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Labeling of VEGFR1D2 through oxime ligation

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ABSTRACT

We reported an useful protocol for the labeling of the second domain of the Vascular Endothelial Growth Factor Receptor 1 (VEGFR1D2), a small protein ligand able to bind VEGF, the main regulator of angiogenesis. We developed a bioconjugation strategy based on the use of oxime-ligation reaction conjugating an aldehyde derivative of the VEGFR1D2 to a molecular probe harboring an alkoxyamine functional group. We applied the synthetic protocol to prepare a biotinylated conjugate of VEGFR1D2 and we demonstrate that the bioconjugate retains its ability to specifically bind its natural ligand, VEGF, with high affinity. The biotinylated VEGFR1D2 could be useful to detect and quantify VEGF for diagnostic purposes as well as a tool for the screening of new molecules targeting VEGFRs for therapeutic applications. The labeling protocol is versatile and can be extended to different molecular probes, such as fluorophores, chelators or multimeric scaffolds, affording a biomedical platform for VEGF targeting.

1. Introduction

The ability to decorate a protein with chemical probes, obtaining a bioconjugate endowed of novel and useful properties, strongly expanded the repertoire of applications of protein molecules in life sciences. For example, proteins modified with fluorophores, chelating units, multimerization scaffolds, drugs or carriers find a plethora of applications both as diagnostic and therapeutic tools [1]. The utility of protein bioconjugates in biomedicine strongly fueled the search for efficient, easy-to-perform and versatile chemical strategies for site-specific functionalization of proteins [2–4]. Although a wide set of chemistries have been proposed to label protein molecules with synthetic probes, site-specific protein modification still remains a not trivial task. The most popular protein labeling chemistries exploit the peculiar reactivity of certain amino acid side-chains, such as thiol function of cysteine [5], amine group of lysine [6,7], thioether of methionine [8] or the indole [9] and imidazole [10] ring respectively of tryptophan and histidine. However, such approaches suffer of a severe limitation, as the protein to be labeled should harbor a single reactive amino acid, leading, otherwise, to a multiple decoration pattern and to heterogeneous preparations in which the uncontrolled introduction of chemical handles may alter protein folding and function. Several efforts aimed at addressing the issue of site-specific protein modification have been performed. A solution to the problem refers to the use of the expanded genetic code methodology, which allows to site-specifically insert an orthogonal functionality into the target protein through recombinant expression [11]. The unnatural amino acid introduced in this way can be exploited for molecular labeling using high selective reactions. This methodology is, however, not easy-to-perform and is often low-yielding, being limited to the small-scale. An alternative approach refers to protein synthesis by chemical ligation reactions. Chemical ligation marked a significant breakthrough in protein bioconjugation, as they ensure a unique level of specificity, are versatile and, notably, are compatible with large-scale protein bioconjugates production [12]. Native chemical ligation and expressed protein ligation have been widely explored for the site-specific and multiple decoration of protein molecules [13,14]. A number of labeling strategies targets protein termini which are usually better exposed protein regions and, in principle, their modification with chemical probes is well tolerated. Several approaches are based on the appendage of a short extra-sequence peptide to the protein N- or C-terminus serving as recognition motif for the enzymatic or chemical tagging [15–18]. The addition of extra sequence amino acids, however, may compromise the structural and functional properties of the protein molecule. Few chemistries have

Abbreviations: VEGFR1D2, vascular endothelial growth factor receptor; VEGF, vascular endothelial growth factor; PCR, polymerase chain reaction; TEV, tobacco etch virus; *SEC*, size exclusion chromatography; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; MS, mass spectrometry; LC, liquid chromatography; Biotin-TEG-ONH_2, biotin-tetra(ethylene glycol)-oxyamine; $rhVEGF_{165}$, recombinant human $VEGF_{165}$

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been reported that take advantage of the peculiar reactivity of the protein *N*-terminal α-amine to site-specifically introduce a label by directly modifying such group [19–23]. Another approach refers to the conversion of the *N*-terminal amino acid into a unique functional group which can be further functionalized using a chemical ligation reaction. We report the use of a chemical ligation based strategy for the covalent modification of the second domain of the Vascular Endothelial Growth Factor Receptor 1 (VEGFR1D2), an Ig-like domain directly involved in the ligand binding *in vivo* [24]. Vascular Endothelial Growth Factor (VEGF)/VEGFR biological system is the main regulator of angiogenesis, a physiological process controlling the sprouting of new blood vessels from an existing one and whose impairment contributes to the onset, development and progression of several human diseases, including cancer, cardiovascular disorders, diabetic complications, retinal degeneration and chronic inflammation. VEGF and its receptors are considered excellent candidates for the monitoring and the treatment of pathological angiogenesis [25–28]. We previously reported a useful protocol for the preparation of the recombinant VEGFR1D2 [29]. We characterized the biophysical and biochemical properties of the recombinant VEGFR1D2, demonstrating that the receptor domain retains the ability to bind VEGF with high affinity, is able to interfere with the VEGF/VEGFR interaction and to inhibit the VEGF-induced biological responses *in vitro*. If suitably modified with convenient molecular probes, VEGFR1D2 can be used as a tool for VEGF targeting with both diagnostic and therapeutic applications. We developed a bioconjugation strategy based on the use of an oxime ligation reaction by which a *N*-terminal aldehyde derivative of the VEGFR1D2 was conjugated to a molecular probe harboring an alkoxyamine function. The aldehyde function was installed through the oxidation with sodium periodate ($NaIO_4$) of the *N*-terminal Ser residue of VEGFR1D2 [30]. As proof of concept, we applied the synthetic protocol to the preparation of a biotinylated conjugate of VEGFR1D2, useful to detect and quantify VEGF for diagnostic purposes as well as a tool for the screening of new molecules targeting VEGFRs for therapeutic applications. We confirmed that the biotinylated VEGFR1D2 retains its ability to bind VEGF selectively and with high affinity and can thus be used for VEGF dosing and for the screening of new compounds targeting VEGF and VEGFRs for biomedical applications. The protocol is versatile and can be extended to different molecular probes, such as fluorophores, chelators or multimeric scaffolds, affording a biomedical platform for VEGF targeting. Such protein ligands represent second generation bioconjugates able to overcome the limitations associated to the use of the expensive anti-VEGF antibodies traditionally employed to detect and quantify VEGF.

2. Results & discussion

2.1. Bioconjugation strategy

The chemical strategy designed for the covalent modification of VEGFR1D2 consisted in two steps, the first one referred to the incorporation of an aldehyde functional group at the protein *N*-terminus (Fig. 1, Step I) which was exploited in the second step for the incorporation of a chemical probe through an oxime ligation reaction (Fig. 1, Step II).

A wide set of strategies exists for the site-specific introduction of aldehyde handles into a protein, both using enzymatic or chemical approaches [31,32] and a growing number of chemical probes targeting aldehyde function are commercially available. As VEGFR1D2 harbors a Ser residue as first amino acid of the polypeptide chain (position 129 of the VEGFR1 full-length sequence), we exploited such residue to insert an aldehyde functionality at the protein *N*-terminus. Indeed, 1,2-amino alcohol function of *N*-terminal Ser (and Thr) can be readily converted into an aldehyde group through an oxidation reaction by treatment with $NaIO_4$ [30]. The reaction is fast and requires mild conditions, as it takes place in aqueous solution at neutral pH in presence of a slight excess of $NaIO_4$ and proceeds using very diluted reactants concentration. The aldehyde group is orthogonal towards all the functional groups commonly present into a protein molecule and, hence, once introduced into the protein, can be chemo-selectively tagged using a portfolio of possible chemistries [31]. We selected the oxime ligation reaction, in which the aldehyde group is reacted with an oxy-amine molecular probe to give an oxime bond. We selected the oxime ligation because such reaction is highly efficient and selective, requires mild conditions, can be performed in aqueous solution at pH around neutrality, water is the only side product generated, reactants can be used at very diluted concentration and, additionally, give rise to a stable conjugate at physiological pH [33]. Overall, our designed labeling strategy is biocompatible, cost-effective and can be easily scaled-up for larger scale production.

2.2. N-terminal serine oxidation to aldehyde

In order to expose the Ser1 at the *N*-terminus of the recombinant VEGFR1D2, we modified by polymerase chain reaction (PCR) the gene coding the protein domain (amino acids 129–229 of VEGFR1) by adding the Tobacco Etch Virus (TEV) protease cleavage site (ENLYFQ \S) upstream the sequence in which the Ser residue in position P1′ of the TEV site corresponded to the Ser1 of VEGFR1D2. In this way, TEV proteolysis allowed the exposition of the *N*-terminal 1,2-amino alcohol function of the recombinant protein needed to introduce the aldehyde function. Besides, no extra amino acids were added to the native protein sequence. Two consecutive steps of PCR were performed to add the TEV protease site upstream the gene. The fusion gene was cloned into the pET28b(+) vector, downstream a poly-His affinity tag useful for the purification of the protein by Ni^{2+} affinity chromatography. Protein expression in bacterial host, purification from inclusion bodies and refolding of the recombinant protein was performed as we previously described [29]. 2 mg of pure protein were obtained from 1 L of culture. Protein purity and identity were verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (MS) (Figs. S1 and S2). The observed mass (MW_{exp}: 11521.19 Da) was in agreement with the calculated value for the protein with the two cysteine residues forming a disulphide bridge (MW_{th}: 11521.28 Da). The protein was analyzed by circular dichroism showing the expected spectrum (Fig. S3) [29]. An aldehyde derivative of VEGFR1D2 was obtained by modifying the Ser1 residue at protein *N*-terminus through an oxidation reaction performed by treatment with $NaIO_4$. $NaIO_4$ selectively reacts with 1,2-amino alcohol function (i.e. *N*-terminal Ser) leading to the formation of two species at the equilibrium, the aldehyde and its diol derivative which originates from the hydration of the aldehyde. Reaction was carried out in aqueous buffer at neutral pH (50 mM phosphate buffer, 120 mM sodium chloride, pH 7.0) using the protein target at diluted concentration (0.1 mM) and a slight excess of $NaIO_4$ (1.5 eq, 0.15 mM). Reaction was performed at room temperature and readily provided VEGFR1D2 protein with its *N*-terminal Ser converted into an *N*-terminal glyoxalamide group. Liquid chromatography-mass spectrometry (LC-MS) analysis of the reaction mixture after 60 min of incubation showed the complete oxidation of the protein, which exists as an equilibrium mixture of the aldehyde and the diol derivatives (MW $aldehyde_{exp}$: 11492.69 Da *vs* MW $aldehyde_{th}$: 11490.28 Da; MW $diol_{exp}$: 11508.75 Da *vs* MW $diol_{th}$: 11508.28 Da) (Fig. 2).

Besides, an additional specie with MW_{exp} of 11472.16 Da, co-eluting with the expected product, was detected in the reaction mixture. Such product could correspond to a cyclic imine adduct originating from the reaction of the protein *N*-terminal aldehyde group with the ε-amine group of one of the Lys residues present within the protein sequence. The formation of the cyclic imine adduct is a reversible reaction, as the molecule may hydrolyze giving back the *N*-terminal aldehyde protein. A prolonged treatment with $NaIO_4$ may result in the collateral oxidation of other amino acids, as Met and Cys [30,32], both present within the protein sequence. Thus, the aldehyde protein was promptly purified by

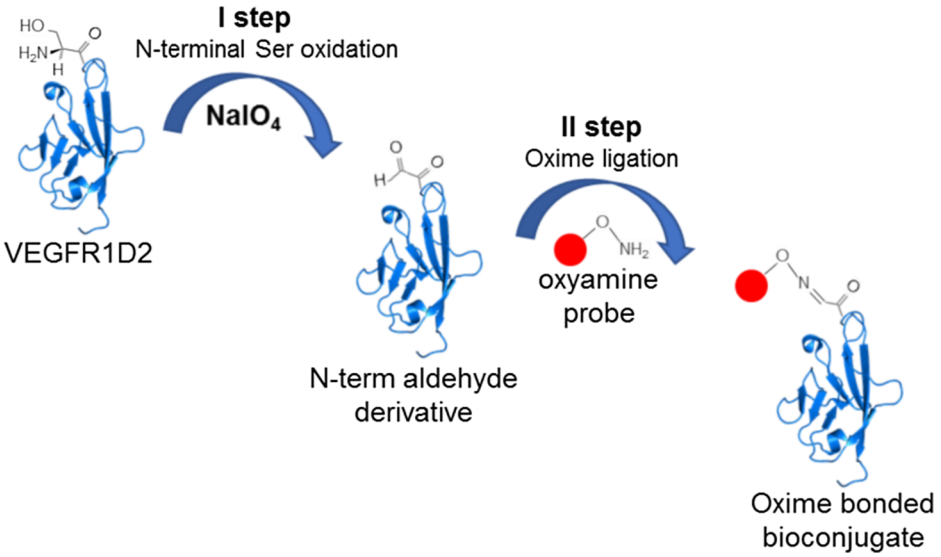

Fig. 1. Chemical strategy designed for the labeling of VEGFR1D2.

size-exclusion chromatography to remove $NaIO_4$ and avoid unspecific oxidation (final yield of 27%).

2.3. Labeling of aldehyde-VEGFR1D2 by oxime ligation

An oxime ligation reaction was performed to derivatize with a biotin unit the aldehyde derivative of VEGFR1D2. To this aim, the aldehyde protein was reacted with a molecule of biotin-tetra(ethylene glycol)-oxyamine (Biotin-TEG-ONH_2), in which the biotin is modified with an oxyamine group to allow the reaction with the aldehyde. A polyethylene glycol spacer links the biotin unit and the oxyamine group in order to improve the solubility of the conjugate and minimize steric hindrance. Oxime ligation proceed with modest rates in slightly acid solution (pH around 5) but is very slow at physiological pH [34]. This limited the utility of this chemistry for the labeling of protein which are not stable under acidic conditions. The rate of oxime ligation reaction at pH value around neutrality can be improved by adding a nucleophilic catalyst, such as aniline [35,36], expanding the applicability of such ligation chemistry also to the modification of protein strictly requiring manipulation at neutral pH. Oxime ligation was carried out in phosphate buffer pH 6.5 using the aldehyde protein at very diluted concentration (10 μM), a 50-fold molar excess of the oxy-amine probe (500 μM) in presence of aniline as catalyst (50 mM). Before adding the oxy-amine probe to the reaction mixture, the aldehyde protein was pretreated with aniline for 30 min at room temperature. Reaction mixture was incubated at 20 °C for 16 h and checked by LC-MS until complete conversion of the aldehyde protein into the biotinylated conjugate (MW_{exp}: 11906.64 Da *vs* MW_{th}: 11906.83 Da). The reaction performed in the same conditions but in absence of the aniline catalyst did not lead to the formation of the bioconjugate (data not shown). Biotinylated-VEGFR1D2 was finally extensively dialyzed in order to remove the unreacted probe and catalyst excess. Protein purity and identity of the biotinylated protein were verified by LC-MS and SDS-PAGE (Fig. 3).

2.4. $rhVEGF_{165}$ binding assays

Protein-protein binding experiments were carried out in order to assess the ability of the biotinylated VEGFR1D2 to bind with high affinity recombinant human $VEGF_{165}$ ($rhVEGF_{165}$), its natural ligand. A direct binding assay was set coating $rhVEGF_{165}$ [37] onto a multi-well plate at the concentration of 10 nM and then incubated with biotinylated VEGFR1D2 at different concentrations in the range 0.005 nM–50 nM. The binding of biotinylated VEGFR1D2 to $rhVEGF_{165}$ was revealed using streptavidin-peroxidase (0.125 ng/mL) and a chromogenic substrate of the peroxidase, in a colorimetric assay. The experiments demonstrated that the biotinylated VEGFR1D2 is able to bind $rhVEGF_{165}$ in a dose-dependent manner (Fig. 4a).

Data analysis provided an apparent dissociation constant (K_d) of 9.3 ± 0.6 nM which is in agreement with the value previously reported [24,29]. Besides, the data suggest that bioconjugation did not alter the binding properties of VEGFR1D2 and support the utility of the biotin-VEGFR1D2 protein as a tool for VEGF dosing instead of using antibody-based kits.

Competition experiments were also performed in order to confirm the specificity of the interaction between biotinylated VEGFR1D2 and $rhVEGF_{165}$ and to set up the experimental conditions for a screening assay. $rhVEGF_{165}$ (10 nM) was coated onto a multi-well plate and then incubated with a solution of biotinylated VEGFR1D2 (10 nM)/unlabeled VEGFR1D2 (0.1 nM–1 μM) (Fig. 4b). The displacement of biotinylated VEGFR1D2 by unlabeled domain was revealed with streptavidin-peroxidase (0.125 ng/mL). Data demonstrated that in the reported experimental condition a competitor can displace the biotinylated VEGFR1D2 from the binding to $rhVEGF_{165}$. In particular as proof of concept we used unlabeled VEGFR1D2 and we obtained an IC_{50} of 4.4 ± 0.9 nM. This result suggests that VEGF recognition is specific and that biotinylated VEGFR1D2 could be used in competition experiments to screen novel compounds inhibiting the interaction between VEGF and VEGFR1D2. Besides, the data suggest that bioconjugation did not alter the binding properties of VEGFR1D2 and VEGF recognition is still specific.

Previously, Goncalves et al. [38] reported a chemiluminescent screening assay to detect VEGFR1 ligands. This method, which rely on the use of the full VEGFR1 ectodomain and biotinylated VEGF obtained from a commercial kit, is comparable with our screening assay. However, considering that VEGF is more complex molecule than VEGFR1D2, we believe that specific biotinylation of VEGFR1D2 is a more convenient and less expensive procedure.

Furthermore, our results well agree with the binding assays reported by Reille-Seroussi et al. [39], which is based on the use of VEGFR1D2-horseradish peroxidase conjugate. This conjugate has the advantage of an easier experimental procedure avoiding some incubation steps. On the other hand, biotinylated VEGFR1D2 could be considered a valuable alternative because of its chemoselective labeling and as consequence its easier purification and characterization.

In definitive, the reported results support the utility of the biotin-

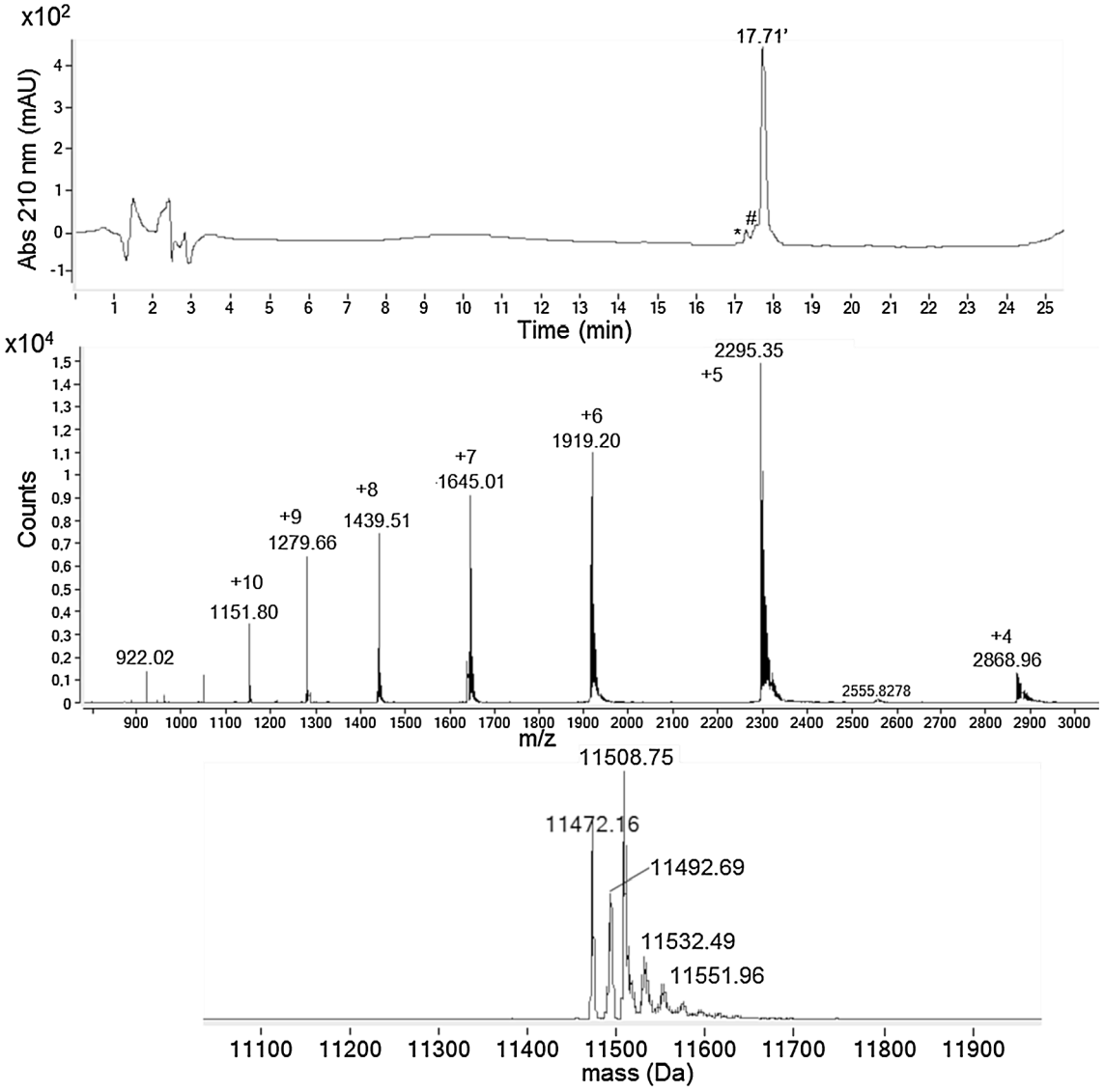

Fig. 2. Analysis by LC-MS ESI-ToF of the oxidation reaction of VEGFR1D2 with $NaIO_4$ after 1 h of incubation at room temperature. The reported chromatographic profile is revealed registering the absorbance at 210 nm. At the bottom of the fig. is reported the deconvolution spectrum. The shoulder of the main peak (#) corresponds to a contamination from the not digested protein (MW_{exp}: 14479.16 Da *vs* MW_{th}: 14479.49 Da); (*) side products showing experimental mass values of 11526.75 Da and 11487.81 Da.

VEGFR1D2 protein as an alternative tool to set up experimental assays to screen novel compounds inhibiting the interaction between VEGF and VEGFR1D2 and for *in vitro* VEGF dosing instead of using antibody-based kits. Antibodies show several disadvantages as high costs of production and complicate site-specific chemical modification, leading to a significant batch-to-batch diversity. In this scenario, small protein ligands retaining high target affinity represent a second generation of bioconjugates able to overcome the limitations associated to the use of antibodies. Furthermore, the reduced size improves their pharmacological properties (i.e. tissue penetration and renal clearance) for *in vivo* application as molecular imaging probes.

3. Conclusions

We reported a useful protocol for the labeling of VEGFR1D2, a small protein ligand able to bind VEGF that represents an interesting tool for the VEGF targeting of VEGF. The labeling strategy consists of the modification of VEGFR1D2 with an aldehyde function which can further react with an oxy-amine probe through an oxime ligation reaction to yield an oxime bonded protein bioconjugate. Exploiting the described protocol, we prepared a biotinylated variant of VEGFR1D2. The protein bioconjugate retained its ability to selectively and efficiently bind VEGF. The biotinylated VEGFR1D2 protein could be useful for VEGF dosing and in drug discovery for the screening of novel molecules targeting VEGF/VEGF receptors. The described chemical strategy is versatile and can be extended to the introduction of different molecular probes for the preparation of a collection of VEGFR1D2 bioconjugates targeting VEGF for diagnostic and therapeutic applications.

4. Experimental section

4.1. Materials

DNA oligonucleotides were synthesized at Sigma-genosys while recombinant plasmids sequencing was commissioned at PRIMM srl. Pfu turbo DNA polymerase and E. *coli* BL21 *Codon Plus* (DE3) RIL cells were purchased from Agilent. Restriction enzymes were from New England Biolabs. pET28b(+) vector was from Novagen. Ni^{2+}-NTA agarose resin was purchased from Qiagen. Tris, NaCl, imidazole, urea, oxidized and

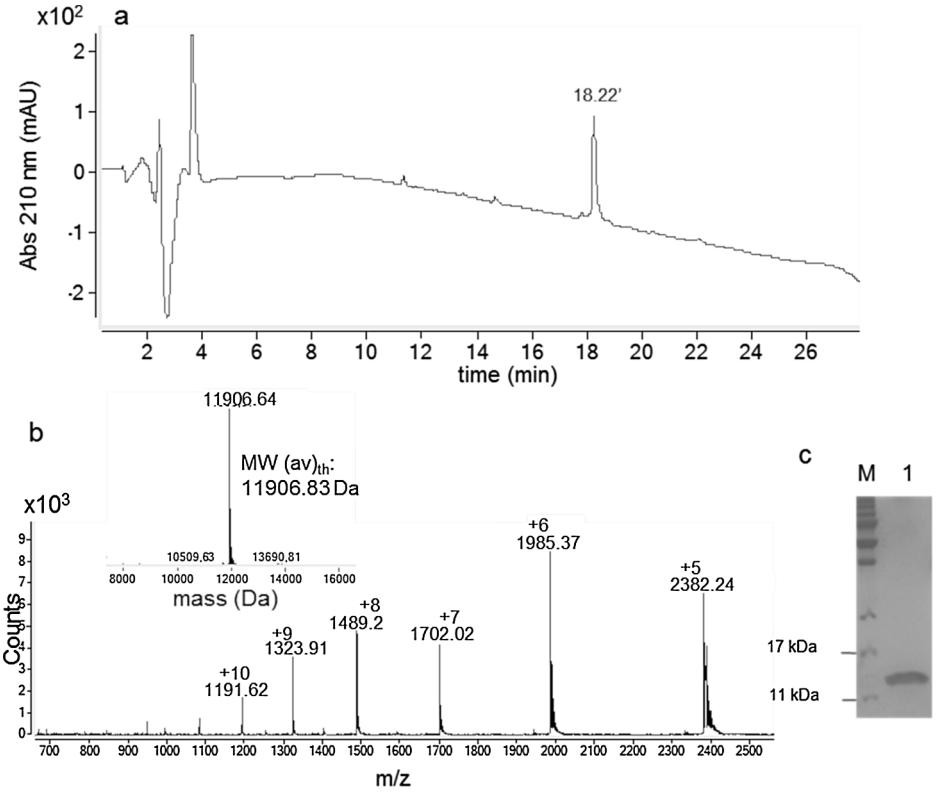

Fig. 3. Analysis by LC-MS ESI-ToF and SDS-PAGE of biotinylated VEGFR1D2. (a) RP-HPLC chromatographic profile revealed registering the absorbance at 210 nm. (b) ESI-ToF spectrum of the species eluted at 18.22 min. In the inset of the mass spectrum is reported the deconvoluted mass. (c) SDS-PAGE on 15% polyacrylamide gel of biotinylated VEGFR1D2 (lane 1); molecular weight marker (lane M).

reduced glutathione, Ethylenediaminetetraacetic acid (EDTA), sodium phosphate buffer were obtained from Applichem. Sodium periodate, Tween-20, phosphate buffer saline (PBS) tablets, streptavidin-peroxidase fusion protein, o-phenylendiamine dihydrochloride tablets (Sigma Fast OPD tablets) and aniline were obtained from Sigma-Aldrich. Biotin-tetra(ethylene glycol)-oxyamine (Biotin-TEG-ONH_2) was from Iris Biotech. Dialysis membrane was purchased from SpectraPor. 96-wells plates MaxiSorp™ were purchased from Nunc. Ultra-pure solvents used for LC-MS analyses were from Romil.

4.2. Methods

4.2.1. Cloning, expression, and purification

The gene coding domain 2 of the Vascular Endhothelial Growth Factor Receptor 1 (VEGFR1D2) (amino acid region 129–229 of full-length VEGFR1 receptor) was amplified in two consecutive PCR steps in order to add at the 5′-terminus of the gene the sequence coding the TEV protease cleavage site. The first gene amplification was performed using the cDNA of the full-length receptor as template (kindly provided by Dr Nicola Normanno, Istituto Nazionale Tumori - IRCCS "Fondazione G. Pascale") and the sequences 5′-AACCTGTATTTTCAGAGTGATACAGGT AGA-3′ and 5′- GTGCTCGAGCTATCAGATTGTATTGGTTTGTGC-3′, as the sense and the antisense primer, respectively. The second PCR step was performed using the product of the first PCR as template, the sense primer 5′-GGAATTCCATATGGAAAACCTGTATTTTCAGAGT-3′, which includes a *Nde*I restriction site upstream the sequence (underlined), and the same antisense primer used in the first amplification step, which includes an *Xho*I restriction site downstream the sequence (underlined). Each PCR reaction was performed in a total volume of 100 µL, using 50 ng of template DNA, 0.3 µM of each primer, 0.25 mM of each dNTP, 5 U of Pfu turbo polymerase, applying an annealing temperature of 42 °C in the first PCR and 45 °C in the second PCR. The hybrid gene coding TEV site-VEGFR1D2, obtained from the second PCR, was digested with the restriction enzymes *Nde*I and *Xho*I and cloned into the pET28b(+) expression vector, downstream the region coding the His_6-tag. Protein expression and purification was carried out as described by Di Stasi et al. [29] with some differences. Briefly, the protein was expressed in E. *coli* BL21 *Codon Plus* (DE3) RIL cells and was extracted from inclusion bodies with a 50 mM Tris, 150 mM NaCl, 10 mM imidazole buffer at pH 7.0 (yield of 90 mg of protein per L of culture). The protein was purified by affinity chromatography on Ni^{2+}-NTA agarose resin, exploiting the poly-His tag at protein *N*-terminus. The protein was refolded on resin, by treatment with buffer solutions at pH 7.0, containing progressively lower concentration of urea as chaotropic agent

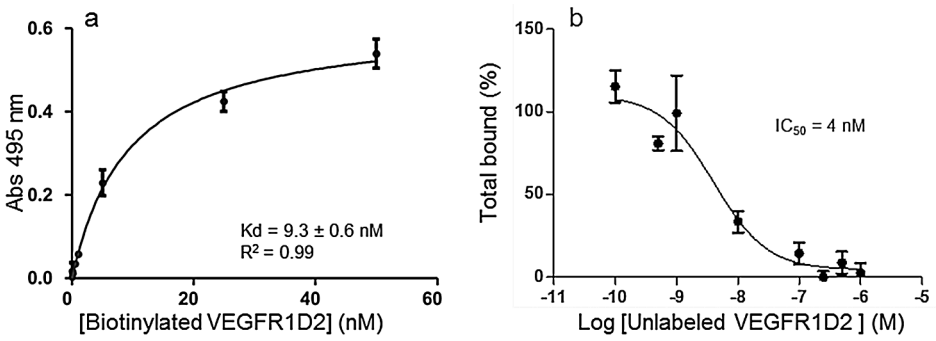

Fig. 4. Binding assays between biotinylated VEGFR1D2 and $rhVEGF_{165}$. (a) Direct binding assay. $rhVEGF_{165}$ was coated on a multi-well plate and incubated with biotinylated VEGFR1D2 at progressively increasing concentration. The value of the dissociation constant (K_d) and the R squared (R^2) are reported. (b) Competition binding assay. $rhVEGF_{165}$ was coated on a multi-well plate and incubated with a mixture of the biotinylated VEGFR1D2 (10 nM) and increasing concentration of the unlabeled VEGFR1D2. Data reported are the mean of three experiments.

(50 mM Tris, 150 mM NaCl containing urea 6.0 M, 4.0 M, 3.0 M, 2.0 M, 1.0 M, 0 M). Once refolded, the protein was eluted from the resin applying a step gradient of imidazole (yield of 8 mg of refolded protein per L of culture). Eluted fractions were analyzed by SDS-PAGE on 15% polyacrylamide gel and those containing pure TEV site-VEGFR1D2 protein were pooled. The protein was dialyzed against 50 mM Tris, 150 mM NaCl, pH 7.0, at 4 °C using a 6000–8000 Da MWCO membrane in order to remove the excess of imidazole. The protein was then digested with TEV protease (protein substrate:TEV protease 1:35 M ratio) in order to remove the affinity tag and expose the *N*-terminal serine residue. Glutathione (3 mM reduced/0.3 mM oxidized) and 0.5 mM EDTA were added to the cleavage mixture and the proteolytic reaction was carried out at 4 °C for 16 h. Finally, size exclusion chromatography (*SEC*) was performed using a $S75_{10/30}$ column (GE Healthcare) and 50 mM sodium phosphate buffer, 120 mM NaCl, pH 7.0 as the eluent in order to purify at the homogeneity the TEV-digested protein and transfer the protein into a more convenient buffer solution for the *N*-terminal Ser oxidation reaction. 2 mg of pure protein were obtained from 1 L of culture.

4.2.2. Mass spectrometry analysis

LC-MS analyses were performed on an Agilent 1200 Infinity Series (Agilent Technologies) equipped with a diode array and an ESI-ToF detector, equipped with a Jupiter C18 column, 150 × 2 mm, 3 μ, 300 Å (Phenomenex) and applying a gradient of CH_3CN (0.05% TFA) in H_2O (0.05% TFA) from 5% to 70% in 20 min.

4.2.3. Circular dichroism studies

Circular dichroism analyses were performed on a Jasco J-715 spectropolarimeter (Jasco corporation) equipped with a Peltier PTC/423S/15 for temperature regulation. CD spectrum was acquired in 10 mM phosphate buffer, pH 6.8, at a protein concentration of 12 μM, in a 0.1 cm path-length quartz cuvette (Hellma), at 20 °C. CD spectrum was registered in the wavelength range 190–260 nm, with a band width of 2 nm, a data pitch of 0.2 nm, a response of 4 *sec*, a scanning speed of 10 nm/min as average of three scans. Thermal denaturation analysis was performed registering the molar ellipticity at 214 nm ($[\theta]_{214}$) in the temperature range 20–95 °C, increasing the temperature at a rate of 0.5 °C/min. The processing of the spectra was obtained by using the Spectra Manager software. Data are displayed in mean residue ellipticity.

4.2.4. N-terminal serine oxidation to aldehyde

The oxidative cleavage of the *N*-terminal 1,2-amino alcohol function of VEGFR1D2 was performed by treatment with $NaIO_4$. 950 μg of VEGFR1D2 (82 nmol) in 800 μL of 50 mM sodium phosphate buffer, 120 mM NaCl, pH 7.0 (protein concentration of 100 μM) were treated with 1.5 eq of $NaIO_4$ by adding to the protein solution 1.2 μL of 0.1 M $NaIO_4$ in 50 mM sodium phosphate buffer, 120 mM NaCl, pH 7.0 (freshly prepared immediately before the use). The reaction mixture was incubated at room temperature for 60 min and then promptly purified by *SEC* on a $S75_{10/30}$ column (GE Healthcare) equilibrated in 50 mM sodium phosphate buffer, NaCl 120 mM, pH 6.5. Eluted peak fractions were pooled and protein concentration (10 μM) was spectrophotometrically estimated by UV absorbance at 280 nm using Lambert-Beer Law and the protein molar extinction coefficient of 11585 $cm^{-1}\,M^{-1}$ (calculated using the ExPASy Bioinformatics Resource Portal at the web site https://web.expasy.org/protparam). 250 μg (22 nmol) of the *N*-terminal aldehyde VEGFR1D2 were recovered, associated to a yield of 27%.

4.2.5. VEGFR1D2 biotinylation by oxime ligation

250 μg (22 nmol) of *N*-terminal aldehyde VEGFR1D2 protein in 50 mM sodium phosphate buffer, NaCl 120 mM, pH 6.5 (protein concentration 10 μM) were preincubated with 50 mM of aniline. To this aim, 10 μL of aniline 10.97 M were added to the protein solution and the mixture was incubated at room temperature for 30 min. Then, 50 eq of Biotin-TEG-ONH2 were added to the reaction mixture (38 μL of a 29 mM solution of Biotin-TEG-ONH2 in 50 mM sodium phosphate buffer, NaCl 120 mM, pH 6.5, freshly prepared) and the ligation mixture was incubated at 20 °C for 16 h. Reaction mixture was extensively dialyzed against 50 mM sodium phosphate buffer, NaCl 120 mM, pH 6.5, at 4 °C using a membrane with MWCO of 6000–8000 Da. Final protein concentration was estimated using the Bradford colorimetric assay (Bio Rad reagent). 250 μg (22 nmol) of the *N*-terminal biotinylated-VEGFR1D2 were recovered, associated to a yield of 100%.

4.2.6. Dose-response binding assay

A 96-wells plate MaxiSorp™ was coated with recombinant $rhVEGF_{165}$, prepared as described in Di Stasi R et al. [37] by incubation with 100 μL/well of 10 nM protein solution in phosphate buffer saline (PBS), at 4 °C for 16 h. After coating, the plate was washed three times with PBS (200 μL/well) and then incubated with the blocking solution (1% bovine serum albumin in PBS, 200 μL/well) for 1.5 h at room temperature. Wells were washed again with PBS and then incubated for 2 h, at room temperature, with 100 μL/well of biotinylated VEGFR1D2 at increasing protein concentration (0.005 nM, 0.05 nM, 0.1 nM, 0.5 nM, 1 nM, 5 nM, 25 nM, 50 nM) in PBS. Wells were washed three times with PBS containing 0.05% Tween-20 and incubated with 100 μL/well of streptavidin conjugated horseradish peroxidase (1 mg/mL) diluted 1:8000 in PBS containing 0.1% BSA. After 1 h of incubation at room temperature, the wells were washed again with PBS containing 0.05% Tween-20. 100 μL of the peroxidase chromogenic substrate solution, containing o-phenylendiamine dihydrochloride 0.4 mg/mL, 0.4 mg/mL urea hydrogen peroxide in 50 mM phosphate–citrate buffer, pH = 5.0 (Sigma Fast OPD tablets), were added to each well. After 10 min, the reaction was stopped by adding 50 μL of 2.5 M H2SO4 in each well. The absorbance was measured at 495 nm, using the plate reader of an EnSpire Multimode Plate Reader (Perkin Elmer). Control blank values obtained incubating the biotinylated-VEGFR1D2 in wells without performing $rhVEGF_{165}$ coating, were subtracted from the absorbance values obtained from coated wells. Data were obtained in triplicate and were analyzed using the software OriginPro (vers. 8.5). Data were globally fitted using the nonlinear curve fit model "one binding site". A R^2 value of 0.99 was obtained.

4.2.7. Competitive binding assay

Recombinant $rhVEGF_{165}$ was coated into the wells of a MaxiSorp™ 96-wells plate as described in the previous paragraph. After wells blocking and washing, 100 μL of a mixture of the biotinylated-VEGFR1D2 (at the fixed concentration of 10 nM) and the unlabeled VEGFR1D2 (at increasing concentrations, 0 nM, 0.1 nM, 0.5 nM, 1 nM, 10 nM, 100 nM, 250 nM, 500 nM, 1000 nM) in PBS was added to the wells. The assay was revealed as described in the previous paragraph. Data were obtained in triplicate and were analyzed using the program OriginPro (vers. 8.5). Data were globally fitted using the nonlinear curve fit "one binding site competition". A R^2 value of 0.94 was obtained.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103160.

References

[1] F. Li, R.I. Mahato, Bioconjugate therapeutics: current progress and future perspective, Mol. Pharm. 14 (5) (2017) 1321–1324.
[2] Y. Gong, L. Pan, Recent advances in bioorthogonal reactions for site-specific protein labeling and engineering, Tetrahedr. Lett. 56 (2015) 2123–2132.
[3] L.H. Qin, W. Hub, Y.Q. Long, Bioorthogonal chemistry: optimization and application updates during 2013–2017, Tetrahedr. Lett. 59 (2018) 2214–2228.
[4] N. Stephanopoulos, M.B. Francis, Choosing an effective protein bioconjugation strategy, Nat. Chem. Biol. 7 (12) (2011) 876–884.
[5] Y. Kim, S.O. Ho, N.R. Gassman, Y. Korlann, E.V. Landorf, F.R. Collart, S. Weiss, Efficient site-specific labeling of proteins via cysteines, Bioconjug. Chem. 19 (3) (2008) 786–791.
[6] E. Basle, N. Joubert, M. Pucheault, Protein chemical modification on endogenous amino acids, Chem. Biol. 17 (3) (2010) 213–227.
[7] R. Singudas, S.R. Adusumalli, P.N. Joshi, V. Rai, A phthalimidation protocol that follows protein defined parameters, Chem. Commun. (Camb) 51 (3) (2015) 473–476.
[8] S. Lin, X. Yang, S. Jia, A.M. Weeks, M. Hornsby, P.S. Lee, R.V. Nichiporuk, A.T. Iavarone, J.A. Wells, F.D. Toste, C.J. Chang, Redox-based reagents for chemoselective methionine bioconjugation, Science 355 (6325) (2017) 597–602.
[9] J.M. Antos, J.M. McFarland, A.T. Iavarone, M.B. Francis, Chemoselective tryptophan labeling with rhodium carbenoids at mild pH, J. Am. Chem. Soc. 131 (17) (2009) 6301–6308.
[10] P.N. Joshi, V. Rai, Single-site labeling of histidine in proteins, on-demand reversibility, and traceless metal-free protein purification, Chem. Commun. (Camb) 55 (8) (2019) 1100–1103.
[11] K. Lang, L. Davis, J.W. Chin, Genetic encoding of unnatural amino acids for labeling proteins, Methods Mol. Biol. 1266 (2015) 217–228.
[12] L. De Rosa, A. Russomanno, A. Romanelli, L.D. D'Andrea, Semi-synthesis of labeled proteins for spectroscopic applications, Molecules 18 (1) (2013) 440–465.
[13] L. De Rosa, A.L. Cortajarena, A. Romanelli, L. Regan, L.D. D'Andrea, Site-specific protein double labeling by expressed protein ligation: applications to repeat proteins, Org. Biomol. Chem. 10 (2) (2012) 273–280.
[14] S.S. Cohen, I. Riven, A.L. Cortajarena, L. De Rosa, L.D. D'Andrea, L. Regan, G. Haran, Probing the molecular origin of native-state flexibility in repeat proteins, J. Am. Chem. Soc. 137 (32) (2015) 10367–10373.
[15] C.S. Theile, M.D. Witte, A.E. Blom, L. Kundrat, H.L. Ploegh, C.P. Guimaraes, Site-specific N-terminal labeling of proteins using sortase-mediated reactions, Nat. Protoc. 8 (9) (2013) 1800–1807.
[16] I. Chen, M. Howarth, W. Lin, A.Y. Ting, Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase, Nat. Methods 2 (2) (2005) 99–104.
[17] B.A. Griffin, S.R. Adams, R.Y. Tsien, Specific covalent labeling of recombinant protein molecules inside live cells, Science 281 (5374) (1998) 269–272.
[18] S.R. Adams, R.E. Campbell, L.A. Gross, B.R. Martin, G.K. Walkup, Y. Yao, J. Llopis, R.Y. Tsien, New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications, J. Am. Chem. Soc. 124 (21) (2002) 6063–6076.
[19] C.B. Rosen, M.B. Francis, Targeting the N terminus for site-selective protein modification, Nat. Chem. Biol. 13 (7) (2017) 697–705.
[20] L. Purushottam, S.R. Adusumalli, U. Singh, V.B. Unnikrishnan, D.G. Rawale, M. Gujrati, R.K. Mishra, V. Rai, Single-site glycine-specific labeling of proteins, Nat. Commun. 10 (1) (2019) 2539.
[21] D. Chen, M.M. Disotuar, X. Xiong, Y. Wang, D.H. Chou, Selective N-terminal functionalization of native peptides and proteins, Chem. Sci. 8 (4) (2017) 2717–2722.
[22] G. Casi, N. Huguenin-Dezot, K. Zuberbuhler, J. Scheuermann, D. Neri, Site-specific traceless coupling of potent cytotoxic drugs to recombinant antibodies for pharmacodelivery, J. Am. Chem. Soc. 134 (13) (2012) 5887–5892.
[23] S.R. Adusumalli, D.G. Rawale, V. Rai, Aldehydes can switch the chemoselectivity of electrophiles in protein labeling, Org. Biomol. Chem. 16 (48) (2018) 9377–9381.
[24] C. Wiesmann, G. Fuh, H.W. Christinger, C. Eigenbrot, J.A. Wells, A.M. de Vos, Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor, Cell 91 (5) (1997) 695–704.
[25] A. Grothey, E. Galanis, Targeting angiogenesis: progress with anti-VEGF treatment with large molecules, Nat. Rev. Clin. Oncol. 6 (9) (2009) 507–518.
[26] G.D. Yancopoulos, Clinical application of therapies targeting VEGF, Cell 143 (1) (2010) 13–16.
[27] L. De Rosa, R. Di Stasi, L.D. D'Andrea, Pro-angiogenic peptides in biomedicine, Arch. Biochem. Biophys. 660 (2018) 72–86.
[28] L.D. D'Andrea, A. Del Gatto, L. De Rosa, A. Romanelli, C. Pedone, Peptides targeting angiogenesis related growth factor receptors, Curr. Pharm. Des. 15 (21) (2009) 2414–2429.
[29] R. Di Stasi, D. Diana, D. Capasso, R. Palumbo, A. Romanelli, C. Pedone, R. Fattorusso, L.D. D'Andrea, VEGFR1(D2) in drug discovery: expression and molecular characterization, Biopolymers 94 (6) (2010) 800–809.
[30] K.F. Geoghegan, J.G. Stroh, Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine, Bioconjug. Chem. 3 (2) (1992) 138–146.
[31] R.J. Spears, M.A. Fascione, Site-selective incorporation and ligation of protein aldehydes, Org. Biomol. Chem. 14 (32) (2016) 7622–7638.
[32] O. El-Mahdi, O. Melnyk, alpha-Oxo aldehyde or glyoxylyl group chemistry in peptide bioconjugation, Bioconjug. Chem. 24 (5) (2013) 735–765.
[33] J. Kalia, R.T. Raines, Hydrolytic stability of hydrazones and oximes, Angew. Chem. Int. Ed. Engl. 47 (39) (2008) 7523–7526.
[34] P.W. Jencks, Studies on the mechanism of oxime and semicarbazone formation, J. Am. Chem. Soc. 81 (2) (1959) 475–481.
[35] A. Dirksen, T.M. Hackeng, P.E. Dawson, Nucleophilic catalysis of oxime ligation, Angew. Chem. Int. Ed. Engl. 45 (45) (2006) 7581–7584.
[36] A. Dirksen, P.E. Dawson, Rapid oxime and hydrazone ligations with aromatic aldehydes for biomolecular labeling, Bioconjug. Chem. 19 (12) (2008) 2543–2548.
[37] R. Di Stasi, D. Diana, D. Capasso, S. Di Gaetano, L. De Rosa, V. Celentano, C. Isernia, R. Fattorusso, L.D. D'Andrea, VEGFR recognition interface of a proangiogenic VEGF-mimetic peptide determined in vitro and in the presence of endothelial cells by NMR spectroscopy, Chemistry 24 (44) (2018) 11461–11466.
[38] V. Goncalves, B. Gautier, C. Garbay, M. Vidal, N. Inguimbert, Development of a chemiluminescent screening assay for detection of vascular endothelial growth factor receptor 1 ligands, Anal. Biochem. 366 (1) (2007) 108–110.
[39] M. Reille-Seroussi, J.F. Gaucher, L.A. Cussac, I. Broutin, M. Vidal, S. Broussy, VEGFR1 domain 2 covalent labeling with horseradish peroxidase: development of a displacement assay on VEGF, Anal. Biochem. 530 (2017) 107–112.