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Site-directed PEGylation as successful approach to improve the enzyme replacement in the case of prolidase

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ABSTRACT

The first aim of this work was to perform site-directed PEGylation of the enzyme prolidase at sulphydril groups by methoxy-polyethylene glycol-maleimide (Mal-PEG, Mw 5000 Da) in order to obtain a safe conjugation product more stable than the native enzyme. Prolidase is a cytosolic aminoacyl-L-proline hydrolase whose deficiency causes the onset of rare autosomal recessive disorder called prolidase deficiency (PD). The second purpose of this work was to investigate whether biodegradable chitosan nanoparticles loaded with PEGylated prolidase could be effective in releasing active enzyme inside fibroblasts as a possible therapeutic approach for PD. The SDS-PAGE analysis and the ESI-MS spectra confirmed the presence of the PEGylated prolidase: in particular the main conjugation product (m/z = about 65,000 Da) corresponded to the enzyme with two residues of Mal-PEG. In this study it was demonstrated the lack of toxicity (MTT assay) and the prolonged activity (40.6 ± 2.6% after 48 h of incubation at 37 °C) of the PEGylated enzyme. The PEGylated prolidase loaded chitosan nanoparticles had spherical shape, narrow size distribution (271.6 ± 45.5 nm), a positive zeta-potential (15.93 ± 0.26 mV) with a good preparation yield (54.6 ± 3.6%) and protein encapsulation efficiency (44.8 ± 4.6%). The ex vivo evaluation of prolidase activity on PD fibroblasts individuated a good level of prolidase activity replaced (about 72% after only 2 days of incubation) up to 10 days with improved morphological cell features.

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1. Introduction

The rationale for the preparation of macromolecular conjugates is based on the assumption that the covalent attachment of polymers to biologically active compounds, such as peptides and proteins, has the capacity to solve or improve many of the problems encountered when these molecules are used as pharmaceuticals (Maeda, 2001; Veronese and Pasut, 2005; Thordarson et al., 2006; Ge et al., 2007). These advantages include shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the reticuloendothelial system (RES), and preventing recognition and degradation by proteolitic enzymes (Harris et al., 2001; Khaled et al., 2003). A highly investigated polymer for covalent modification of biological macromolecules is polyethylene glycol (PEG), one of the most popular materials used for the preparation of these conjugates (Veronese, 2001; Roberts et al., 2002). Due to its structural simplicity and to the chemical inactivity of the polyether backbone in the biological environments, it has a very low toxicity, low immunogenicity and antigenicity and shows high persistence in the blood compartment after administration into living organisms (Bailon and Berthold, 1998). PEG conjugation also increases the apparent size of the polypeptide thus reducing the renal filtration and altering distribution (Caliceti and Veronese, 2003). In addition, owing to its excellent solubility in aqueous solutions it has no deleterious effects on protein conformation and shows an exceptional ability to preserve activities of enzymes (Werle and Bernkop-Schnürch, 2006). As a matter of fact, PEGylation plays today an important role in drug delivery by enhancing the potential of peptides and proteins as therapeutic agents (Duncan, 2003). In particular site-directed PEGylation to specific groups on proteins has been designed to selectively couple a definite number of PEG chains to proteins with the advantage to obtain a stable and well-defined bioconjugates (Roberts et al., 2002; Greenwald et al., 2003). PEGylation of free cysteine residues in proteins is one of the main approaches for site-specific modification because usually the number of free cysteines on the protein surface is lower than other conjugable groups (i.e. lysine residues) and, for this reason, reagents that specifically react with cysteines have been

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widely synthesized (Harris and Chess, 2003; Manjula et al., 2003; Shaunak et al., 2006; Balan et al., 2007). Reaction of PEG derivatives containing maleimide with protein thiol groups is a particularly useful reaction for the preparation of bioactive conjugates, it is highly specific and occurs under mild conditions (Lee et al., 2001; Trakas and Tzartos, 2001; Wang et al., 2004; Guo et al., 2005; Veronese et al., 2007). On account of these favourable properties, methoxy-polyethylene glycol-maleimide (Mal-PEG) was chosen in our laboratory as the agent best suited for the chemical modification of prolidase. Prolidase is an enzyme composed of two identical subunits, each of them of 492 residues corresponding to Mr 56,000, and, even if its structure is not almost well-defined, it contains six cysteine residues into its primary structure (Endo et al., 1989).

Prolidase is a cytosolic esopeptidase whose deficiency causes the development in humans of a rare autosomal recessive disorder known as prolidase deficiency (PD) (Myara et al., 1984; Royce and Steinmann, 2002).

Looking for an enzyme replacement therapy for the treatment of this disorder, native prolidase loaded into micro- and nanoparticulate systems and conventional liposomes had previously used in our laboratory (Genta et al., 2001; Perugini et al., 2005). Based on the results obtained from ex vivo studies performed on cultured fibroblasts of PD patients, the delivery efficiency of the active enzyme from the microparticulate carriers to the cells was very poor (Lupi et al., 2004). By contrast liposomes, although being very efficient in delivering of prolidase inside fibroblasts, were not able to preserve enzyme activity for period of time longer than 6 days (Perugini et al., 2005). Further studies on the utilization of nanoparticles as carriers of prolidase revealed a good protection of the enzyme against the degradation (Colonna et al., 2007) and the chance of a suitable internalization and replacement of the prolidase activity into fibroblasts obtained from PD patients for a prolonged period of time (Colonna et al., 2006).

With the aim of establishing a resolving enzyme replacement therapy for the PD treatment, this work had the purposes to perform the site-directed PEGylation of the prolidase at the sulphydril groups by PEG-Mal in order to obtain a safe conjugation product more stable than the native enzyme, and then to use the PEGylated prolidase encapsulated in chitosan nanoparticles as drug delivery system for PD therapy.

2. Materials and methods

2.1. Materials

Chitosan glutamate (Protasan G213, Mw 300,000 Da, degree of acetylation 15%, glutamic acid content 30–50%) was obtained from Pronova Biomedical (N). Prolidase (Mw 54,000–56,000 Da), methoxy-polyethylene glycol-maleimide (Mal-PEG, Mw 5,000 Da), tripolyphosphate pentasodium salt (TPP, Mw 367.9 Da), Cibacron Brilliant Red 3B-A (Mw 995.23 Da, dye content 50%), glycine-proline (Gly-Pro, Mw 172.18 Da), 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2*H*-tetrazolium (MTT, Mw 335.43 Da) were purchased from Sigma–Aldrich Chemical Company (UK).

2.2. Methods

2.2.1. PEGylation of prolidase

1.5 mg of freeze-dried native prolidase (100 IU) were solubilized in 3 ml of 10 mM Tris–HCl, 1.2 mM $MnCl_2$, 0.1 mM glutathione reduced form (TMG) freshly prepared at pH 7.8. The PEGylation of sulphidryl groups was performed in solution-phase in presence of a reducing agent (1.2 mM glutathione reduced form) under nitrogen atmosphere.

The final molar ratio Mal-PEG:cysteine considered for the PEGylation process was 3:1; 159 μl of Mal-PEG solution (10 mg/ml) were added to prolidase solution under magnetic stirring: the reaction was carried out for 16 h at r.t. and avoiding the light. PEGylated conjugates were purified by a gel filtration column, Sephacryl S-200 column (2.5 cm × 100 cm) equilibrated with 10 mM Tris–HCl in order to remove the non-reacted PEG-derivatives. The solutions were freeze-dried for 24 h in the dark.

For some samples, PEGylation under denaturing conditions was achieved performing a preventive denaturation of the native enzyme by incubation for 4 h with a solution of 6 M guanidine hydrochloride in 10 mM phosphate buffer (pH 7.8) and then the PEGylation reaction was allowed to proceed using the conditions reported above.

2.2.2. Characterization of PEGylated prolidase

SDS-PAGE electrophoresis (SDS-PAGE) and electrospray ionization mass spectrometry (ESI-MS) were used to characterize the PEGylated conjugates.

2.2.2.1. SDS-PAGE electrophoresis. In order to check the extent of PEGylation reaction, PEGylated conjugates were analyzed by SDS-PAGE and compared with native prolidase and reference markers: proteins were visualized by dipping the gel in a solution containing 1 volume of 0.2% Coomassie Brillant Blue R250 in 45% methanol and 10% acetic acid plus 4 volumes of 0.1 M picric acid. To identify PEGylated conjugates, the gel was dipped in 40 ml of 2 M perchloric acid. After 10 min, 10 ml of 5% $BaCl_2$ were added to this solution followed by 2 ml of 0.1 N iodine solution (Kurfürst, 1992). The bands corresponding to PEGylated conjugates appeared brown coloured.

2.2.2.2. Mass spectrometry analysis (ESI-MS). Analytes were separated on a HPLC system (Thermo Finnigan, San Josè, CA, USA) using a C18 column (100 mm × 2.1 mm I.D., flow rate 0.3 ml/min) coupled to an LCQ-DECA ion trap mass spectrometer (Thermo Finnigan, San Josè, CA, USA) equipped with an electrospray ionization (ESI) ion source and controlled by Xcalibur software 1.1. Experiments were performed in positive ion mode under the following constant instrumental conditions: source voltage, 5.0 kV; capillary voltage, −14 V; capillary temperature, 200 °C; tube lens voltage, −5 V.

2.2.3. Stability evaluation of native and PEGylated prolidase

The activity levels of native and PEGylated prolidase were determined by the capillary electrophoresis method previously reported (Viglio et al., 2006).

Samples were run in triplicates as follows: native and PEGylated prolidase solutions were incubated at 37 °C for 2, 4, 6, 8, 24 and 48 h in 2 ml of 50 mM Tris–HCl (pH 7.8). The theoretical concentration of prolidase solution in the medium considered was 0.4 IU/ml. One milliliter of each sample considered was preincubated overnight at 37 °C with an activation buffer consisting of 50 mM Tris–HCl, 1 mM $MnCl_2$, 0.75 mM glutathione reduced form, freshly prepared at pH 7.8. Following preincubation, the prolidase reaction was initiated by adding 0.4 mM glycine-L-proline (Gly-Pro) for an additional 1 h at 37 °C. To stop the reaction, 70 μl of 2.7 M trichloroacetic acid was added to the mixture. The supernatant was filtered using a Microcon concentrator (Millipore, MA, USA) for 5 min at 6000 rpm. The prolidase activity was determined by a capillary electrophoretic method (CE) previously set up 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. Each determination was performed in triplicate and data were expressed as mean ± S.D. The enzyme activity was calculated as the amount (μmol) of Gly-Pro hydrolyzed/min at 37 °C (IU). The enzyme activity was expressed as residual enzyme activity percentage (REA %), that is the percentage of the starting activity of each enzyme form considered.

2.2.4. Evaluation of PEGylated conjugates toxicity

The effects of PEGylated conjugates and of PEGylation mixture components on cell viability were assessed with a 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2*H*-tetrazolium (MTT) assay (Mosmann, 1983), using 96-well cell culture cluster in which 10,000 fibroblasts from healthy subjects and PD patients were plated in contact to different concentrations of native and PEGylated prolidase, ranging from 0.075 to 0.6 IU/ml, and Mal-PEG, from 1.1 to 8.8 μg/ml. Briefly, fibroblasts were cultured in wells in Dulbecco's-modified Eagle's medium (DMEM) supplemented with Foetal Calf Serum (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 percentage of untreated cells (control).

2.2.5. Preparation and characterization of PEGylated prolidase loaded chitosan nanoparticles

Placebo nanoparticles were prepared by combining ionotropic gelation and ultrasonication treatment (Tang et al., 2003; Colonna et al., 2007). One milliliter of TPP solution, at constant rate of 0.5 ml/min, was added to 2.5 ml of 1 mg/ml aqueous 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 in TMG (200 IU/ml) to the chitosan solution before adding cross-linking agent. 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.

Morphologic characterization of nanoparticles was performed by transmission electron microscopy (TEM 208 S, Philips, The Netherlands). Nanoparticle suspensions were diluted 10-fold with distilled water, an aliquot of 15 μl was then deposited on a Formvar coated grid (300 mesg, AGAR Scientific, UK) and stained with 1 M uranyl acetate solution as the negative staining material. The excess of reagent was removed by means of filter paper.

Diameter, polydispersity and *Z*-potential of chitosan nanoparticles were measured using a NICOMP 380 ZLS apparatus (Particle Sizing Systems, CA, 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 (Muzzarelli, 1998).

The amount of prolidase encapsulated in the nanoparticles was determined by UV spectrophotometry (Beckmann DU7500, USA) at 280 nm and it was calculated from the difference between the total amount of enzyme in the nanoparticles preparation and the amount of non-entrapped prolidase remaining in the clear supernatant after the centrifugation.

2.2.6. In vitro PEGylated prolidase release test

PEGylated prolidase loaded nanoparticles were incubated in 2 ml of 50 mM Tris–HCl (pH 7.8) at 37 °C under intermittent agitation. The final concentration of prolidase in the medium was 0.75 IU/ml.

After predetermined time intervals (1, 2, 4, 6, 8, 24 and 48 h) the samples were centrifuged at 10,000 rpm for 10 min, and 1 ml of supernantant was removed. The amount of PEGylated prolidase released was determined spectrophotometrically at 660 nm by Lowry Assay (Hartree, 1972). Analyses were carried out in triplicate.

2.2.7. Ex vivo evaluation of prolidase activity on cultured skin fibroblasts from PD patients incubated with PEGylated 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 containing 1% insulin-transferrin-sodium selenite media supplement (ITS + 3) added with 25 or 100 μl of prolidase loaded nanoparticles suspension, corresponding to 0.75 or 3 IU of enzyme. In order to set-up the optimal incubation time, fibroblasts were incubated with prolidase loaded nanoparticles for 2, 5, 6, 8, 10 and 12 days. After the period of incubation the medium was removed and stored and the cell layers washed three times with phosphate saline buffer (PBS, 7.4 pH), resuspended with PBS containing protease inhibitor (4 mM sodium ethylendiaminetetracetic 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 using the capillary electrophoretic method developed in our laboratory (Viglio et al., 2006).

For all ex vivo experiments enzyme activity has been reported as the amount of Gly-Pro hydrolyzed (μmol)/mg of protein at 37 °C.

2.3. Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). 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. Characterization of PEGylated prolidase

SDS-PAGE and ESI-MS have been used to evaluate the formation of prolidase and Mal-PEG conjugates. The results of electrophoretic runs performed on native prolidase before and after its reaction with Mal-PEG are shown in Fig. 1, lanes 2 and 3, respectively. The appearance in lane 3 of a large band (band B), reactive

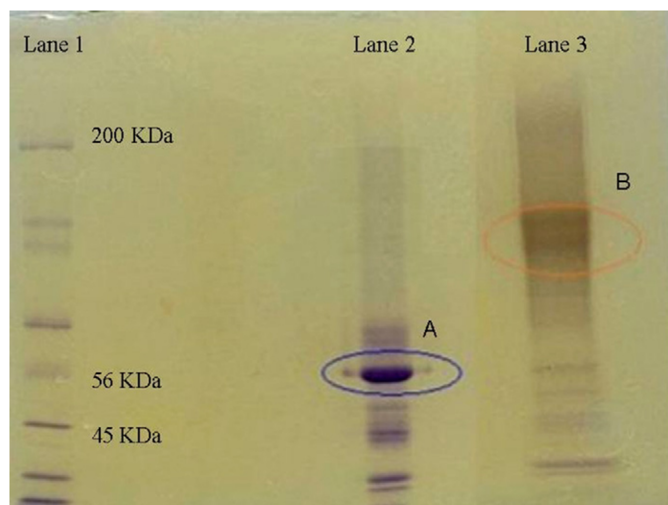

Fig. 1. SDS-PAGE electrophoresis slab after Coomassie Brilliant Blue staining for proteins and after specific staining for PEG-conjugated: protein standards (lane 1), native prolidase (lane 2), PEGylated native prolidase (lane 3).

toward the selective staining for PEGylated compounds, and the concomitant disappearance of the band corresponding to native prolidase (Fig. 1, lane 2, band A) allowed to observe the formation of conjugates between Mal-PEG and prolidase. In any case, the molecular mass of the PEGylated prolidase could not be determined owing to the band broadening and to the electrophoretic mobility that, for these conjugates, is known not to be strictly related to the Mw (Kurfürst, 1992). Thus, to gain more information on molecular mass of prolidase conjugates, the reaction mixture was submitted to RP-HPLC coupled with ESI-MS. Not surprisingly, the separation of the reaction products let to obtain a chromatogram (Fig. 2), in which five different peaks (indicated as peaks 1–5) could be highlighted. Our interest was previously focused on the presence of a major peak (Fig. 2, peak 2) that accounted for 68.19% of the total protein eluted. Based on MS data shown in panel A of Fig. 3, its apparent molecular mass (Mr = 65,490 Da) corresponded to that expected by the conjugation of two residues of Mal-PEG (10,000 Da) with a monomer of prolidase (Mr ≅ 55,000 Da). However, the finding of other minor peaks allowed to hypothesize that, although in a lower extent, other products could be originated by this reaction. In particular, MS analysis (Fig. 3, panel B) allowed to identify peak 1 (Mr = 54,910 Da; 5.20% of the total protein eluted) as unreacted prolidase and preliminary MS data suggested that peaks 3 and 4 could represent the conjugation products of prolidase with higher number of Mal-PEG monomers (3 and 5, respectively). Given the low concentration of material under peak 5, no information could be collected for this specimen. Details concerning these peaks are summarized in Table 1.

To improve the reactivity of the thiol groups of prolidase in the native state, we studied also the conjugation under partially denaturing conditions. The fact that a very similar pattern of peaks (unreported data), in terms of Mr and relative abundance (%), was obtained when denaturated prolidase was used for conjugation reaction with Mal-PEG clearly indicated that both native and denaturated enzyme had an identical reactivity and stoichiometry of reaction.

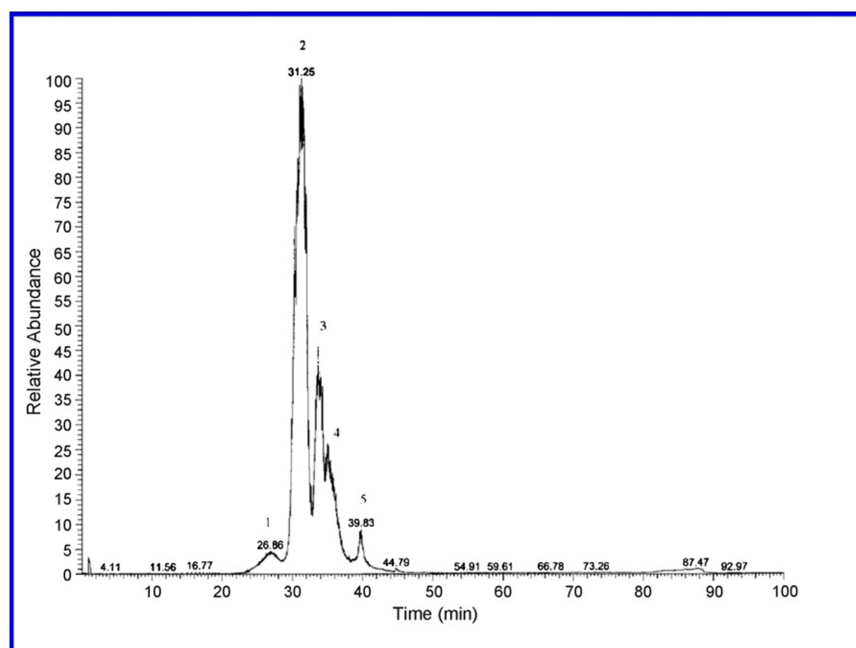

Fig. 2. ESI-MS chromatogram of the reaction mixture between prolidase and Mal-PEG: five different peaks (indicated as peaks 1–5) could be highlighted.

Table 1
Principal characteristics of the five major peaks of the PEGylated prolidase ESI-MS spectrum

Peak no.	Retention time (min)	Mean Mw (Da)	Conjugated Mal-PEG monomer no.	Relative abundance (%)
1	25.6	54,000	–	5.20
2	31.5	65,000	2	68.19
3	32.9	70,000	3	13.95
4	33.8	80,000	5	9.70
5	39.3	n.d.	n.d.	2.94

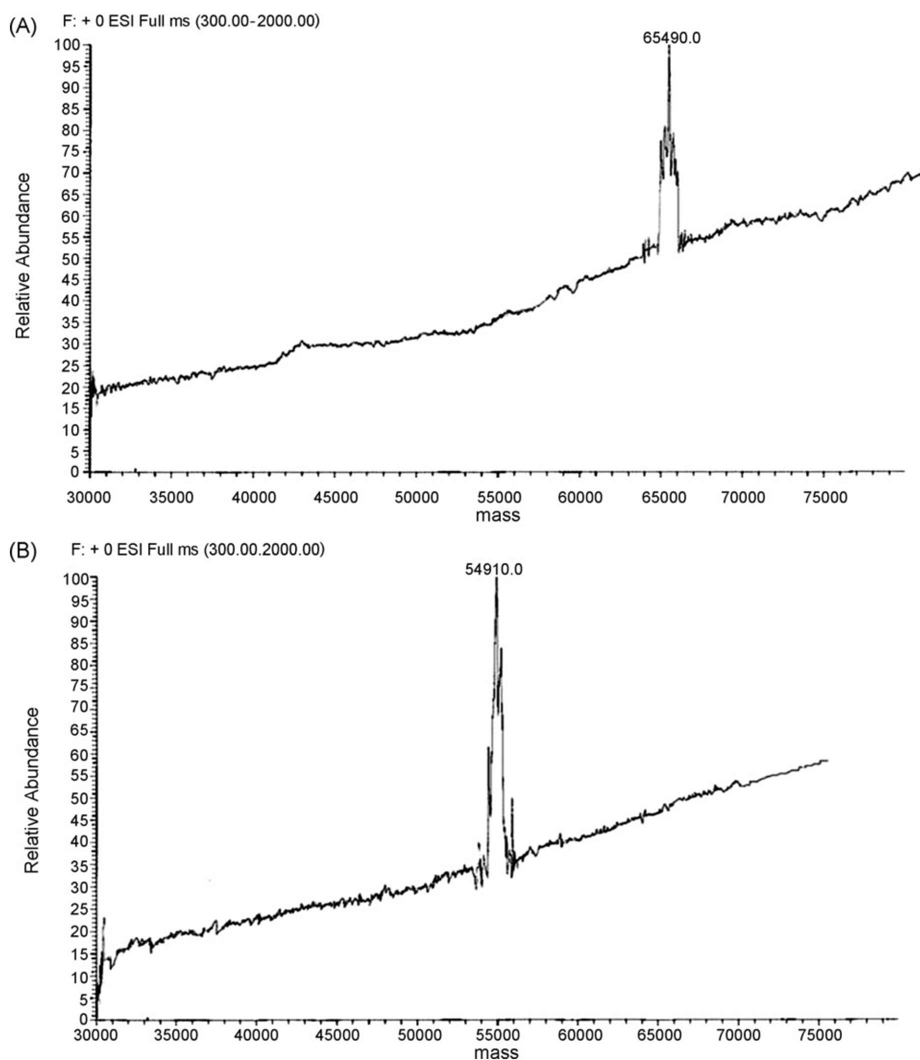

Fig. 3. Particular of the ESI-MS analysis of PEGylated prolidase: (A) magnification of the peak 2, that accounted for 68.19% of the total protein eluted and had the apparent molecular mass of 65,490 Da; (B) magnification of the peak 1, identified with the unreacted prolidase with the apparent molecular mass of 54,910 Da and corresponding to 5.20% of the total protein eluted.

3.2. Stability evaluation of native and PEGylated prolidase

The evaluation of enzymatic activity was carried out by CE on samples of native and PEGylated prolidase at concentration of 0.4 IU/ml. PEGylation procedure performed under the conditions described in the experimental section did not determine any loss of enzyme activity; indeed the conjugated prolidase exhibited a stability much higher than the native enzyme. As shown in Fig. 4, where the behaviour of these two species are compared, after 8 h of incubation at 37 °C in 50 mM Tris–HCl (pH 7.8) native prolidase lost more than 50% (REA % = 56.85 ± 3.41%) of its initial activity following a biphasic kinetic of inactivation.

By contrast, PEGylated enzyme was inactivated following a pseudo-first-order kinetic, that allowed the enzyme to save more than 80% (REA % = 81.56 ± 2.61%) of its initial activity after 8 h of incubation under the same experimental conditions described above. After 48 h of incubation, the residual activity of the native enzyme was less than 1%, while PEGylated species kept 40.68 ± 2.62% of their initial level. Based on these data, it is possible to conclude that the PEGylation procedure set up in the first part of this work was able to preserve and prolong the prolidase activity.

3.3. Evaluation of PEGylated conjugates toxicity

The toxicity of PEGylated prolidase and of all compounds used in the PEGylation process was assessed by MTT test on healthy

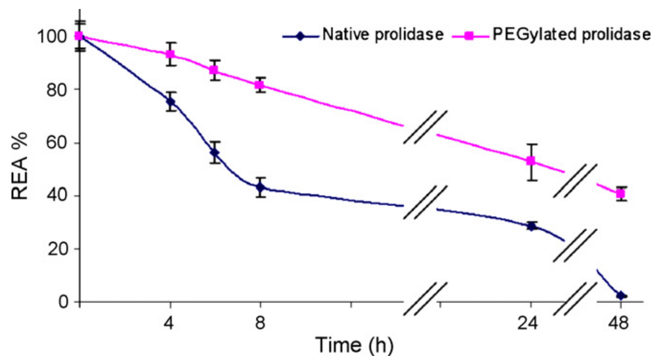

Fig. 4. Stability profile of native and PEGylated prolidase after incubation at 37 °C for scheduled times. The enzyme activity was expressed as residual enzyme activity percentage (REA %), that is the percentage of the initial activity of each enzyme form considered. Data shown are the mean ± S.D. ($n = 3$).

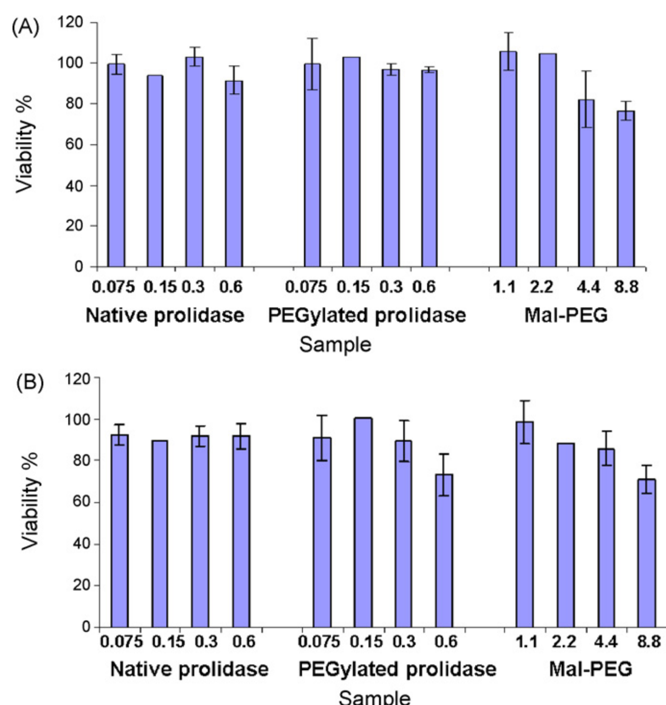

Fig. 5. The influence of different amounts of PEGylated prolidase on healthy (A) and PD (B) fibroblasts viability (MTT test). Data shown are the mean ± S.D. ($n = 6$).

and PD fibroblasts: different concentrations of native and PEGylated enzyme in the range of 0.075–0.6 IU/ml and the corresponding concentration of Mal-PEG (1.1–8.8 μg/ml) were added to the cell culture media. The quite broad range of concentration tested was selected regarding to the potential loading of the conjugates into chitosan nanoparticles (Colonna et al., 2006), while the amounts of Mal-PEG tested were calculated in relation to the total amount of PEGylating agent employed into enzyme samples considered. Data obtained by the MTT assay on healthy and PD fibroblasts (Fig. 5A and B) showed that the native and PEGylated enzyme did not interfere with cellular metabolism: PD fibroblasts highlighted a slight reduction in viability (viability % = 73.3 ± 10.3%) after incubation with the higher concentration of PEGylated prolidase tested, even if this result is neutralized by a broad standard deviation of the collected data.

Mal-PEG seemed to be a very safe PEGylating agent: all the sample tested on both types of fibroblasts considered did not affect the cellular viability up to the concentration of 4.4 μg/ml of medium and the only sample causing a significant decrease in viability percentage (viability % = 76.7 ± 4.72%) was the Mal-PEG concentration of 8.8 μg/ml.

In any case, the viability studies confirmed the good biocompatibility of the PEGylated prolidase, so it was possible to employ the conjugation products for encapsulation into nanoparticles and for the ex vivo studies of the replaced prolidase activity.

3.4. Characterization of PEGylated prolidase loaded nanoparticles and in vitro release test

After performing its characterization, PEGylated prolidase was loaded into the chitosan nanoparticles. The preparation method based on TPP ionic gelation combined with one cycle of ultrasonication was set up in a previous work (Colonna et al., 2007). The obtained PEGylated prolidase showed spherical shape (Fig. 6) and a narrow size distribution: the mean diameter of nanoparticles

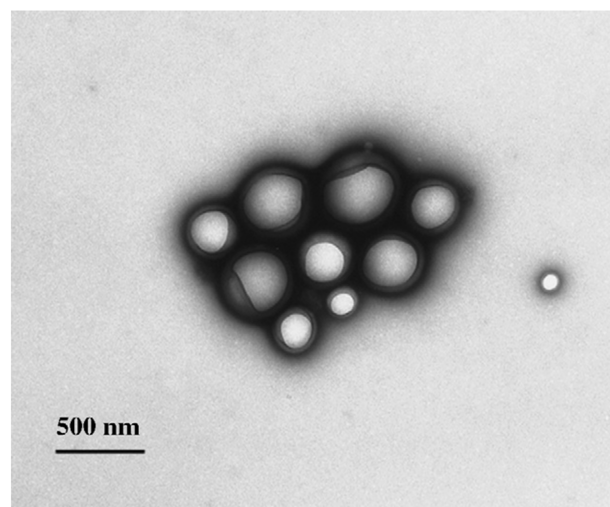

Fig. 6. TEM photomicrograph of PEGylated prolidase loaded chitosan nanoparticles. TEM photomicrograph (magnification 85,000×) shows the image of few distinctive single particles, each possessing a typical spherical shape and similar nanometric dimension.

was about 270 nm (271.6 ± 45.5 nm) with a positive zeta-potential of about 16 mV (15.93 ± 0.26 mV). The yield of process was quite satisfactory (54.21 ± 5.71%) and the results concerning the actual enzyme content revealed a good protein encapsulation efficiency of about 45% (44.8 ± 4.6%).

In vitro release studies were performed in 50 mM Tris–HCl (pH 7.8) at 37 °C in 48 h. As shown in Fig. 7 the release profile of PEGylated prolidase revealed a faster release up to the 6th hour (about 50%), with a “burst” effect of about 25% in the 1st hour, followed by a subsequent gradual release up to 48 h.

In a previous work the biocompatibility and the capability of preserving enzymatic activity of these drug delivery systems were assessed (Colonna et al., 2007). Thus, in this study the advantage of the PEGylation process and the encapsulation of the PEGylated prolidase into nanoparticles were matched to performed the ex vivo evaluation of the potential enzyme replacement.

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

The effect on enzymatic activity replacement due to PEGylated prolidase loaded nanoparticles was investigated by CE. Cultured skin fibroblasts from PD patients were plated in contact with different amount of nanoparticles: the volumes of nanoparticles suspension tested in this study were chosen considering results obtained by a previous work in which the native enzyme was considered (Colonna et al., 2006). Twenty-five microliters (NP25) or

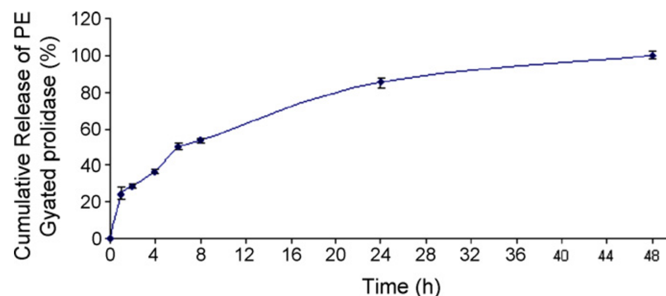

Fig. 7. In vitro release profile of PEGylated prolidase from chitosan nanoparticles (mean ± S.D., $n = 3$).

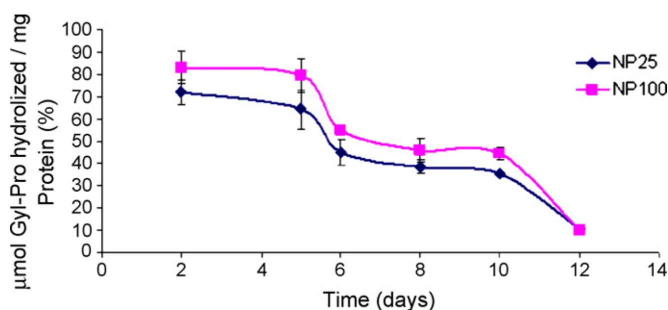

Fig. 8. Prolidase activity (expressed as μmol Gly-Pro hydrolized/mg Protein (%)) detected in the cellular extracts after different time of incubation of PD fibroblasts with prolidase loaded chitosan nanoparticles. The legend is referring to 25 or 100 μl of nanoparticles suspension as NP25 and NP100, respectively. Data shown are the mean ± S.D. (n = 6).

100 μl (NP100) of suspension were added to fibroblasts flasks and at fixed times cellular extracts were collected.

Prolidase is a cytosolic enzyme and had not any specific membrane carrier. In order to investigate whether the presence of PEG molecules was able to create an a-specific interaction with cellular membrane and to promote the up-take of free enzyme, PEGylated prolidase solution (0.4 IU/ml medium) were added to culture media: the hydrolytic activity was fully recovered in the media but not in the cell extracts, even if the presence of PEGylated conjugates seemed to improve the morphology of PD fibroblasts (unreported data).

After incubation with PEGylated prolidase loaded nanoparticles, the prolidase activity was assayed in the cell extracts: Fig. 8 shows the results of the restored enzymatic activity during a long time of incubation (up to 12 days). A similar profile of restored activity was obtained by NP25 and NP100; indeed, it was not possible to reveal a restored enzymatic activity proportional to amount of nanoparticles incubated with PD cells, even if the cellular answer can be considered significantly different till to 10 days (P < 0.05).

Nanoparticles were quickly up-taken by cells and PEGylated prolidase released from nanoparticles was able to drastically reduced the amounts of endogenous Gly-Pro. After only 2 days of incubation NP25 and NP100 were effective to restore about 72% and 83% of the prolidase activity in the cells, and these percentages were kept almost constant up to 5 days. These values are sensibly higher than the maximum values (66.85 ± 4.51%) of restored activity obtained by 5 days of incubation of PD cells with an amount of native prolidase loaded nanoparticles corresponding to NP100. Moreover, it is possible to highlight a faster cellular answer to the incubation with prolidase loaded nanoparticles when the enzyme is PEGylated. Starting from 2 days of incubation the enzymatic restored activity is increased of about 44% and 40%, respectively for NP25 and NP100, using PEGylated prolidase with respect to the co-incubation of cells and native prolidase loaded nanoparticles. Because the main nanoparticles features (mean diameter, Z-potential value and loading efficiency) and the initial activity of the modified enzyme did not significantly change, it is suitably possible to suppose that when PEGylated, the prolidase modifies its overall charge at working pH during the encapsulation procedure, leading to a weaker interaction with chitosan chains. Indeed, native prolidase posses an isoelectric point of about 4.65 and shows a negative overall charge at working pH (about 7.8). The PEGylation may modify this charge distribution leading to a faster release from the nanoparticles and a ready activation of the enzyme in the cytoplasm.

Longer incubation times do not induce any better enzyme replacement, in fact after 6 days of incubation NP25 and NP100 showed a heavy reduction in hydrolytic activity percentages: these values decreased up to 45% and 55%, respectively, but they were maintained up to 10 days. These percentages are more than five-fold the value (9.61 ± 2.22%) obtained by 10 days of incubation of PD cells with nanoparticles loaded with native prolidase. This brilliant result could be explained by the presence of PEGylated prolidase: the PEGylated prolidase resulted more stable than the native enzyme and the biphasic profile of the replaced enzymatic activity resembled to the enzyme stability profile reported in Fig. 4.

After 12 days of incubation the recovered enzymatic activity fell to very low values (10.31 ± 2.72%) and morphological features of cellular sufferance were highlighted: this experimental evidence could be explained not only by the enzyme inactivation but also by the culture media exhaustion and by the growth contact inhibition, as confirmed by cellular counts (unreported data).

After the co-incubation with PEGylated prolidase loaded nanoparticles, PD fibroblasts obtained an improvement in the morphological features in terms of regular shape, nuclear organization and reduction of cytoplasmatic swelling (unreported data).

4. Conclusion

The site-directed PEGyilation of prolidase at sulphydril groups allowed to obtain a mixture of PEGylated products that presented the native enzyme conjugated with two residues of PEG-Mal as major product revealed by ESI-MS spectra (m/z = about 65,000 Da; relative abundance (%) = 68.19%). The PEGylation of the prolidase performed in mild condition did not modify its initial activity and significantly prolonged the enzyme activity when incubated in solution at 37 °C. Indeed PEGylated prolidase kept about 41% of its initial activity up to 48 h (REA = 40.68 ± 2.62%), while the native enzyme becomes completely inactive.

Moreover, the MTT assay revealed that PEGylated prolidase at the concentrations tested did not interfere with healthy and PD fibroblasts growth.

The loading of the modified enzyme into chitosan nanoparticles let to the cell internalization of the prolidase in the active form, showing the recovery of the fibroblasts regular shape and of a suitably prolonged (up to 10 days) hydrolytic cytoplasmatic activity (up to about 72% after only 2 days of co-incubation with nanoparticles).

This study demonstrated the efficacy of PEGylated prolidase loaded chitosan nanoparticles in restoring normal prolidase activity inside cells, thus it could create the chance of a suitably effective enzyme replacement therapy for PD.

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