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Research paper

Supramolecular association of recombinant human growth hormone with hydrophobized polyhydroxyethylaspartamides

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Abstract

The protein delivery properties of polymer supramolecular assemblies were investigated by using recombinant human growth hormone (rh-GH) and two polyhydroxyethylaspartamide (PHEA) derivatives: (a) PHEA-C_{16} obtained by PHEA random grafting with hexadecylalkylamine; (b) PHEA-PEG_{5000}-C_{16} obtained by PHEA random co-grafting with hexadecylalkylamine and 5 kDa poly(ethylene glycol). The two polymers possessed similar self-assembling properties: critical micelle concentration (CMC) and particle size. The protein loading (protein/polymer, w/w, %) was $12.1 \pm 1.3\%$ and $8.5 \pm 0.4\%$ with PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}, respectively. The rh-GH/polymer association constant calculated by Scatchard analysis was $1.87 \times 10^5\ M^{-1}$ with PHEA-C_{16} and $0.27 \times 10^5\ M^{-1}$ with PHEA-PEG_{5000}-C_{16}. The Klotz analysis showed that 5 PHEA-C_{16} and 9 PHEA-PEG_{5000}-C_{16} polymer chains associated with one protein molecule. The protein dissociation from the PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} supramolecular complexes was complete in about 350–450 and 450–550 h, respectively. With both polymers, the protein release was faster as the protein/polymer ratio increased. Pharmacokinetic studies were performed by subcutaneous administration to rats of protein/polymer solutions at different w/w ratios (1:75 and 1:150). Both polymer formulations slowed the protein absorption. The protein bioavailability increased as the protein/polymer complex stability decreased and the protein/polymer w/w ratio increased indicating that efficient protein delivery can be achieved by proper polymer choice and formulation composition.

Keywords: Protein delivery; Supramolecular assembly; Growth hormone; Polyhydroxyethylaspartamide

1. Introduction

Throughout the last decades, various depot–payout and bioconjugation technologies, namely micro/nanoparticles and polymer bioconjugates, have turned out to overcome a few issues associated with protein formulation and delivery such as low stability, inconvenient pharmacokinetics and poor immunological properties [1–5]. However, several drawbacks are associated with micro/nanoparticles, such as low drug loading, burst and incomplete drug release, low protein stability during processing and long-term storage. On the other hand, polymer conjugation often provokes a dramatic reduction of protein biological activity that, although balanced by the increased systemic exposure, may in some cases compromise its therapeutic value [6,7].

Soluble supramolecular assemblies obtained by physical protein/polymer associations are deemed a valuable alternative tool to micro/nanoparticle formulation and polymer bioconjugation. A variety of soluble polymers can be properly designed to yield protein/polymer aggregates,

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complexes, micelles, supercolloids and other supramolecular structures, which can be exploited for medical and pharmaceutical applications.

Protein/polymer associations can be induced either by specific or non-specific interactions. Lactosyl and biotin functionalized rotaxanes, for example, can selectively interact with galectin and streptavidin, respectively, by a cooperative multivalent binding which yields highly stable complexes [8,9]. Less specific polymer/protein interactions can be obtained by using polyion macromolecules, similar to the charged vectors used for oligonucleotide delivery [10,11]. As an example, lysozyme was found to form a rather stable complex with a poly(ethylene glycol)-poly(aspartic acid) copolymer and the micelles, composed by 42 polymer chains and 36 molecules of protein, were found to possess a diameter of about 50 nm [12].

Amphiphilic polymers with different compositions and architecture used for delivery of sparingly soluble drugs seem to offer interesting opportunities for production of soluble colloidal protein delivery systems by physical assembly. Their self-assembling properties can, in fact, provide for the formation of micelle-like nanoparticles that entrap and stabilize drugs into their hydrophobic core, while exposing hydrophilic functions to the surface, preventing interaction with circulating proteins or hydrophobic surfaces [13–17].

Polymer grafting with hydrotropic moieties such as nicotinamide, salicylic acid and polycyclic moieties can generate macromolecules that can interact with proteins. Cholesterol-bearing pullulan, for example, associates spontaneously with many globular proteins in water to incorporate them into soluble microgels formed by 0.9:1 protein/polymer molar ratio complexes with size ranging from 13 to 17 nm diameter depending on the degree of polymer composition [18,19].

Recently, comb-like hydrophobically modified poly(glutamate)s have been specifically designed for protein delivery. Poly-(L-glutamate)-poly-(L-leucine) copolymer and hexadecylalkylamine-grafted poly-(L-glutamate) have been found to associate with various cytokines, namely IL-2 and interferons, forming soluble supramolecular complexes. *In vivo* studies demonstrated that the drug is slowly released from these supramolecular systems by displacement mechanisms, resulting in prolonged protein release: after intravenous injection the polymer formulated protein displayed about sevenfold higher $t_{1/2}$ as compared to the unformulated protein [20,21].

Polyhydroxyethylaspartamide (PHEA) is a polymer potentially exploitable for preparation of protein/polymer supramolecular delivery systems. This polymer as well as few derivatives has been found non-immunogenic, non-toxic and biocompatible in many cell culture studies and after oral administration or ocular application [22–24]. Because of its multifunctional character, PHEA can be properly functionalized with hydrophobic and hydrophilic moieties, endowing bioconjugates with peculiar physicochemical properties, namely hydrophilic/hydrophobic balance, hydrodynamic volume and self-assembling features [24]. By virtue of their ability to form polymer micelles, alkyl- and alkyl/PEG-grafted PHEAs have been demonstrated to be suitable for delivery of poorly soluble and degradable drugs [25]. Furthermore, similar to plain PHEA, cell toxicity studies demonstrated that PEG and alkyl chain grafted PHEAs do not induce toxic effects indicating their good biocompatibility [24].

In order to evaluate the potential of hydrophobized PHEAs in protein delivery, the association of recombinant human growth hormone (rh-GH) with hexadecylalkylamine-grafted PHEA (PHEA-C_{16}) and 5 kDa poly(ethylene glycol)/hexadecylalkylamine co-grafted PHEA (PHEA-PEG_{5000}-C_{16}) was investigated. *In vitro* studies were performed to gain information about the main parameters that dictate the physicochemical properties of the polymer/protein supramolecular assembly. *In vivo* studies were carried out to investigate the pharmacokinetic behaviour of the new protein delivery systems.

2. Materials and methods

Recombinant human growth hormone (rh-GH) was a kind gift of Bio-Ker (Pula, Italy). Hexadecylalkylamine, Nb2-11 cell line, penicillin, streptomycin, trypsin solution, Fisher medium, horse serum and foetal bovine serum were from Sigma (St. Louis, MO, USA). Linear 5 kDa monoamine-poly(ethylene glycol) was provided by Nektar (Huntsville, AL, USA) or by Fluka (Buchs, Switzerland). [^{3}H]-succinimidyl propionate and [^{3}H]-ethanolamine came from Amersham International (Amersham, UK). Instagel for radioactivity determinations was obtained from Canberra Packard (Groningen, The Netherlands). All the other solvents and chemicals of technical grade were from Fluka (Buchs, Switzerland).

In vivo studies were carried out using male rats weighing 200–250 g. Care and handling of animals used for the pharmacokinetic studies were in accordance with the provisions of the European Economic Community Council Directive 86/209 (recognized and adopted by the Italian Government with the approval decree D.M. No. 230/95-B) and the NIH publication No. 85-23, revised in 1985.

2.1. PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} synthesis

Hexadecylalkylamine-grafted poly(hydroxyethylaspartamide) (PHEA-C_{16}) and the 5 kDa poly(ethylene glycol)/hexadecylalkylamine co-grafted poly(hydroxyethylaspartamide) (PHEA-PEG_{5000}-C_{16}) were prepared according to the synthesis protocol reported elsewhere [22]. Briefly, PHEA-C_{16} was obtained by PSI reaction with hexadecylalkylamine in DMF solution at 60 °C for 7 h followed by reaction with ethanolamine in DMF solution for 3 h, keeping the reaction temperature between 22 and 26 °C. PHEA-PEG_{5000}-C_{16} was obtained by PSI reaction with 5 kDa PEG-NH_2 in DMF solution at 60 °C for 30 h

followed by reaction with hexadecylalkylamine and ethanolamine as reported above. The final product composition was determined by elemental analysis, IR spectrophotometry and ^{1}H NMR. The molecular weight and polydispersity index were determined according to the procedure reported in the literature [26]

2.2. PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} critical micelle concentration

Phenantrene/polymer samples were prepared by mixing 100 μg of phenantrene with 1 ml of a 1, 0.8, 0.5, 0.25, 0.1, 0.05 or 0.025 mg/ml solution of PHEA-C_{16} or PHEA-PEG_{5000}-C_{16} in distilled water. The samples were maintained overnight under shaking at 37 °C. The mixtures were centrifuged at 5000 rpm for 5 min and the solutions were analysed by spectrofluorimetry (λ_{ex} 294 nm, λ_{em} 365 nm). Each experimental point was repeated five times and the results were analysed according to the method reported in the literature [27].

2.3. Light scattering analysis

The light scattering analyses were performed by using a single angle photon correlation spectrometer (Nicomp 380, Particle Sizing Systems, Santa Barbara, CA) equipped with a 632.5 nm laser. Polymer and protein/polymer samples were prepared by using PHEA-C_{16} or PHEA-PEG_{5000}-C_{16} solutions at different polymer concentrations (0.2–2 mg/ml) in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2, maintained overnight at room temperature. The protein/polymer samples were prepared by using 12% and 8% (w/w) rh-GH/PHEA-C_{16} and rh-GH/PHEA-PEG_{5000}-C_{16}, respectively. The solutions were centrifuged at 5000 rpm for 5 min and filtered using 0.22 or 0.45 μm cut-off membranes and finally analysed.

2.4. PHEA-C_{16}/rh-GH and PHEA-PEG_{5000}-C_{16}/rh-GH associations

Polymer/rh-GH samples were prepared by addition of 20 μl of 20 mg/ml polymer solutions in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4 to 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 μl of 2 mg/ml rh-GH solution in the same buffer. Buffer volumes were added to reach a final sample volume of 200 μl. The samples were maintained overnight at room temperature and then centrifuged at 5000 rpm for 3 min, filtered with a 0.22 μm cut-off filter and finally analysed by gel permeation HPLC using a Bio-Gel SEC 40XL (300 × 7.8 mm) column (Bio Rad, Hercules, CA, USA). The column was isocratically eluted with 63 mM phosphate buffer, pH 7.4/isopropanol 3%. The detector was set at 276 nm. The non-associated and associated protein were determined by analysis of the corresponding protein peak area according to a standard titration curve obtained by using fixed protein amounts ($y = 1518.1x$; $R^2 = 0.999$).

A molecular weight calibration curve obtained using rh-GH (21 kDa), superoxide dismutase (31 kDa), bovine serum albumin (69 and 138 kDa monomer and dimer, respectively), gamma globulin (158 kDa) and urease (480 kDa) was used to determine the apparent molecular weight of the polymer associated protein.

2.5. Scatchard and Klotz evaluation

The experimental data obtained by the association studies described above (bound protein and free protein) were evaluated according to the Scatchard [28] and Klotz equations [29] to calculate the protein/polymer affinity constants (k) and the interaction site number (n):

Scatchard: $[\text{rh-GH}]_b/[\text{rh-GH}]_f = nk - k[\text{rh-GH}]_b$

Klotz: $1/[\text{rh-GH}]_b = (1/nk)(1/[\text{rh-GH}]_f) + 1/n$

where $[\text{rh-GH}]_f$ is the molar concentration of the free rh-GH and $[\text{rh-GH}]_b$ is the molar concentration of the bound rh-GH (polymer associated protein).

2.6. rh-GH dialysis studies

^{3}[H]-radiolabelled rh-GH was prepared according to the procedure reported elsewhere [30]. ^{3}[H]-rh-GH/polymer samples were prepared by dissolving 15, 75 or 150 mg of polymer in 1 ml of 1 mg/ml ^{3}[H]-rh-GH solution in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4. After 12 h of incubation at room temperature, the solutions were placed into a 100-kDa cut-off dialysis Float-A-lyzer tube (Spectrum Laboratories, Los Angeles, CA, USA) and dialysed against 11 ml of the same buffer at room temperature. At scheduled times, 1 ml of external buffer was withdrawn and replaced with fresh buffer. The sampled volumes were analysed by RP-HPLC using an analytical (250 × 4.6 mm) C-18 Luna column (Phenomenex, Torrance, CA, USA) eluted with H_2O/0.05%TFA (eluent A) and acetonitrile/0.05%TFA (eluent B) in a gradient mode: 0–3 min 45% eluent B, 3–23 min from 45% to 70% eluent B. The UV detector was set at 210 nm. The protein amount was determined on the basis of a protein titration curve ($y = 15995x$; $R^2 = 0.9965$). Fifty microlitre samples were also analysed by radioactivity after addition of 3 ml of Instagel cocktail. The radioactivity data were compared to a protein/radioactivity titration curve.

2.7. PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} microdialysis

^{3}H-radiolabelled PHEA-C16 and PHEA-PEG_{5000}-C_{16} were prepared according to the procedure reported elsewhere [24]. rh-GH/polymer samples were prepared by dissolving 15, 75 or 150 mg of ^{3}[H]-radiolabelled polymer in 1 ml of 1 mg/ml protein solution in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4. After overnight incubation at room temperature, the solutions were placed into a 100-kDa cut-off dialysis Float-A-lyzer tube and were

dialysed against 11 ml of the same buffer at room temperature. At scheduled times, 5 ml of the external volume was withdrawn and replaced with fresh buffer. One hundred microlitre samples were added to 3 ml of Instagel cocktail addition and analysed by B-counter to determine the polymer content in the withdrawn volumes. The radioactivity data were compared to a polymer/radioactivity titration curve.

2.8. Cell culture studies

Nb2-11 cells were cultured as a monolayer in a 75 cm^2 tissue culture treated flask at 37 °C under humidified atmosphere containing 5% CO_2 using Fisher medium supplemented with 10% (v/v) horse serum, 10% foetal calf serum, 0.05 mM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin. The cells were routinely harvested by treatment with 500 μg/ml trypsin in 0.02 M phosphate buffer, 0.15 M NaCl, 200 μg/ml EDTA, pH 7.4, and the detached cells collected by centrifugation at 350 rpm were washed three times in serum free Fisher medium containing 10 mM Hepes, 0.05 mM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin. The cells were seeded in the same serum free Fisher medium and grown for 24 h and then in 96-well tissue culture treated plates at a density of 4×10^4 cells/well. Serially diluted free hGH or hGH/PHEA-PEG_{5000}-C_{16} or hGH/PHEA-C_{16}solutions (1:15, 1:75 and 1:150 protein/polymer w/w ratio) were added to each well at a final protein concentration ranging from 0 to 9 ng/ml. The cell proliferation was assessed after 46 h by using the "Cell Proliferation Biotrak ELISA System, version 2" from Amersham Biosciences. The absorbance values measured by a microplate reader at 450 nm were plotted as function of the rh-GH concentration in the assay.

2.9. Pharmacokinetic studies

Four rat groups were subcutaneously administered on the back with physiological solutions containing rh-GH/PHEA-C_{16} (1:75 and 1/150 w/w), rh-GH/PHEA-PEG_{5000}-C_{16} (1:75 and 1:150 w/w) and rh-GH alone (reference). The rh-GH dose of the protein/polymer formulations was 2.5 mg rh-GH/kg while the rh-GH dose of the reference was 0.5 mg rh-GH/kg. Blood samples were withdrawn from the animals treated with the protein/polymer formulation before treatment and after 1, 2, 4, 8, 24, 48, 72 and 96 h from administration. The animals treated with rh-GH (reference) were sampled before protein administration and after 5, 15, 30 min, 1, 2, 3, 4, 6 and 12 h after administration. The blood samples were centrifuged and the rh-GH concentration in serum was determined by ELISA, using the Active® Human Growth Hormone ELISA kit (Diagnostic Systems Laboratories Inc., Webster, TX, USA). The experimental values obtained with rh-GH alone (reference) were normalized to a 2.5 mg/kg dose. The data were elaborated in order to calculate the mean concentration value, standard deviation (±SD) and the main pharmacokinetic parameters: half-life ($t_{1/2}$), maximal concentration (c_{max}), time of maximal concentration (t_{max}) and area under the curve (AUC_{0-t}).

3. Results

3.1. Polymer synthesis and characterization

The synthesis of PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} was carried out in order to obtain polyhydroxyethylaspartamide derivatives with similar physicochemical properties [22]. The data summarized in Table 1 show that the two polymers possess comparable degrees of hexadecylalkylamine grafting. Since both copolymers are formed by the same mean number of repeating units (about 130–135), both PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} contain about 10 hexadecylalkylamine moieties per polymer chain. Similarly, the 1.7 mol % PEG content in PHEA-PEG_{5000}-C_{16} corresponds to 2–3 PEG molecules per PHEA chain. Accordingly, the 5 kDa PEG contribution to the final molecular weight of PHEA-PEG_{5000}-C_{16} was in the range of 10–15 kDa, which is in good agreement with the difference between the mean molecular weights of PHEA-PEG_{5000}-C_{16} and PHEA-C_{16}determined by gel permeation chromatography [26].

The CMC studies were performed by monitoring changes in the phenantrene fluorescence intensity as a function of the polymer concentration, according to the method reported in the literature [27]. The CMC values were calculated by fitting two portions of the fluorescent data above and below the apparent break near the CMC using a linear model and taking the intersection of the two fitted equations. The data reported in the table show that the PEG attachment did not affect the self-assembling properties of

Table 1
Physicochemical properties of PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}: molecular weight (M_w), degree of hexadecylalkylamine substitution (hexadecylalkylamine/monomer, mole/mole %), degree of PEG substitution (PEG/monomer, mole/mole %), micelle diameter and critical micelle concentration (CMC)

	M_w (kDa)	Polydispersity index	Hexadecylalkylamine content (DD, mol%)	PEG content (DD, mol%)	Micelle diameter (nm)	CMC (μM)
PHEA-C_{16}	22.3	1.8	7.4	–	23	46
PHEA-PEG_{5000}-C_{16}	34.2[a]	1.6[a]	6.6[a]	1.7[a]	16	33

[a] Ref. [26].

the PHEA derivatives as the two polymers displayed similar CMC and the micelles possessed similar size which was evaluated by light scattering analysis.

3.2. Polymer/protein association

The rh-GH association with PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} was evaluated by mixing a fixed polymer amount with increasing protein amounts in physiologic buffer; the resulting mixtures were analysed by gel permeation chromatography. The polymer associated rh-GH was eluted with a retention time of 13.3 min, while the free rh-GH was eluted at 15.5 min. The addition of small protein amounts to the polymer solution yielded the formation of the supramolecular protein/polymer association. and only the peak corresponding to the bound protein was observed. As the protein concentration increased, the peak corresponding to the polymer associated protein increased to a maximal value corresponding to the polymer saturation, while the non-associated rh-GH was eluted as free protein.

The peak areas corresponding to both bound and free protein were analysed to determine the association profiles reported in Fig. 1. The drug loading, calculated on the basis of the maximal protein association (polymer saturation) at the association profile plateau, was $12.1 \pm 1.3\%$ and $8.5 \pm 0.4\%$ (w/w) for PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}, respectively.

The gel permeation chromatography showed that the apparent molecular weight of the two polymer/protein assemblies was in the range of 120–180 kDa. Negligible differences were observed using different protein/polymer ratios.

The light scattering analysis did not show significant differences between the size of the self assembled polymers and the corresponding protein/polymer assemblies.

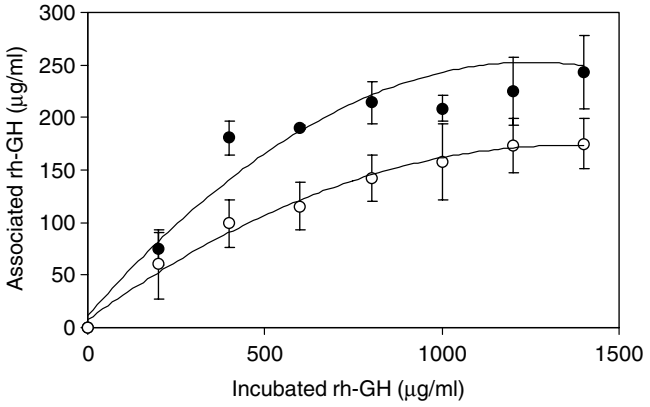

Fig. 1. Association profiles obtained by incubation of different amounts of rh-GH with 2 mg/ml of PHEA-C_{16} (●) or PHEA-PEG_{5000}-C_{16} (○). Each point reports the mean value and the standard deviation (±SD) calculated from five experimental values.

3.3. Scatchard and Klotz analysis

Fig. 2 reports the Scatchard and Klotz profiles obtained with PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}. The association constants (k) calculated by the regression Scatchard plots were $1.87 \times 10^5\ M^{-1}$ and $0.27 \times 10^5\ M^{-1}$ for PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}, respectively. The binding sites were determined by the Klotz analysis and showed a better experimental data correlation (R^2) in comparison to the Scatchard plots. The binding sites were 0.2 and 0.11 for PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}, respectively, which corresponded to 5 associated PHEA-C_{16} chains and 9 PHEA-PEG_{5000}-C_{16} chains per protein molecule.

3.4. Protein and polymer release

The protein and polymer release from the supramolecular associations was determined by dialysis. The use of 100 kDa cut-off membrane could allow for the dialysis of the protein (21 kDa) and polymer (22.3 and 34.2 kDa PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}, respectively) while the protein/polymer assembly could be retained in the donor compartment.

Fig. 3 reports the protein release profiles obtained by dialysis of protein/polymer mixtures at different w/w ratios: 1:15, 1:75 and 1:150. In the case of PHEA-C_{16}, the time required for complete protein release increased from 450 to 550 h as the protein/polymer ratio decreased. The release rate constants inversely correlated with the protein/polymer ratio, and were 0.254, 0.234 and 0.227 h^{-1} for the 1:15, 1:75 and 1:150 protein/polymer ratios, respectively. In the case of PHEA-PEG_{5000}-C_{16}, the protein release was faster in comparison to PHEA-C_{16}, as complete release was obtained in 350–450 h depending on the protein/polymer ratio. Also in this case the higher protein/polymer ratio formulation displayed higher protein release rate constant (0.484 h^{-1} with a 1:15 protein/polymer ratio), while similar release rate constant values were obtained with 1:75 and 1:150 ratios: 0.349 and 0.354 h^{-1}, respectively.

Fig. 4 reports the PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} release profiles obtained with a 1:75 protein/polymer ratio. PHEA-C_{16} was rapidly released throughout the first 48 h incubation and then the release rate decreased over time. Less than 30% of the polymer was dialysed in 550 h, when all the protein was completely released. PHEA-PEG_{5000}-C_{16} showed a smoother release when compared to PHEA-C_{16}. In this case, only about 17% of polymer was released in 450 h.

Nb2-11 cell line was used to evaluate the biological activity of rh-GH. The cell proliferation profiles reported in Fig. 5 showed that similar rh-GH concentration/cell proliferation profiles were obtained with free and formulated rh-GH, regardless of the polymer or the protein/polymer ratio used in the formulation, indicating that the protein was released from the supramolecular assembly in the

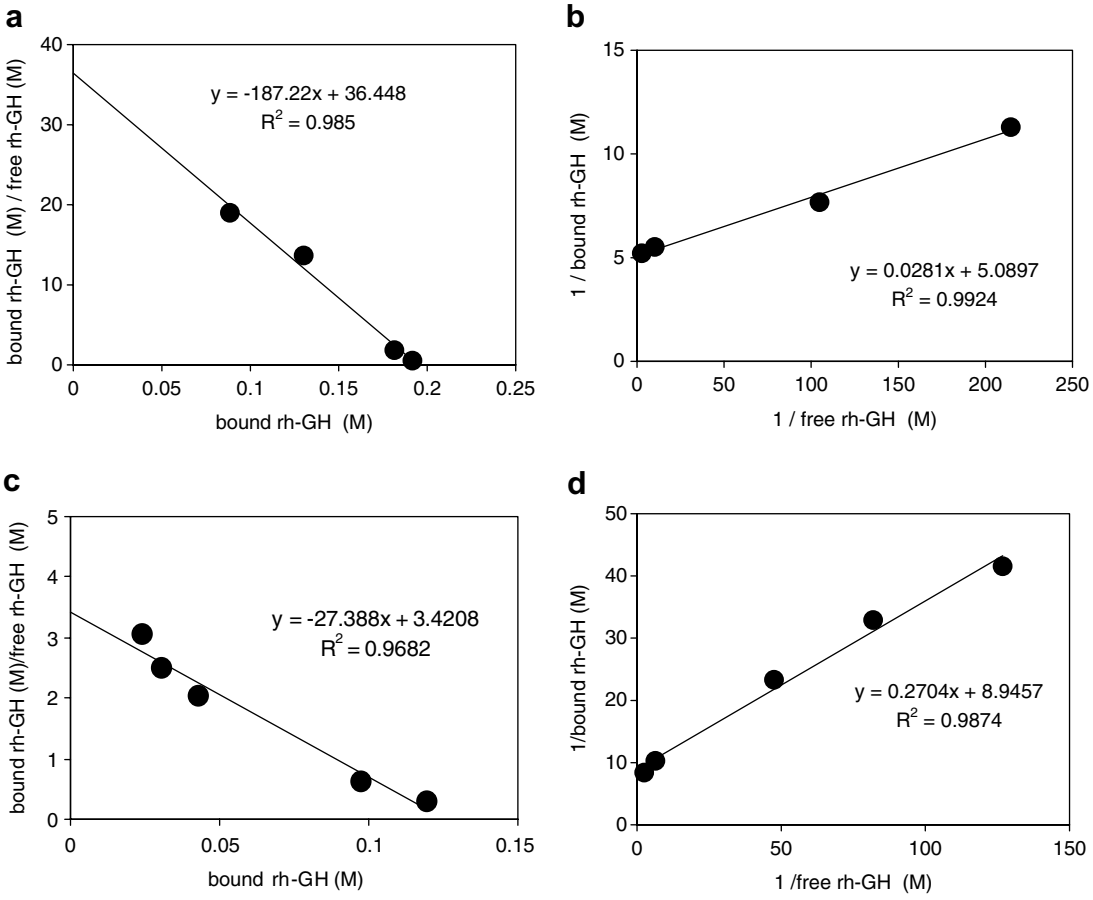

Fig. 2. Scatchard and Klotz plots of rh-GH/PHEA-C_{16} and rh-GH/PHEA-PEG_{5000}-C_{16} associations: (a) rh-GH/PHEA-C_{16} Scatchard plot; (b) rh-GH/PHEA-C_{16} Klotz plot; (c) rh-GH/PHEA-PEG_{5000}-C_{16} Scatchard plot; (d) rh-GH/PHEA-PEG_{5000}-C_{16} Klotz plot.

active form. Accordingly, similar IC_{50} values were calculated for the native rh-GH, and the protein formulated with PHEA-C_{16} and PHEA-PEG_{5000}-C_{16}, which was in the range of 350–550 pg/ml.

3.5. Pharmacokinetic studies

The higher dose of the polymer formulated rh-GH as compared to the non-formulated one was chosen on the assumption that the former should display a longer lasting behaviour as compared to the latter. Actually preliminary studies were carried out to adjust the formulated protein dose to yield an effective maximal concentration (c_{max}).

The pharmacokinetic profiles obtained by subcutaneous administration to rats of free rh-GH (normalized to the dose of 2.5 mg/kg) and those of 1:75 and 1:150 rh-GH/polymer associations are reported in Fig. 6. The main pharmacokinetic parameters, where the data referred to free rh-GH have been also normalized to the dose of 2.5 mg/kg, are summarized in Table 2.

The PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} association significantly reduced the rh-GH maximal concentration (c_{max}) and increased the maximal concentration time (t_{max}). The two polymer formulations showed a maximal concentration plateau in the range of 4–24 h that rapidly decreased during the following 24 h.

The polymer formulation was found to significantly alter the rh-GH AUC at the last point (AUC_{0-t}). The 1:150 rh-GH/PHEA-C_{16} formulation decreased the rh-GH AUC_{0-t} by about 12%, while the 1:75 rh-GH/ PHEA-C_{16} formulation increased the protein AUC_{0-t} by about 95%. Both 1:75 and 1:150 rh-GH/PHEA-PEG_{5000}-C_{16} ratio formulations yielded higher AUC_{0-t} as compared either to rh-GH/PHEA-C_{16} formulations or to rh-GH alone. The 1:75 rh-GH/PHEA-PEG_{5000}-C_{16} formulation increased the AUC_{0-t} of rh-GH by 160%, while the 1:150 rh-GH/PHEA-PEG_{5000}-C_{16} increased the protein AUC_{0-t} by about 55%.

4. Discussion

PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} were selected for preparation of protein delivery systems as they possess amphiphilic properties that can be properly exploited to form supramolecular associations with protein drugs. In fact, hydrophobic interactions are well known to take place at protein membrane interfaces, cavities and binding clefts and to play important roles in a variety of biological pro-

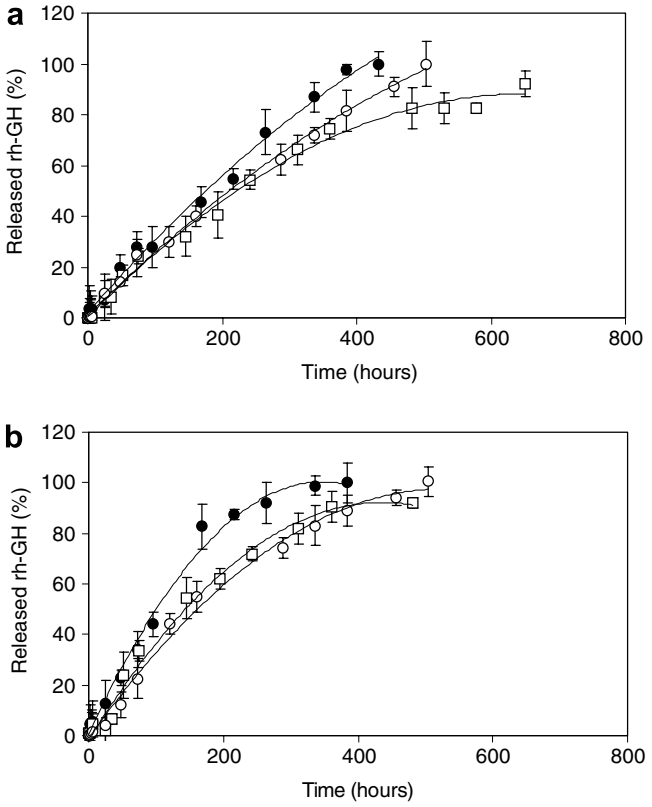

Fig. 3. rh-GH release profiles from PHEA-C_{16} (a) and PHEA-PEG_{5000}-C_{16} (b) associations: 1:15 (●), 1:75 (○) and 1:150 (□) protein/polymer weight ratio. Each point reports the mean value and the standard deviation (±SD) calculated from five experimental values.

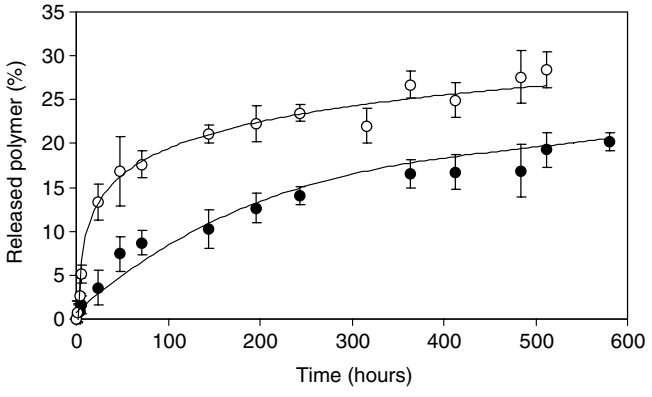

Fig. 4. PHEA-C_{16} (○) and PHEA-PEG_{5000}-C_{16} (●) release profiles from 75:1 protein/polymer ratio associations. Each point reports the mean value and the standard deviation (±SD) calculated from five experimental values.

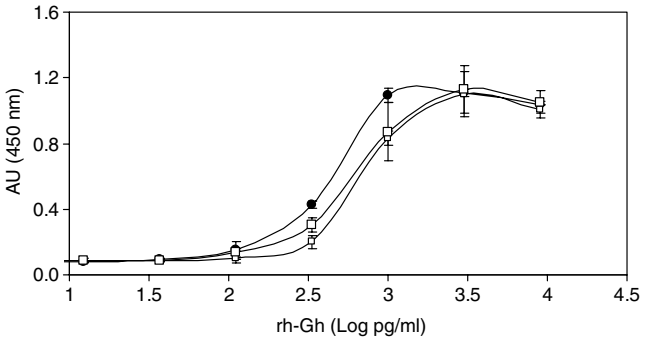

Fig. 5. Biological activity profiles obtained by incubating Nb2-11 cells with different concentration of rh-GH (●), 1:75 rh-GH/PHEA-C_{16} (□) and 1:75 rh-GH/PHEA-PEG_{5000}-C_{16} (■) w/w ratio. The mean values and the standard deviation (±SD) were calculated from at least ten experimental values.

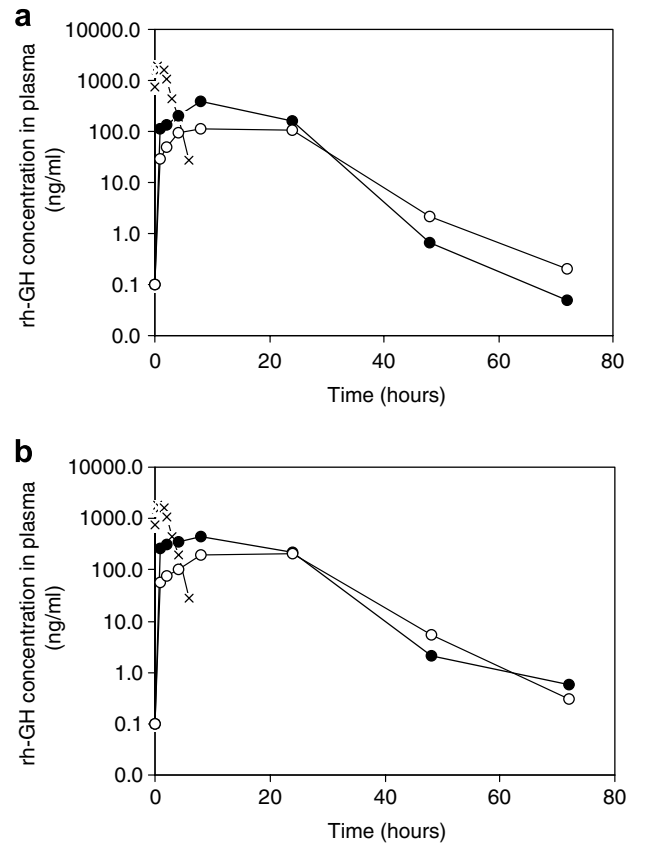

Fig. 6. Pharmacokinetic rh-GH profiles obtained by subcutaneous administration of rh-GH in the absence of polymers (×), 1:75 rh-GH/polymer (●) and 1:150 rh-GH/polymer (○) w/w ratio. Panel a reports the pharmacokinetic profiles obtained with PHEA-C_{16} and panel b the profiles obtained with PHEA-PEG-C16. Each point reports the mean value and the standard deviation (±SD) calculated from three to five experimental values.

cesses, namely ligand/protein recognition, receptor mediated triggering and cell membrane functions. Hydrophobic interactions between proteins and detergents are also well suited for probing protein binding sites [17,31,32].

The PHEA derivatization with linear alkyl moieties was motivated by the evidence that the requirements for hydrophobic binding between detergents and proteins are expected to be better met by aliphatic rather than ring-bearing detergents. In fact, a few studies showed that alkyl chains interact with proteins by hydrocarbon chain penetration deeper and deeper into the interior of proteins as a function of the hydrocarbon chain length. The limit to how much the hydrocarbon chain can be adapted by the

Table 2
Main pharmacokinetic parameters calculated with rh-GH, rh-GH/PHEA-C_{16} and rh-GH/PHEA-PEG_{5000}-C_{16} at different protein/polymer w/w ratio (1:75 and 1:150): maximal concentration (c_{max}), time of maximal concentration (t_{max}), area under the curve (AUC)

	Protein/polymer w/w ratio	c_{max} (ng/ml)	t_{max} (h)	AUC_{0-t} (ng ml^{-1} h^{-1})
Free rh-GH		1870	0.5	4227
rh-GH/PHEA-C_{16}	1:75	391	8	8229
rh-GH/PHEA-C_{16}	1:150	112	8	3705
rh-GH/PHEA-PEG_{5000}-C_{16}	1:75	452	8	10,938
rh-GH/PHEA-PEG_{5000}-C_{16}	1:150	201	8	6545

protein does not appear to be exceeded by the C14. Therefore, the hexadecyl aliphatic moiety was considered suitable for protein interaction [33].

The derivatization of about 7% of the polyaspartamide repeating units with the alkyl moiety was found to endow a soluble derivative (PHEA-C_{16}), although at high concentrations the polymer yielded highly viscous dispersions. The linear PEG co-grafting was, instead, pursued to enhance the polymer solubility, which prevents *in vivo* aggregations, conveys to the supramolecular system *in vivo* stealth properties thus impairing monocyte or polymorphonucleate phagocytosis as well as the approaching of proteolytic enzymes [34,35]. PEG could decrease the PHEA solution viscosity, which may prevent its application in drug delivery. Actually, the maximal concentration for the polymer solution syringe ability was about 150 mg/ml for PHEA-PEG_{5000}-C_{16} and 90 mg/ml for PHEA-C_{16}. On the other hand, the PEG grafting was not found to significantly alter the self-assembling properties of the polymers, which determine the supramolecular association either with low molecular weight drugs or proteins [36]. The two polymers displayed similar CMC and particle size (Table 1), which were in good agreement with the values already reported in the literature [26].

The ability of PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} to form protein/polymer associations was investigated by using recombinant human growth hormone (rh-GH) as a protein model. Similar to many other cytokines and hormones, this single domain globular protein possesses discrete hydrophobic sites that are pivotal for molecular stability and biological activity. Though rh-GH possesses a certain structural rigidity, it contains dynamically flexible helices and less structured loops, which can undergo transitory environmentally induced conformational changes. The reversible partial unfolding of the loops exposes the hydrophobic surface of the helices, resulting in increased hydrophobicity without substantial alteration of the molecular conformation. These hydrophobic surfaces, which play a leading role in the receptor interactions, are potential interaction sites for docking hydrophobic molecules anchored to hydrophilic polymers [37].

The association studies described in Figs. 1 and 2 and summarized in Table 2 showed that both PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} polymers interact efficiently with rh-GH, forming large supramolecular structures with an apparent hydrodynamic size of 120–180 kDa as determined by gel permeation chromatography. The light scattering studies demonstrated that the protein/polymer assemblies have a similar size to the protein-unloaded polymeric micelles, indicating that few protein molecules are accommodated into the loose core of the polymeric micelles without substantial alteration of the morphological properties of the polymer associations.

The Scatchard and Klotz data, as well as the *in vitro* protein release studies described in Fig. 3, showed that PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} display different assembly behaviour with rh-GH. About 5 PHEA-C_{16} and 9 PHEA-PEG_{5000}-C_{16} chains were calculated to interact with the rh-GH molecule, indicating that PHEA-C_{16} underwent more extensive protein interaction as compared to PHEA-PEG_{5000}-C_{16}. Possibly, by virtue of its high hydrophobic character, the PHEA-C_{16} alkyl chains could lie on the protein surface, establishing multi-point interactions that soon saturate the protein surface. On the other hand, the PEG chains grafted to the PHEA backbone could prevent the extensive PHEA-PEG_{5000}-C_{16} interaction with the protein surface. As a consequence of the few PHEA-PEG_{5000}-C_{16} binding points, a high number of polymer chains interacted with the protein. However, both the 1:5 and 1:9 protein/polymer stoichiometric ratios seem to be very low, considering the fact that each polymer chain bears about 10 hexadecylalkylamine chains potentially available for protein binding. Therefore, it is conceivable that in both cases only few hexadecylalkylamine moieties per polymer chain involved in the supramolecular assembly were effectively engaged in the protein interaction, while the remaining alkyl functions were involved in intra- or inter-polymer interactions. Furthermore, since the PHEA derivatives have been demonstrated to associate with an aggregation number ranging between 10 and 20 polymer molecules [26], 1–3 protein molecules were associated into the polymer micelle-like system. According to the literature reports, this supramolecular protein/polymer assembly can typically be referred to as a non-specific binding, where cooperative phenomena are involved and the interaction is not confined to single protein sequences [33,38].

The higher rh-GH/PHEA-C_{16} association constant as compared to that of rh-GH/PHEA-PEG_{5000}-C_{16}seems to confirm the impairing effect of the grafted PEG has on the protein/polymer association. Probably, similar to what is observed with PEGylated proteins, the higher polymer hydration conveyed by the PEG chains partially hampered the polymer approach to macromolecules and prevented the hormone interaction as tight as in the case of the

PEG-less polymer. Furthermore, the higher number of PHEA-C_{16} alkyl chains engaged in the protein interaction as compared to PHEA-PEG_{5000}-C_{16} may provide for a more efficient association cooperative effect.

The different association constant of the two polymers was found to reflect the different protein release from the supramolecular assembly reported in Fig. 3, which determines its *in vivo* availability and bioavailability and, in turn, its pharmacological performance. The protein release from the more stable PHEA-C_{16} complex was significantly slower than that from PHEA-PEG_{5000}-C_{16} association, and it was increasingly slower as the protein/polymer ratio decreased. In fact, both dialysis and cell culture studies showed that the rh-GH release took place by dilution and diffusion mechanisms, whereas other similar supramolecular systems were reported to undergo displacement mechanisms [20,21]. Therefore, association/dissociation equilibrium shifting towards the associated form as the association constant increased or the protein/polymer ratio decreased slowed the protein release from the supramolecular association. It is interesting to note that PHEA-C_{16} formulations could provide for a slower and sustained protein release as compared to PHEA-PEG_{5000}-C_{16}, also when high protein/polymer ratios were used.

In vitro cell culture studies reported in Fig. 5 showed that comparable protein concentration/activity profiles were obtained with free rh-GH and the rh-GH/polymer formulations: the assayed products have also comparable EC_{50} values, being their limits of confidence overlapped. This result is attributable to the high rh-GH/polymer dilutions used in the biological assay that achieved complete protein release from the supramolecular formulation. Furthermore, the release of biologically active rh-GH indicates that the penetration of alkyl moieties into hydrophobic protein cavities or the interaction with hydrophobic surfaces did not locally disrupt the rh-GH structure.

With respect to the polymer, dialysis studies showed that PHEA-PEG_{5000}-C_{16} underwent slower release from the supramolecular association than PHEA-C_{16}. This behaviour could be ascribed to the higher hydrodynamic volume of the PEGylated PHEA, which slowed the polymer diffusion through the dialysis membrane. It should be noted that in all cases only a small polymer amount was released at the end-point, when the protein was completely dissociated, probably because the polymer-to-polymer interactions were stronger than the polymer-to-protein interactions.

Finally, pharmacokinetic studies showed that the protein/polymer association could efficiently provide for sustained rh-GH release (Fig. 6 and Table 2). The polymer association decreased the maximal rh-GH concentration (c_{max}) and increased the maximal concentration time (t_{max}), indicating that the supramolecular formulations efficiently slowed the protein absorption and provided for prolonged protein delivery. Compared to the *in vitro* release data, the pharmacokinetic profiles obtained in rats show a considerable lower effectiveness in rh-GH long lasting formulation that is probably due to the fast rh-GH renal clearance. However, it should be considered that the rh-GH half-life in plasma is 0.5 h in rats and 3.6 h in man. In order to evaluate if these formulations could provide at least one week release in humans, pharmacokinetic studies have been undertaken in primates (monkeys), in which unformulated hGH has a plasma half-life of 2.6 h. Preliminary results showed that in this animal model, the formulated rh-GH displayed over 2 days half-life thus confirming the delivery effectiveness of this systems.

However, the pharmacokinetic performance of the polymer-formulated rh-GH strictly depended on the protein/polymer association properties. Using a 1:75 rh-GH/polymer ratio, the protein bioavailability (AUC_{0-t}) obtained with PHEA-C_{16} and PHEA-PEG_{5000}-C_{16} increased by 95% and 160%. Therefore, the lower the protein/polymer association constant the higher the protein bioavailability, suggesting that the protein release from the more stable supramolecular association was too slow to obtain an efficient therapeutic system as it did not guarantee the maintenance of proper protein levels for prolonged time. Also, the protein permanence in administration site could reflect in enhanced protein degradation or inactivation, which reduces the protein bioavailability. These results were confirmed by using different protein/polymer ratios. Indeed, the protein availability decreased dramatically as the protein/polymer ratio decreased, which shifted the association equilibrium towards the associated form and prolonged the protein residence in the administration site. Therefore, only 55% higher rh-GH bioavailability was obtained with the 1:150 rh-GH/PHEA-PEG_{5000}-C_{16} formulation, while in the case of the 1:150 rh-GH/PHEA-PEC_{16} product the protein bioavailability decreased by 15% as compared to the non-formulated rh-GH.

5. Conclusions

The results reported in this study show that amphiphilic polymers can be properly designed to prepare supramolecular micelle-like formulations for protein delivery. These supramolecular systems represent an interesting formulation alternative to polymer bioconjugation or micro/nanoparticle encapsulation whose development is often limited by technological and biopharmaceutical problems.

Polyhydroxyethylaspartamide offers a variety of possibilities for functionalization with both hydrophobic and hydrophilic moieties to yield tailor-made derivatives suitable for delivery of specific protein candidates, namely cytokines and hormones that present hydrophobic surfaces and cavities in their structure involved in membrane receptor interactions. Alkyl- and alkyl/PEG-grafted polyhydroxyethylaspartamides, for example, were found to form supramolecular assemblies with rh-GH, providing for prolonged protein release.

However, the study evidenced that the polymer composition plays a key role in determining the biopharmaceutical properties of the new drug delivery system.

In particular, the polymer physicochemical properties dictate the affinity for the protein, which affects both drug loading and release. Though high affinity is required to achieve high protein loading and prolonged release, tight protein/polymer interactions may negatively affect the protein bioavailability. The high protein/polymer affinity can in fact yield slow protein release in the injection site, thus enhancing the first pass effect, namely protein degradation and inactivation.

Therefore, the nature and number of the substituents to multifunctional polymers must be properly selected to yield self-assembling polymers with specific physicochemical and balanced biopharmaceutical properties, which allow for obtaining efficient protein delivery.

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