. Okamoto, Effects of molecular weight and deacetylation degree of chitin/chitosan on wound healing, Carbohydr. Polym. 67 (2007) 640–644.
[3] F.L. Mi, Y.C. Tan, H.F. Liang, H.W. Sung, In vivo biocompatibility and degradability of a novel injectable-chitosan-based implant, Biomaterials 23 (2002) 181–191.
[4] S.A. Agnilotri, N.N. Mallikarjuna, T.M. Aminabhavi, Recent advances on chitosan-based micro- and nanoparticles in drug delivery, J. Control. Release 100 (2004) 5–28.
[5] K.A. Janes, M.P. Fresneau, A. Marazuela, A. Fabra, M.J. Alonso, Chitosan nanoparticles as delivery systems for doxorubicin, J. Control. Release 73 (2001) 255–267.
[6] K.A. Janes, P. Calvo, M.J. Alonso, Polysaccharide colloidal particles as delivery systems for macromolecules, Adv. Drug Deliv. Rev. 47 (2001) 83–97.
[7] L. Illum, I. Jabbal-Gill, M. Hinchcliffe, A.N. Fisher, S.S. Davis, Chitosan as a novel nasal delivery system for vaccines, Adv. Drug Deliv. Rev. 51 (2001) 81–96.
[8] S. Mansouri, P. Lavigne, K. Corsi, M. Benderdour, E. Beaumont, J.C. Fernandes, Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy, Eur. J. Pharm. Biopharm. 57 (2004) 1–8.
[9] N.G.M. Schipper, S. Olsson, J.A. Hoogstraate, A.G. deBoer, K.M. Varum, P. Artursson, Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement, Pharm. Res. 14 (7) (1997) 923–929.
[10] N.A. Peppas, Y. Huang, Nanoscale technology of mucoadhesive interactions, Adv. Drug Deliv. Rev. 56 (2004) 1675–1687.
[11] Z. Ma, L.-Y. Lim, Uptake of chitosan and associated insulin in Caco-2 cell monolayers: a comparison between chitosan molecules and chitosan nanoparticles, Pharm. Res. 20 (11) (2003) 1812–1819.
[12] O. Harush-Frenkel, N. Debotton, S. Benita, Y. Altschuler, Targeting of nanoparticles to the clathrin-mediated endocytic pathway, Biochem. Biophys. Res. Commun. 353 (2007) 26–32.
[13] L. Myara, C. Charpentier, A. Lemonnier, Prolidase and prolidase deficiency, Life Sci. 34 (1984) 1985–1998.
[14] P.M. Royce, B. Steinmann (Eds.), Connective Tissue and its Heritable Disorders, Wiley-Liss, New York, 2002, pp. 727–743.
[15] S. Viglio, L. Annovazzi, B. Conti, I. Genta, P. Perugini, C. Zanone, B. Casado, G. Cetta, P. Iadarola, The role of emerging techniques in the investigation of prolidase deficiency from diagnosis to the development of a possible therapeutical approach, J. Chromatogr. B 832 (2006) 1–8.
[16] A. Lupi, P. Perugini, I. Genta, T. Modena, B. Conti, B. Casado, G. Cetta, F. Pavanetto, P. Iadarola, Biodegradabile microspheres for prolidase delivery to human cultured fibroblasts, J. Pharm. Pharmacol. 56 (2004) 597–603.
[17] P. Perugini, K. Hassan, I. Genta, T. Modena, F. Pavanetto, G. Cetta, C. Zanone, P. Iadarola, A. Asti, B. Conti, Intracellular delivery of liposome-encapsulated prolidase in cultured fibroblasts from prolidase-deficient patients, J. Control. Release 102 (2005) 181–190.
[18] C. Colonna, B. Conti, P. Perugini, F. Pavanetto, T. Modena, I. Genta, Chitosan glutamate nanoparticles for protein delivery: development and effect on prolidase stability, J. Microencapsulation 24 (2007) 553–564.
[19] R.A.A. Muzzarelli, The colorimetric determination of chitosan, Anal. Biochem. 260 (1998) 255–257.
[20] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[21] E.E. Hartree, Determination of protein: a modification of Lowry method that gives a linear photometric response, Anal. Biochem. 48 (1972) 422–427.
[22] A. Forlino, A. Lupi, P. Vaghi, A. Icaro Cornaglia, A. Calligaro, E. Campari, G. Cetta, Mutation analysis of five new patients affected by prolidase deficiency: the lack of enzyme activity causes necrosis-like cell death in cultured fibroblasts, Hum. Genet. 111 (2002) 314–322.
[23] M. Huang, E. Khor, L.-Y. Lim, Uptake and cytotoxicity of chitosan molecules ad nanoparticles: effects of molecular weight and degree of deacetylation, Pharm. Res. 21 (2) (2004) 344–353.