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RESEARCH PAPER

Multivalent and Flexible PEG-Nitrilotriacetic Acid Derivatives for Non-covalent Protein Pegylation

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ABSTRACT

Purpose A new approach for non-covalent protein PEGylation is translated from immobilized metal ion affinity chromatography, and based on metal coordination bonds between a chelating agent linked to PEG, nitrilotriacetic acid (NTA), and the ring nitrogen of histidines in a protein.

Methods PEG-NTA conjugates were synthesized differing in the number of NTA units and in the polymer structure. Three derivatives were investigated in association experiments with five model proteins. The most promising complex, PEG8-$(NTA)_8$–Cu^{2+}–G-CSF (granulocyte colony stimulating factor), was thoroughly characterized and the pharmacokinetic profile was evaluated in rats.

Results The experiments demonstrated that only PEG8-$(NTA)_8$, bearing eight NTA molecules on flexible PEG arms, associated strongly with those proteins having several histidines. The protein secondary structure was not affected in the complex. PEG8-$(NTA)_8$–Cu^{2+}–G-CSF showed a K_D of 4.7 nM, as determined by surface plasmon resonance, but the association was not stable *in vivo*.

Conclusions PEG8-$(NTA)_8$ is the first derivative able to associate with native proteins and form soluble complexes with a nanomolar K_D. The study highlights the need of a multivalent and flexible coordination and encourages further investigations to increase the stability of PEG8-$(NTA)_8$ complexes *in vivo* either through the use of protein mutants or His-tag proteins.

KEY WORDS bioconjugation · drug delivery · PEGylation · protein delivery

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INTRODUCTION

Proteins and peptides are promising therapeutic agents, and their clinical use is continuously increasing (1). On the other hand, the full exploitation of the potentials of such drugs is often hampered by several problems: i) short *in vivo* half-life, especially for peptides and small proteins; ii) immunogenicity, particularly relevant for heterologous proteins; iii) instability, either due to aggregation or chemical degradation; and iv) degradation by proteases. Polymer conjugation has been proposed as a solution that can address these shortcomings. In particular, since the pioneering studies of Abuchowski and Davis (2, 3), poly (ethylene glycol) (PEG) has become so far the polymer of choice, demonstrating the feasibility of this approach in preventing immunogenicity and in prolonging *in vivo* half-life of conjugated proteins (4). PEG chains attached to a protein surface can mask sites that are either immunogenic or susceptible to enzyme degradation and, at the same time, increase the hydrodynamic volume of the conjugate, thus reducing renal clearance and prolonging the *in vivo* half-life (4).

PEGylation has achieved important commercial successes as demonstrated by the several PEG products already approved for human use (4). Despite the advantages of this

technique, PEG-protein conjugates still present some limitations that prevent full exploitation of protein drugs. In particular, conjugated proteins sometimes present a marked activity reduction owing to either steric entanglement of PEG chains for the protein/receptor recognition or to modification of protein active site. Although this loss is often counterbalanced by a prolongation of the *in vivo* half-life, it still represents a crucial parameter to seek relevant improvements of therapeutic effectiveness for PEG-protein conjugates. Several approaches have been investigated to circumvent this problem. For example, "releasable PEGs" have been proposed because they can release the native protein, in its fully active form, thanks to cleavable spacers (5–9). It is important that, after polymer release, no tag remains attached to the protein surface, because they might become potential immunogenic sites. Although this is a fascinating approach, its exploitation still presents some difficulties, in particular for large-scale production, purification and storage of the conjugate, presenting an intentionally hydrolytically unstable bond/spacer.

Here, we propose for the first time a different approach of protein PEGylation based on a multiarm PEG conjugated with a chelating agent, nitrilotriacetic (NTA). NTA is able to associate with histidine side chain of proteins through reversible coordination bonds mediated by metal ions (Fig. 1). The rationale comes from the immobilized metal ion affinity chromatography (IMAC), routinely used to purify protein in molecular biology (10, 11). NTA is widely used with hexahistidine (His6)-tagged proteins for purification, fluorescent labeling (12–14), and surface immobilization (15). As demonstrated in previous studies, mono-NTA derivatives and (His6)-tagged proteins have a weak interaction—the K_D is about 10 μM—but it can be significantly increased by exploiting the multivalency of derivatives bearing several molecules of NTA (16–18). Szoka and colleagues recently proposed a new tris-NTA derivative, based on a dendritic lysine scaffold, that fits perfectly with the structure of His6 tag and yields a K_D of about 20 nM (18–20). This approach is suitable for His6-tagged proteins, and it has been successfully used for the preparation of surface plasmon resonance (SPR) chips with high NTA density (21). On the other hand, since these tris-NTA have been designed to fit the His6 tag, it is conceivable that their rigid and grouped structure might not reach a high affinity binding to native proteins without the specific tag. Therefore, these tris-NTA can be exclusively used with His6-tagged proteins. It should be highlighted that for an *in vivo* use, the His6 tag might represent a potential site of immunogenicity. Furthermore, the risk of cross-immunogenicity should also be considered when different His-tag proteins would be available in therapy.

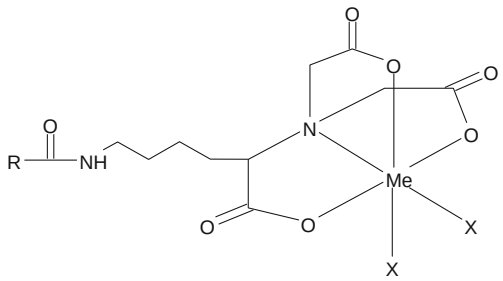


Fig. 1 Schematic representation of metal–NTA complex for octahedral configuration about a hexacoordinate central metal ion. ***X*** might be H_2O, HO^-, buffer ion or protein ligand (*e.g.* ring nitrogen of histidine or tryptophan, *etc.*), ***R*** can be an insoluble resin in case of IMAC or a PEG chain as in this study.

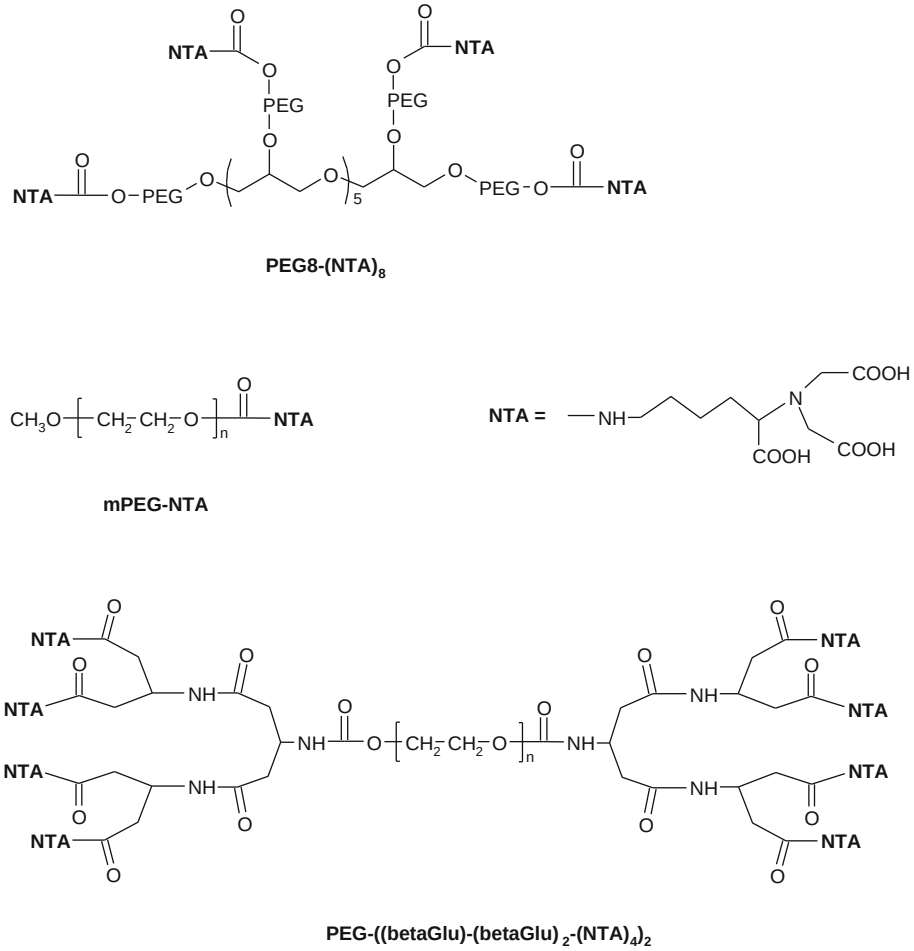
In this work, different PEG-NTA derivatives were synthetized, differing in the number of linked NTA moieties and for the flexibility of the structure (Fig. 2). The most promising conjugate, a multi–arm PEG derivative bearing eight NTA molecules (PEG8-$(NTA)_8$), was tested in association studies towards model proteins: hemoglobin, human growth hormone (hGH), granulocyte colony stimulating factor (G-CSF), interferon α-2b (IFN) and insulin (INS). The advantage of this derivative resides in the flexibility of the eight PEG arms that allow the NTA to reach and fit with the native histidines of a given protein even when these amino acids are located in dispersed sites on the protein surface. Native G-CSF strongly associates *in vitro* with PEG8-$(NTA)_8$, using copper as metal, but the complex did not show a half-life prolongation of G-CSF *in vivo*. In this study, Cu^{2+} was chosen instead of Ni^{2+} because it has lower toxicity, even if Ni^{2+} would form more stable complexes. In fact, as reported in the "Guideline on the Specification Limits for Residues of Metal Catalysts or Metal Reagents" of the committee for medicinal products for human use, the limit of parental exposure is ten times higher for Cu (250 μg/day) than for Ni (22).

To our knowledge, this study is the first example of multivalent NTA derivatives in which the chelating agents are linked to flexible arms, allowing complexation to native histidines-rich proteins (Fig. 3).

MATERIALS AND METHODS

Lyophilized rh-GH and rh-G-CSF were supplied by Bio-Ker (Pula, Cagliari, Italy). Diol PEG (MW 10,000 Da), mPEG-OH (MW 5,000 Da) and 8-arm PEG (MW 14,380 Da) were purchased from Nektar (Huntsville, AL). Nα,Nα-bis(carboxymethyl)-L-lysine hydrate (NTA), N,N-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), 2,4,6-trinitrobenzensulfonic acid (TNBS), *p*NO_2-phenyl chloroformate, and organic solvents were purchased from Aldrich Chemie (Steinheim-West-Germany). Salts of analytical grade were from Merck.

Fig. 2 Chemical structures of the studied PEG-chelating agent derivatives.



Protein concentrations were determined spectrophotometrically on a Perkin-Elmer Lambda-25 spectrophotometer. The concentrations of stock solutions of proteins and PEG-proteins conjugates were evaluated from their absorbance at 280 nm.

Gel-filtration chromatography was performed with a Shimadzu (Kyoto, Japan) analytical HPLC system, using a BIOSEP SEC S3000 column (250×7.8 mm) for GPC analysis. Elution was carried out at a flow rate of 0.5 ml/min with 10 mM tris-HCl, 50 mM NaCl, pH 7.4. For RP-HPLC analysis, a Phenomenex Jupiter column (250×4.6 mm; 5 µm; 300Å), eluted with a water +0.1% TFA/acetonitrile +0.1% TFA gradient, was used. The effluent was monitored by recording the absorbance at 226 nm or at 280 nm.

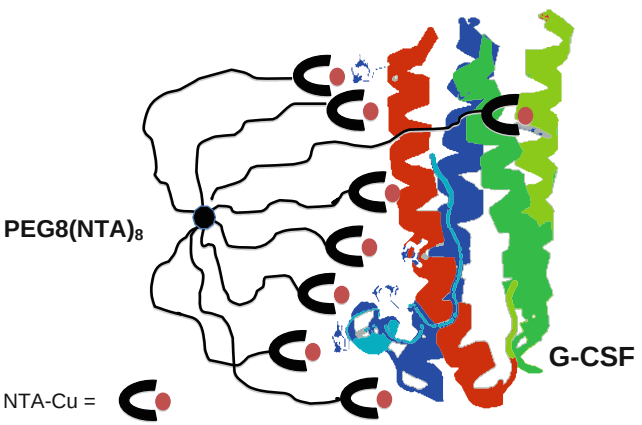


Fig. 3 Schematic representation of PEG8-$(NTA)_8$ association with G-CSF.

The kinetic interaction assay was carried out using a surface plasmon resonance (SPR) biosensor, Biacore 3000 (Biacore, Uppsala, Sweden). All the SPR experiments were conducted at 25°C in 10 mM TRIS, 50 mM NaCl, pH 7.4 with 0.005% Tween 20.

Far-UV circular dichroism (CD) measurements were made on a Jasco J-710 spectropolarimeter (Tokyo, Japan) equipped with a Peltier temperature control unit at 20°C. Measurements of native and complex G-CSF were made in 10 mM phosphate buffer pH 7.0 at the concentration solutions of 0.1 mg/mL as spectrophotometrically determined at 280 nm (absorption coefficients of G-CSF: 0.88 mL cm^{-1} mg^{-1}). The spectra were collected over the wavelength range of 190–250 nm with an average of two scans. The data at each wavelength were averaged for 8 s. The sample cell path

length was 1 mm. The CD data were converted to mean residue ellipticity, expressed in deg cm^2 $dmol^{-1}$ by applying the following formula: $\Theta = \Theta_{obs}(MRW)/10L[C]$, where Θ is the observed ellipticity in degrees, MRW is the mean residue weight of the peptide (molecular weight divided by the number of residues), [C] is the peptide concentration in mg/ml, and L is the optical path length in centimetres.

Dynamic light scattering (DLS) measurements were performed at 25°C using a Malvern Zetasizer Nano S instrument, equipped with a 633 nm laser and operating in backscatter detection (scattering angle of 173°). G-CSF and PEG8-$(NTA)_8$–Cu^{2+}–G-CSF solutions (1 mg/ml protein equiv.) were prepared per duplicate using PBS. The solutions were sonicated for 10 min at 4°C and filtered through a 0.45 μm cellulose membrane filter before analysis. Micelle size distribution by volume was measured (diameter, nm) for each sample (n≥3).

Synthesis of PEG8-$(NTA)_8$

The hydroxyl groups of 8-arm PEG (287.6 mg; 0.02 mmol; MW 14,380 Da) were activated by *p*NO_2-phenyl chloroformate (96.75 mg; 0.48 mmol; MW 201.56 Da) in anhydrous CH_2Cl_2 added with 69.7 μl of Et_3N (0.50 mmol; MW 101.19 Da). After 24 h under stirring, the reaction mixture was filtered on a gooch to remove $Et_3NH^+Cl^-$ and dropped in 150 ml of diethyl ether. The white precipitate, PEG8-(*p*NO_2-phenyl carbonate)$_8$, was recovered by filtration and dried under vacuum. The degree of activation was 95.6%, as determined by evaluating the absorption of *p*NO_2-phenol (ε_{400}=17,000 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$) released from an accurately weighted solution of the activated polymer in NaOH 0.2 N, after 30 min incubation.

Activated 8-arm PEG (250 mg; 0.016 mmol; MW 15,700 Da) was reacted with NTA (100.23 mg; 0.38 mmol; MW 262.26 Da) in 1:1 water/acetonitrile mixture (v/v), pH 8.5 by 0.2 N NaOH, to yield the final product PEG8-$(NTA)_8$. After 3 h the reaction mixture was extracted with diethyl ether (4×50 ml) to eliminate *p*NO_2-phenol. The product was recovered from the aqueous solution by extraction with chloroform (6×50 ml). The organic phase, dried over anhydrous Na_2SO_4, was concentrated to about 3 ml and dropped into 150 ml of diethyl ether. The white precipitate, PEG8-$(NTA)_8$, was recovered by filtration and dried under vacuum. Yield: 76.2%.

The product was first characterized by RP-HPLC chromatography and by Snyder assay to verify the absence of free NTA (23).

^{1}H-NMR (300 MHz, D_2O, δ ppm) analysis of PEG8-$(NTA)_8$: δ 1.4–2.0 (bs, -NH-CH_2-C<u>H</u>$_2$-C<u>H</u>$_2$-C<u>H</u>$_2$-CH, NTA), δ 3.72 (bs, O-C<u>H</u>$_2$-C<u>H</u>$_2$-, 8-arm PEG), δ 3.96 (s, 40 H, -C<u>H</u>(N-(C<u>H</u>$_2$-COOH)$_2$)-COOH, NTA).

Synthesis of mPEG-NTA

mPEG-OH (500 mg; 0.1 mmol; MW 5,000 Da) was dissolved in anhydrous CH_2Cl_2 and activated by *p*NO_2-phenyl chloroformate (60.47 mg; 0.3 mmol; MW 201.56 Da). NTA was coupled exploiting the same chemical route as for PEG8-$(NTA)_8$. Yield: 81.8%.

The product was characterized, as reported for PEG8-$(NTA)_8$, by RP-HPLC chromatography and by Snyder assay to verify the absence of free NTA (23).

^{1}H-NMR (300 MHz, D_2O, δ ppm) analysis of mPEG-NTA: δ 1.44–1.68 (bs, 4 H, -NH-CH_2-C<u>H</u>$_2$-C<u>H</u>$_2$-CH_2-CH, NTA), δ 1.92 (bs, 2 H, -NH-C<u>H</u>$_2$-CH_2-CH_2-CH_2-CH, NTA), δ 3.41 (s, 3 H, O-C<u>H</u>$_3$, PEG), δ 3.76 (bs, O-C<u>H</u>$_2$-C<u>H</u>$_2$, PEG), δ 3.92 (s, 5 H, -C<u>H</u>(N-(C<u>H</u>$_2$-COOH)$_2$)-COOH, NTA).

Synthesis of PEG-((βGlu)-(βGlu)$_2$-$(NTA)_4)_2$

PEG-((βGlu)-(βGlu)$_2$-$(NHS)_4)_2$ was synthesized from diol PEG as reported elsewhere (24). Briefly, the hydroxyl groups of diol PEG (1 g; 0.1 mmol; MW 10,000 Da) were activated by *p*NO_2-phenyl chloroformate (120.94 mg; 0.6 mmol; MW 201.56 Da) using the procedure reported above. Then the activate intermediate, PEG-(*p*NO2-phenyl carbonate)$_2$ (950 mg; 0.092 mmol), was coupled to beta-glutamic acid (βGlu; 40.59 mg; 0.276 mmol; MW 147.13 Da), previously dissolved in a water/acetonitrile mixture (3:2 v/v) pH 8 by Et_3N. The carboxylic groups of PEG-((βGlu)-$(COOH)_2)_2$ were activated by NHS/DCC and then coupled again to βGlu, whose carboxylic group was in turn activated by NHS/DCC obtaining PEG-((βGlu)-(βGlu)$_2$-$(NHS)_4)_2$. In the final step, NTA was linked to the activated residues of intermediate polymer, as above reported, yielding PEG-((βGlu)-(βGlu)$_2$-$(NTA)_4)_2$. Yield: 35.2%.

The polymer was characterized as described for PEG8-$(NTA)_8$.

^{1}H-NMR (300 MHz, D_2O, δ ppm) analysis of PEG-((βGlu)-(βGlu)$_2$-$(NTA)_4)_2$: δ 1.4–2.2 (bs, -NH-C<u>H</u>$_2$-C<u>H</u>$_2$-C<u>H</u>$_2$-CH_2-CH, NTA and -NH-C<u>H</u>-(C<u>H</u>$_2$-COOH)$_2$, βGlu), δ 3.8 (bs, 909 H, O-C<u>H</u>$_2$-C<u>H</u>$_2$, PEG), δ 3.93 (s, 40 H, -C<u>H</u>(N-(C<u>H</u>$_2$-COOH)$_2$)-COOH, NTA).

Measurement of PEG-NTA/Proteins Interaction by Gel Permeation Chromatography

1:1 mixtures of Cu^{2+}/chelating agent moiety for each one of the synthesized PEG-chelating agent polymers (*i.e.* PEG8-$(NTA)_8$, mPEG-NTA and PEG-((βGlu)-(βGlu)$_2$-$(NTA)_4)_2$) were prepared in 10 mM TRIS, 50 mM NaCl, pH 7.4. In particular, 500 μl of mPEG-NTA solution (2.6 mg/ml) were mixed with 500 μl of $CuSO_4$ solution (0.076 mg/ml). Similar mixtures of PEG8-$(NTA)_8$–Cu^{2+} (1 : 8) and PEG-((βGlu)-(βGlu)$_2$-$(NTA)_4)_2$–Cu^{2+} (1 : 8) were obtained by mixing

500 µl of PEG8-$(NTA)_8$ solution (4.0 mg/ml) or 500 µl of PEG-((βGlu)-$(βGlu)_2$-$(NTA_4)_2$ solution (3.1 mg/ml) with 500 µl of $CuSO_4$ solution (0.306 mg/ml).

Different amounts of these mixtures, containing the PEGylated chelating agent derivatives and Cu^{2+}, were added separately to stock solutions of each investigated protein. After 1 h at room temperature, the formation of PEG–Cu^{2+}–protein complexes was analyzed by GPC as described above. As control, PEG8-$(NTA)_8$ (1 mg/ml) was added to G-CSF solution (1 mg/ml) without $CuSO_4$, and the mixture was analyzed by GPC.

For HSA displacement experiment, PEG8-$(NTA)_8$–Cu^{2+}-G-CSF complex (1 mg/mL in G-CSF eq.) was mixed with an excess of HSA (15 mg/ml) at room temperature. After 1 h, the mixture was analyzed by GPC.

Kinetic Interaction Analysis Between PEG8-$(NTA)_8$–Cu^{2+} and Proteins by an SPR Biosensor

Immobilization of rh-GH or rh-G-CSF on a CM5 sensor chip was conducted by the amine coupling method (BIAapplication handbook; Biacore). The amounts of immobilization were around 150 resonance units (RU). The real-time interaction was measured by injecting different concentrations (0, 120, 240, 480 and 960 nM) of mixtures of PEG8-$(NTA)_8$–Cu^{2+} over these surfaces at flow rate of 100 µl/min. Several concentrations of PEG8-$(NTA)_8$ (0, 120, 240, 480 and 960 nM) were also injected to check interactions of polymer/protein in absence of Cu^{2+}. The sensor surfaces were regenerated by injecting 0.5 M EDTA pH 8.0 solution for 30 s after the dissociation phase. The sensorgrams were analyzed using the BIAevaluation software (Biacore). The association and dissociation phases were separately fit to obtain association and dissociation rate constants (k_{on} and k_{off}). The equilibrium dissociation constant (K_D) was calculated as $K_D = k_{off}/k_{on}$. Individual values were obtained from three independent experiments.

Pharmacokinetic Studies of PEG8-$(NTA)_8$–Cu^{2+}–G-CSF in Rats

Pharmacokinetic study in rats has been performed on male Sprague–Dawley rats, weighing about 300–350 g (Harlan Nossan). G-CSF content, in serum samples, were quantified by ELISA using the human G-CSF Immunoassay Kit (Invitrogen, Camarillo, CA, USA). Eight rats were randomly divided in two groups, four rats each. Each group was administered once at 0.1 mg/Kg b.w. (protein eq.) G-CSF or PEG8-$(NTA)_8$–Cu^{2+}–G-CSF intravenously in the tail vein. Blood samples were withdrawn at pre-dose, 5, 15, 30, 60, 90 min and 2, 3, 4, 6 hours after administration. The animal procedures were approved by the Ethic Committee of the University of Padua and the Italian Health Ministry, and all animals received care according to the DLGS 116/92 and in compliance with the "Guide for the Care and the Use of Laboratory Animals."

RESULTS

Preparation and Characterization of PEG-NTA Derivatives

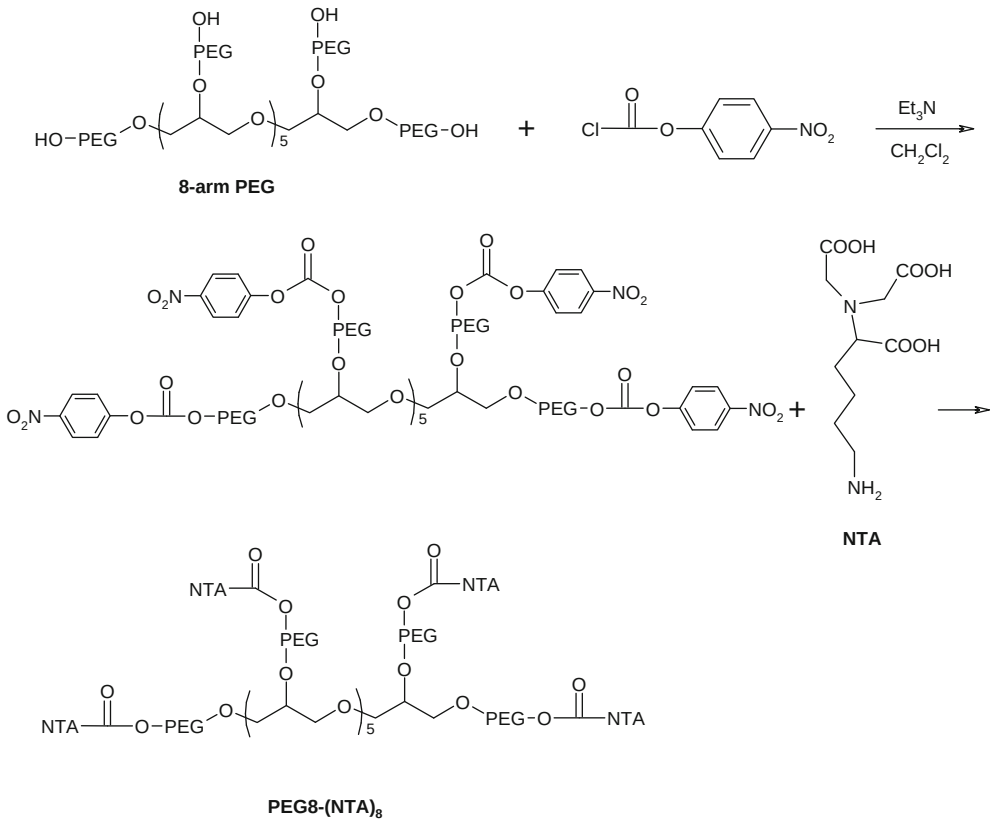
The chemical structures of PEG-chelating agent derivatives are shown in Fig. 2. As an example, the synthesis of PEG8-$(NTA)_8$ is reported in scheme 1. Overall yields of the products were 76.2, 81.8 and 35.2%, for PEG8-$(NTA)_8$, mPEG-NTA and PEG-((βGlu)-$(βGlu)_2$-$(NTA_4)_2$, respectively. Characterization by both RP-HPLC and Snyder assay confirmed the absence of free NTA in the purified derivatives. Percentages of bound NTA, estimated by ^{1}H-NMR spectroscopy, were 93.1, 97.2 and 89.6% for PEG8-$(NTA)_8$, mPEG-NTA and PEG-((βGlu)-$(βGlu)_2$-$(NTA_4)_2$, respectively.

Investigation of PEG-NTA/Proteins Interaction by GPC

The formation of a soluble interaction complex between the PEG-chelating agent derivatives and a protein was mediated by a divalent metal ion, as in the case of IMAC (25). In this case, Cu^{2+} was used and the chemical structure of the coordination complex is showed in Fig. 1. The ability of PEG-NTA–Cu^{2+} derivatives to form stable protein complexes was verified using several proteins, namely hemoglobin, G-CSF, hGH, IFN and INS. The investigation by GPC chromatography showed that mPEG-NTA–Cu^{2+} and PEG-((βGlu)-$(βGlu)_2$-$(NTA_4)_2$–Cu^{2+} were not able to form any complex with the studied proteins, while PEG8-$(NTA)_8$–Cu^{2+} yielded conjugates, with a significant increased hydrodynamic volume, in the case of hemoglobin and G-CSF (Fig. 4a, b). For hemoglobin the appropriate polymer/protein ratio was 2:1; in fact, the use of 1:1 ratio still showed the presence of free protein. For G-CSF the most appropriate ratio was found to be 1:1. The polymer/protein interaction is mediated by Cu^{2+} because the absence of metal ions prevented the formation of a polymer–protein conjugate (pattern 3, Fig. 4b). On the other hand, under the above-reported conditions, INS, hGH and IFN did not form any conjugate even at a high PEG8-$(NTA)_8$–Cu^{2+}/protein molar ratio.

The stability of PEG8-$(NTA)_8$–Cu^{2+}–G-CSF complex was also tested by adding human serum albumin (HSA) to the conjugate solution to verify whether this protein could displace G-CSF by competition. As shown in Fig. 5, G-CSF was not released after the addition of a high amount of HSA.

Scheme I Synthesis of PEG8-(NTA)$_8$.



Structural Investigation by Far UV-CD

Comparison of far UV-CD spectra of native G-CSF and PEG8-(NTA)$_8$–Cu^{2+}–G-CSF complex demonstrated that the secondary structure of the protein is preserved after the coordination by the PEGylated metal chelating derivative (Fig. 6).

Dynamic Light Scattering Studies on PEG8-(NTA)$_8$–Cu^{2+}–G-CSF

Dynamic light scattering was used to investigate the size of the PEG8-(NTA)$_8$–Cu^{2+}–G-CSF complex. G-CSF and PEG8-(NTA)$_8$–Cu^{2+} carrier have a diameter of 4.53 and 5.75 nm, respectively. The complex PEG8-(NTA)$_8$–Cu^{2+}–G-CSF showed a diameter of 11.94 nm, confirming that this approach does not promote the formation of aggregates.

The Specific Interaction between PEG8-(NTA)$_8$–Cu^{2+} and G-CSF Confirmed by SPR Analysis

To further validate the specific interaction of PEG8-(NTA)$_8$–Cu^{2+} with G-CSF, an SPR biosensor assay was used. The data demonstrated that PEG8-(NTA)$_8$–Cu^{2+} binds to G-CSF but not to hGH (Fig. 7a). The interaction of PEG8-(NTA)$_8$ and G-CSF was not observed in the absence of $CuSO_4$, demonstrating that the complex was formed through the NTA chelating Cu^{2+} and also confirming the data obtained by GPC. To measure the binding affinity for the complex formation, different concentrations of PEG8-(NTA)$_8$–Cu^{2+} were injected over the G-CSF-loaded sensor surface (Fig. 7b). Association and dissociation rate constants of 1.5×10^5 M^{-1} s^{-1} and 7.1×10^{-4} M^{-1} s^{-1} were measured that allowed us to calculate a K_D of 4.7 nM. This value is similar to the K_D (14 nM) reported for the hexahistidine binding to Ni-NTA (26).

Pharmacokinetic Studies of PEG8-(NTA)$_8$–Cu^{2+}–G-CSF in Rats

Pharmacokinetics of G-CSF and PEG8-(NTA)$_8$–Cu^{2+}–G-CSF were investigated after *i.v.* injection of 0.1 mg/kg of protein equivalent in rats. Both G-CSF and the complex were cleared rapidly from the body (half-life of 1.81 and 1.95 h, respectively), showing no significant difference in the pharmacokinetic profiles.

DISCUSSION

We designed a series of PEG-NTA derivatives for non-covalent protein conjugation through the formation of metal coordination bridges between a chelating agent, NTA, and suitable protein ligands, such as the imidazole ring of histidines (Figs. 1 and 2). Previously, tris-NTA

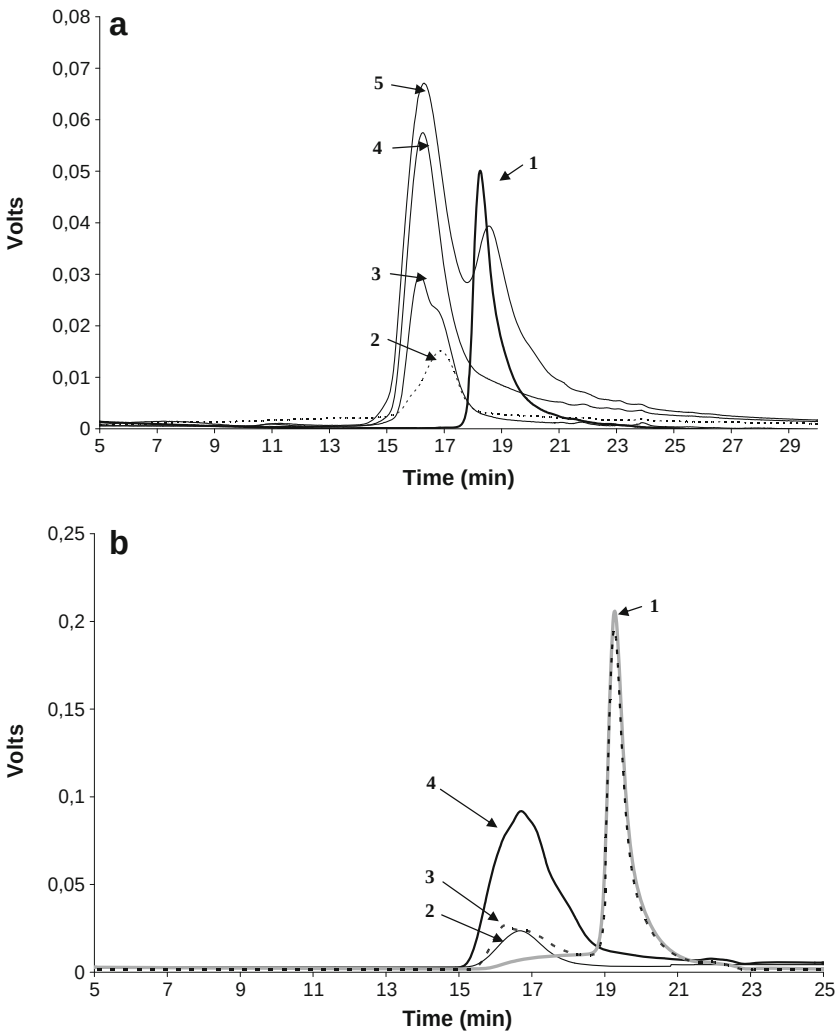


Fig. 4 GPC elution profiles of PEG8-$(NTA)_8$–Cu^{2+}–protein conjugates. (**a**) PEG8-$(NTA)_8$–Cu^{2+}–hemoglobin complex formation, 2 mg/ml hemoglobin (pattern 1), 2 mg/ml PEG8-$(NTA)_8$–Cu^{2+} (pattern 2), 2 mg/ml hemoglobin +2 mg/ml PEG8-$(NTA)_8$–Cu^{2+} (pattern 3), 4 mg/ml hemoglobin +2 mg/ml PEG8-$(NTA)_8$–Cu^{2+} (pattern 4), 6 mg/ml hemoglobin +2 mg/ml PEG8-$(NTA)_8$–Cu^{2+} (pattern 5), detection at 280 nm. (**b**) PEG8-$(NTA)_8$–Cu^{2+}–G-CSF experiment, 1 mg/ml G-CSF (pattern 1, grey line), 1 mg/ml PEG8-$(NTA)_8$–Cu^{2+} (pattern 2), 1 mg/ml G-CSF +1 mg/ml PEG8-$(NTA)_8$ (pattern 3, dotted line), 1 mg/ml G-CSF +1 mg/ml PEG8-$(NTA)_8$–Cu^{2+} (pattern 4), detection at 226 nm.

derivatives have been specifically design to incorporate the His6 tag, with the aim to increase the strength of the binding through a multivalent coordination. The data reported here confirm that a single chelating agent per polymer chain (*i.e.* the mPEG-NTA), which yields a single coordination metal bridge with a His in a protein sequence, is not sufficient to form a stable protein/polymer complex. On the other hand, NTA molecules coupled to a backbone

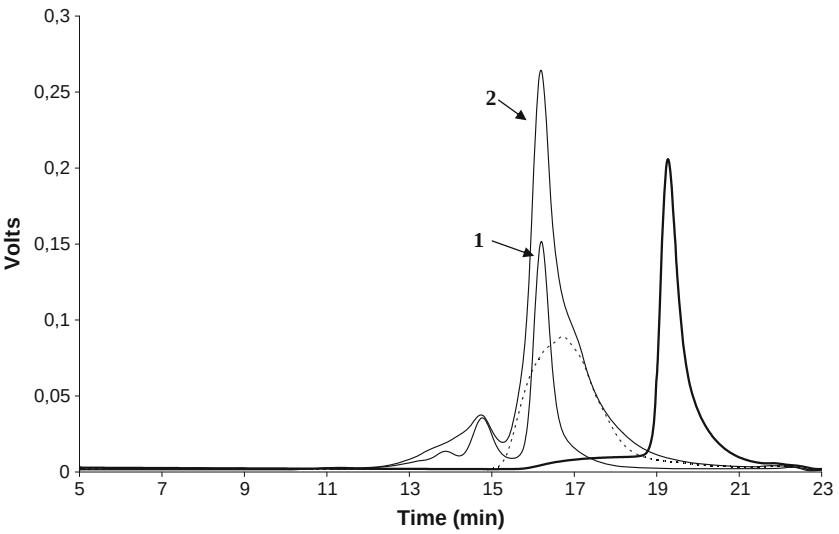


Fig. 5 Experiment of PEG8-$(NTA)_8$–Cu^{2+}–G-CSF stability in presence of HSA. Bold line = G-CSF alone; dotted line = PEG8-$(NTA)_8$–Cu^{2+}–G-CSF conjugate; HSA; (pattern 1) PEG8-$(NTA)_8$–Cu^{2+}–G-CSF (1 mg/ml G-CSF eq.) and HSA (15 mg/ml) (pattern 2). Detection at 226 nm. Peaks eluting at 13–15 min are dimer and aggregates already present in the starting HSA.

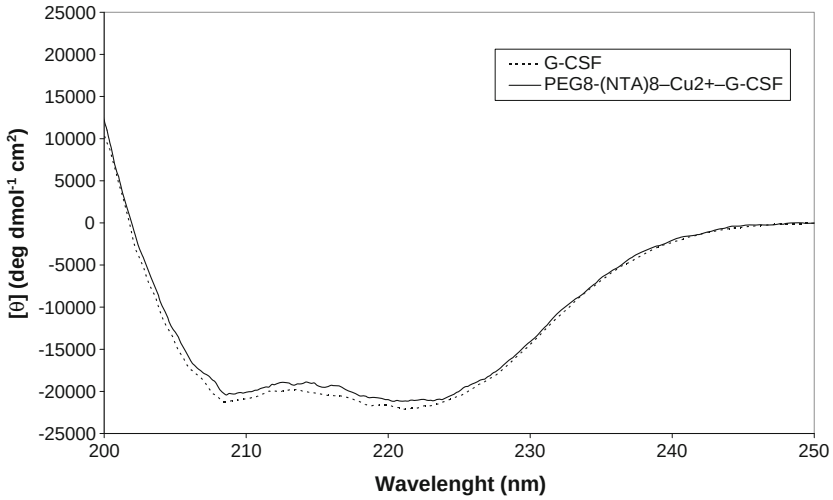


Fig. 6 Far UV-CD spectra of G-CSF and PEG8-$(NTA)_8$–Cu^{2+}–G-CSF.

with a rigid structure or coupled in close proximity might not yield high affinity binding to proteins without a His6 tag because these NTA derivatives cannot simultaneously reach histidines in spatially distant positions on the protein surface. This hypothesis has been corroborated with PEG-((βGlu)-$(βGlu)_2$-$(NTA)_4)_2$ that is not able to coordinate native G-CSF, although the polymer presents a set of four NTA molecules per each PEG end chain. Likely, the evident proximity of the chelating agents and the rigidity of the dendrimeric structure did not fit with the five histidines of G-CSF, precluding the possibility to obtain a multivalent interaction. Differently, PEG8-$(NTA)_8$, which possesses the same number of chelating agent as PEG-((βGlu)-$(βGlu)_2$-$(NTA)_4)_2$ but individually coupled to flexible PEG arms, is able to associate with native G-CSF through a metal coordination. As expected, another important requirement for this type of interaction is that a given protein must possess several and well solvent-exposed ligands, (especially histidines, see Figs. 1 and 3) to ensure a stable multivalent coordination with the NTA–Cu^{2+}moieties linked to a polymer. Among the five proteins tested, hemoglobin, G-CSF, hGH, IFN and INS, only the first two were coordinated by PEG8-$(NTA)_8$–Cu^{2+} because they satisfy the above requirements.

The multivalency and flexibility of PEG8-$(NTA)_8$–Cu^{2+} might lead to the formation of complexes of large size owing to the potential ability to recruit several protein units per polymer chain. This possibility can be reasonably excluded by analyzing the GPC elution profiles of Fig. 4a, where increasing amounts of hemoglobin were mixed with a fixed amount PEG8-$(NTA)_8$–Cu^{2+}. As shown, the height of PEG8-$(NTA)_8$–Cu^{2+}–hemoglobin peak increased with the hemoglobin additions until a certain point over which a further addition of hemoglobin formed a new peak corresponding to the free protein. On the other hand, if the polymer coordinates several proteins, the requirement for stability, multivalency coordination, would be lost. In the case of G-CSF, the complex was stable when the polymer/protein ratio was 1:1. Furthermore, the stability of the complex was confirmed by HSA displacement experiment, in which a high amount of HSA did not induce the release of G-CSF.

In this study, Cu^{2+} was preferred for its lower toxicity instead of the commonly used Ni^{2+}. A risk with the use of Cu^{2+} is the oxidation of protein's histidines, but this effect was excluded by using RP-HPLC and mass-spectrometry analyses of the G-CSF incubated with PEG8-$(NTA)_8$–Cu^{2+} (data not shown). We speculate that the oxidation did not occur for several reasons, including low concentration of Cu^{2+} used and chelation of the metal by NTA.

The kinetics of PEG8-$(NTA)_8$–Cu^{2+}–G-CSF interaction have been studied by SPR analysis. The complex showed a high affinity with a K_D value at nanomolar level (4.7 nM), which is comparable to that of some therapeutic antibodies with their target antigens. In spite of this high affinity, the complex PEG8-$(NTA)_8$–Cu^{2+}–G-CSF did not show a significant half-life increase *in vivo* with respect to native G-CSF. Likely, as reported by Szoka with tris-NTA lipid derivatives and His6-tagged proteins, also in this case dissociation or competition with plasma proteins reduced the stability of the complex *in vivo* (20). As shown by SPR investigation, PEG8-$(NTA)_8$ can still dissociate from the protein with a finite off-rate. It could be interesting to design mutants of the target protein seeking to increase the stability of the conjugate *in vivo*. For example, recombinant proteins having an increased number of histidines should be able to recruit all eight NTA moieties of PEG8-$(NTA)_8$, thus decreasing the dissociation rate of the complex. In fact, a recent kinetic analysis of oligo His tags and Ni^{2+}–NTA surface has demonstrated that a peptide with a higher number of histidine residues has the slower off-rate for the binding to Ni^{2+}–NTA surface (26). It was also found that not only the number of histidine residues but also their position in the primary sequence is important for the optimal binding. These data suggest that we might be able

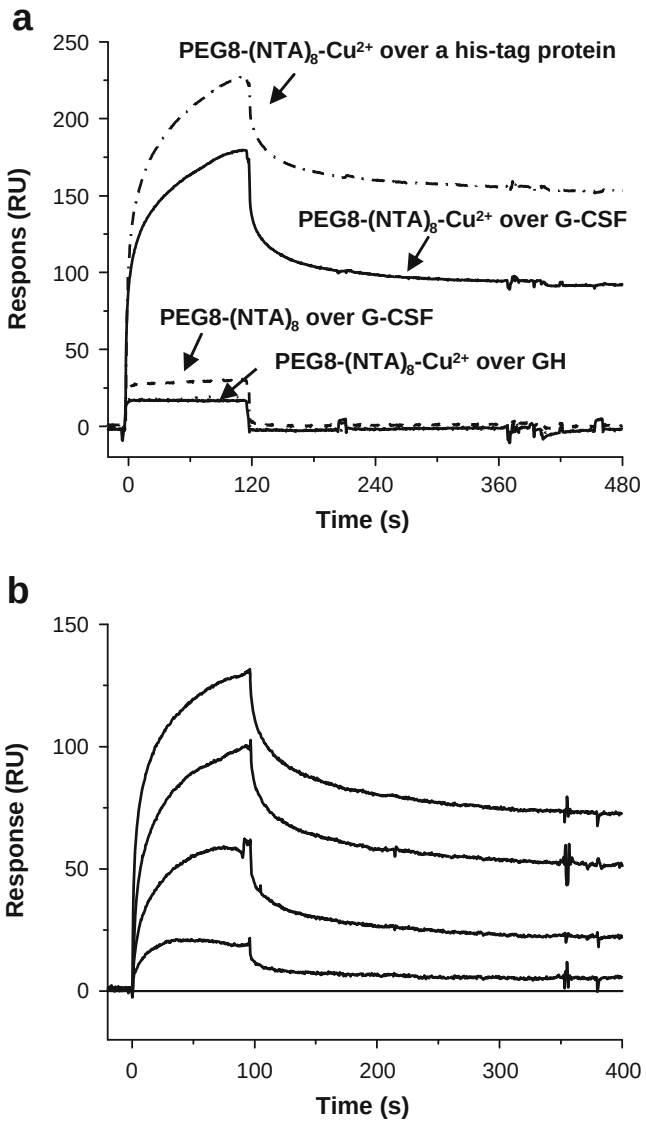


Fig. 7 Real-time interaction between PEG8-$(NTA)_8$–Cu^{2+} and G-CSF. (**a**) Specific binding of PEG8-$(NTA)_8$–Cu^{2+} to G-CSF. In this assay, a mixture of 1 μM of PEG8-$(NTA)_8$ and 8 μM $CuSO_4$ was injected over G-CSF, hGH and his-tagged thioredoxin (positive control) (27) covalently immobilized on separate biosensor surfaces, or over no protein surface (negative control). To confirm the role of Cu^{2+}, PEG8-$(NTA)_8$ alone was also injected. (**b**) Dose-dependent interaction of PEG8-$(NTA)_8$–Cu^{2+} with G-CSF on the biosensor surface. The observed association and dissociation rate constants have been calculated separately by using association phase (0–40 s) and dissociation phase (160–400 s), respectively.

to strengthen or tune the polymer/protein coordination by incorporating histidine residues in proper positions.

On the other hand, it is possible that a combination of recombinant His-tag proteins and the use of Ni^{2+} instead of Cu^{2+}would significantly increase the strength of the binding, although such a combinantion could raise some safety concerns in terms of immunogenicity and toxicity, respectively.

PEG8-$(NTA)_8$ showed that two requirements are also mandatory for the success of this approach: i) the multivalency of the association, reached by using both a polymer with several chelating agents and a protein with several histidines or eventually a His tag, and ii) the flexibility of NTA moieties, allowing the formation of coordination bonds even with histidines located distantly from each other on the protein surface.

CONCLUSION

The work reported here demonstrated that the PEG-enhanced delivery system needs not only several metal coordination bonds to reach a stable polymer/protein complex, by a cooperative action, but also requires that the NTA moieties have to be linked to long and flexible polymer arms to allow the recruitment of histidine located far away on the protein surface. In this study, these two prerequisites were fulfilled only by PEG8-$(NTA)_8$, whose highly flexible PEG arms guaranteed the necessary mobility of NTA moieties, which could therefore well adapt to the three-dimensional structure of the target protein. On the other hand, a suitable protein for this approach should present some basic requirements, such as a proper number of histidine residues, eventually thanks to the presence of a His tag and a good surface exposure of these histidines. These parameters should offer the possibility to recruit all the NTA moieties of PEG8-$(NTA)_8$ for a strong multivalent coordination.

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