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Multivalency – a way to enhance binding avidities and bioactivity – preliminary applications to EPO‡

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Abstract: Multivalency has advantages over monovalency for binding interactions and even for activity. In particular, avidity is higher since the off-rate of a multivalent species is much slower than that of a monomer. This is particularly profitable for ligand-binding receptors that require dimerization for activity, such as the receptor of erythropoietin (EPOR). Peptides that mimic the action of erythropoietin (EPO) have been described with no sequence similarity with the human hormone: erythropoietin mimetic peptide (EMP) and EPO receptor peptide (ERP). These two peptides have similar activity but interact through different sites on the EPOR. Here, we describe the construction of several new synthetic homo- and hetero-dimers based on EMP-ERP sequences. To link the monomeric molecules together, several monodisperse polyamide linkers of different lengths were synthesized with dialdehyde functionalities. Chemoselective oxime chemistry was used to obtain homogeneous constructs. Certain chemical incompatibilities were dealt with via a protection approach. The oximes are stable under normal conditions and so lend themselves to biological testing. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: multivalency; oxime; peptide synthesis; erythropoietin

INTRODUCTION

Natural polyvalent molecules exist of which IgM with its ten binding sites is probably the most well-known example. Multivalency has been approached from a theoretical point of view on many occasions, including in a recent article describing the thermodynamic parameters [1]. Experimentally, the pentameric recombinant 'peptabody' [2] showed 10^5 times tighter binding than the constituent monomeric peptides and was proved to be biologically active. Besides proteins, multivalent molecules may be nonpeptidic organic chemicals, nucleotides [3], antibiotics [4], etc. Hetero- and homodimeric synthetic peptides have been described, as well as molecules with higher multiplicity, by many groups including our own.

Synthetic or recombinant dimers can bind to and activate those receptors that require dimerization to activate. Examples of synthetic dimers that bind to and activate receptors include the peptide mimetic of thrombopoietin [5] and a series of erythropoietin mimetic peptides [5,6]. Erythropoietin (EPO) is a 166-amino acid protein that regulates the production of red blood cells [7]. The recombinant human erythropoietin (rhEPO) is used clinically for the treatment of anemia [8], and a hyperglycosylated analog that has a prolonged duration of action can also be administered [9]. Other EPO analogs have been designed by pegylation for obtaining prolonged *in vivo* activity [10,11]. The receptor of erythropoietin (EPOR) is a good example of a receptor that requires dimerization for activity [12]. The receptor may be activated not only by EPO but also by monomeric and dimeric erythropoietin mimetic peptides (EMPs): 13–20-amino-acid peptides having no sequence similarity with EPO, which was discovered by phage display [5]. The erythropoietin receptor peptide (ERP), a 23-residue peptide with the sequence identical to a site on the EPO receptor, is also active on EPO-responsive cells but involves another mode of action [13]. The EPO itself has been dimerized in an attempt to improve activity, either by recombinant techniques (with a 17-residue linker [14]) or chemically [14–16]. The aim of such studies is to find a molecule that has improved pharmacological properties as a therapeutic for the treatment of chronic anemia.

Several dimers of EMP have been described [6] that are more active than the corresponding monomers although considerably less active than the recombinant protein. While the distance between the monomer units in synthetic dimers may sometimes be quite short [5], in other cases a linking moiety of considerable length is required for receptor activation [3,17]. As exemplified by studies on other multimeric molecules, the distance between the monomeric peptides is crucial for activity [3,18]. We have previously described a simple, stepwise, solid-phase procedure for the synthesis of linking moieties that have a defined structure in spite of their great length [17]. These flexible linkers, produced by coupling commercially available diacids and diamines in a controlled manner,

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have a repeat unit—[NH–Y–NHCO–X–CO]$_n$–, where the value of n depends on the number of coupling cycles performed. The combination X = —CH_2CH_2–, Y = —$CH_2CH_2CH_2$–$(OCH_2CH_2)_3$–CH_2– has particularly favorable solubility properties and is resistant to proteolytic digestion. In the present study, we describe the synthesis of several new EMP and ERP constructs. By synthesizing the polyamide linkers, we were able to design linkers with different lengths that were monodisperse, in comparison with commercially available PEGs. To attach the peptides on each side of the linkers, we decided to use chemoselective oxime chemistry, which requires very mild conditions and is robust and biocompatible.

Combining the advantages of defined linker length and monodispersity with the specificity of the oxime chemistry, we synthesized several homogeneous EMP homodimers and even EMP-ERP heterodimers.

MATERIALS AND METHODS

Materials

Peptide-synthesis-grade DMF and DIEA were purchased from Biosolve. DCM, NMM, *N*-methylpyrrolidinone (NMP), diethyl ether, dimethylsulphoxide (DMSO), carbonyldiimidazole (CDI), trifluoromethanesulphonic acid (TFMSA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and DIC were purchased from Fluka, Switzerland. TFA was from Halocarbon, New Jersey. Acetonitrile CHROMASOLV gradient grade for high-pressure liquid chromatography (HPLC) was from Sigma–Aldrich. HBTU was from Iris Biotech GmbH Germany and HATU was from GL Biochem (Shanghai) Ltd. HOBt was from NovaBiochem (Switzerland) and the amino acids were from Orpegen (Germany) or from NovaBiochem.

Analytical High-Pressure Liquid Chromatography

Analytical reverse-phase high-pressure liquid chromatography (RP-HPLC) was performed at 0.6 ml/min on Waters equipment using a Macherey–Nagel C_8 column (4 × 250 mm, 300 Å 5-μm particle size). Solvent A was 0.1% TFA in HPLC grade water. Solvent B was 90% acetonitrile with 0.1% TFA. Elution was done with a 40-min linear gradient 0–80% B.

Preparative High-Pressure Liquid Chromatography

Preparative RP-HPLC was performed at 15 ml/min on Waters equipment using a Vydac C_8 column (22 × 250 mm 300 Å 10–15 μm particle size). Elution with previously described solvents A and B of the peptides was done with an appropriate linear gradient, usually 1%/min. UV monitoring was at 214 nm. The peaks were collected manually and the product was recovered by lyophilization.

Mass Spectrometry

MALDI-TOF mass spectrometry was performed in linear mode using sinapinic acid as matrix, on a Voyager-DE STR (Applied Biosystems) instrument equipped with delayed extraction. External calibration was performed using porcine insulin (Novo Nordisk).

Solid Phase Peptide Synthesis

Solid phase peptide synthesis [19] was performed on a modified ABI 433A machine using Boc chemistry and *in situ* neutralization, as previously described [20]. Peptides were prepared on a 0.2 mmol scale on MBHA cross linked with 1% DVB resin (0.9 mmol/g, Senn Chemicals, Switzerland). Boc-amino acids were protected by the following groups: Arg(Tos), Asn(Xan), Asp(OcHx), Cys (4-MeBzl), Glu(OcHx), His(Dnp), Lys(Z(2Cl)), Ser(Bzl), Thr(Bzl), Trp(For), Tyr(Z(2Br)). After chain elongation, a Boc-aminooxyacetyl group (Boc-AoA) was manually coupled as its *N*-hydroxysuccinimide ester (Boc-AoA-OSu) used in 1.2 molar excess in DMSO with NMM as the base [21]. Certain protecting groups were removed prior to acid cleavage (Dnp with 20% 2-mercaptoethanol and 10% DIEA; formyl with 20% piperidine; Boc with neat TFA) with HF containing 5% *p*-cresol for 60 min at 0 °C. The peptides were precipitated and then washed with cold diethyl ether. The crude peptide was exposed to high vacuum overnight and purified by preparative RP-HPLC, and the purified product was lyophilized. EMP sequences after resin cleavage were AoA-GGLYACHMGPMTWVCQPLRG-amide for the *N*-terminally modified peptide (referred to as AoA-EMP), and GGLYACHMGPMTWVCQPLRGK(AoA)-amide for the *C*-terminally modified peptide (referred to as EMPK-AoA). For the synthesis of the *C*-terminally modified EMP, Boc-Lys(Fmoc) was used to initiate the synthesis. Deprotection of the Fmoc group was performed with 20% piperidine in DMF after synthesis completion and Boc-AoA-OSu was coupled manually with 1.2 equivalents. The ERP peptide had the sequence QRVEILEGRTECVLSNLRGRTRY. Five 'PEG-succ' units were manually coupled to the *N*-terminus of the ERP and finally a Boc-Ser(Bzl)-OH was added (see details in the following text).

Disulfide Bond Formation of EMP

Twenty milligrams of the peptide was dissolved in 20 ml water and the pH adjusted to 7 with 1% ammonia solution. Two hundred and forty-five microliters of a 3% H_2O_2 solution was added and the mixture left to react for 30 min. After acidification with acetic acid, the solution was directly injected into preparative RP-HPLC for purification.

Synthesis of a PEG–Polyamide Linker

PEG-like dialdehyde linkers of various lengths were synthesized as described in Ref [17]. Briefly, 0.3 mmol of SASRIN resin (1.02 mmol/g, 200–400 mesh, Bachem Switzerland) was acylated with succinic anhydride, 4 mmol in 8 ml of DMF containing 0.5 M of DMAP to which 0.4 ml of DIEA was added. Free carboxyl groups were activated with CDI for 30 min. The activated carboxyl group was then aminolyzed with 4,7,10-trioxa-1,13-tridecanediamine (commercial PEG diamine from Fluka) in the presence of HOBt for 60 min in DMF [17]. The growing molecule was again acylated with succinic anhydride, 4 mmol in 8 ml of DMF containing 0.5 M HOBt to which 0.4 ml of DIEA was added. The polyamide chain was further grown by successive activation, aminolysis and acylation steps. Each 'acylation–activation–aminolysis' cycle will add a 'PEG-succ' unit

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(–$NHCH_2(CH_2CH_2O)_3CH_2CH_2CH_2NH$-$COCH_2CH_2CO$–) to the resin. After addition of the desired number of units, Boc-Ser(Bzl)-OH was coupled to the free amine. For this, 1.2 mmol (4 equivalents) of Boc-L-Ser(Bzl) was activated with 1.2 mmol (4 equivalents) HBTU dissolved in DMF. Two millimols (8 equivalents) of DIEA was added, and the solution reacted for 40 min. Ninhydrin test (Fluka) was performed to verify that the acylation coupling had succeeded [22]. If the test was positive, the coupling was repeated for another 40 min. The products were cleaved from the resin with 1% TFA in DCM and neutralized in pyridine/methanol (9/1) before being precipitated with cold diethyl ether. HPLC preparative purification was employed. The linker was then coupled [17] to the commercial PEG diamine: 4 equivalents of linker with 4 equivalents of HATU and 8 equivalents DIEA were preactivated for 5 min before reaction with 1 equivalent of the commercially available diamine. After 4 h incubation, the product was isolated by RP-HPLC. The symmetrical linker with the formula Boc-Ser(Bzl)-$[PEG\text{-}succ]_{4,6,8}$-PEG-$[PEG\text{-}succ]_{4,6,8}$-Ser(Bzl)-Boc was obtained. The Boc and Bzl protecting groups were removed by dissolving 10 mg of the linker in 300 µl TFA for 4 min followed by the addition of 30 µl of TFMSA for 25 min. TFA was evaporated with air and the product precipitated and washed with cold ether, and dried in a desiccator under high vacuum.

Serine Oxidation to a Glyoxylyl Function

The *N*-terminal serine is oxidized with periodate to a glyoxylyl function as described in Refs [23,24]. The molecule is dissolved in a 50 mM Imidazole buffer pH 7 at a final Ser concentration of 200 µM. In the presence of 50 molar excess of methionine, each serine is oxidized with 4 equivalents of sodium periodate. After 5 min, 1000 equivalents (over periodate) of ethylene glycol is added and the solution is brought to pH 4–5 with acetic acid. The solution is then injected into RP-HPLC for purification.

Alkylation of the Cysteine of ERP

Ten milligrams of peptide was dissolved in 2.3 ml of 100 mM phosphate buffer pH8. To this, 6.5 mg of TCEP was added and the solution was incubated for 30 min at 37 °C. Then 8.5 mg of iodoacetamide was added and incubated for 90 min at 37 °C. The solution was acidified before injection into RP-HPLC for purification.

Oximation of the Dialdehyde Linker with Aminooxy EMP Derivatives

Two hundred microliters of a 14 mM aldehyde solution (HPLC fraction concentrated) was quickly added to 200 µl (1.5 excess over aldehyde groups) of a 20 mM solution of aminooxy-peptide (dissolved in 10 mM acetate buffer, pH 4.6 with 50% acetonitrile) [21]. The reaction mixture was stirred for 15 h at room temperature and purified by RP-HPLC, as previously described.

RESULTS AND DISCUSSION

Synthesis of EMP Homodimers

The EPO dimer analogs were assembled using the highly specific oxime reaction between an aldehyde and an aminooxy compound [21]. On the basis of a previously described strategy [17], we decided to synthesize EMP dimers of precise lengths, with the aminooxy monomeric peptides attached to the

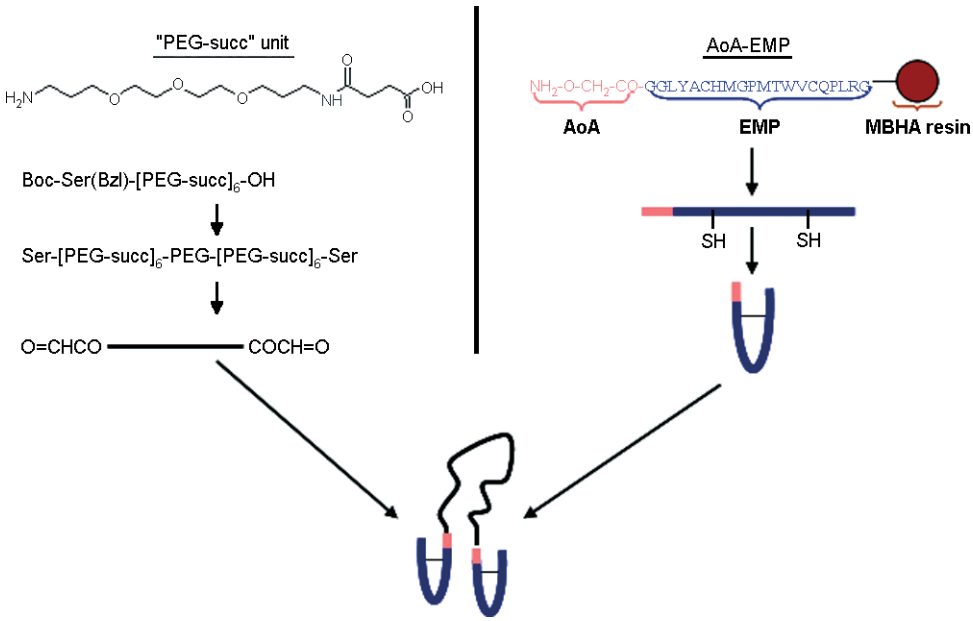

Figure 1 Strategy for *N*-EMP medium dimer synthesis. On the left panel the dialdehyde linker was synthesized by successive addition of "PEG-succ" units on resin, release from the resin and further acylation of the carboxylic linker parts with a diamine. Finally, Ser were oxidized to glyoxylyl function. On the right panel, AoA-EMP was synthesized by SPPS, and the disulfide bond formed after resin cleavage. Finally, both molecules were reacted together to form the *N*-terminal EMP dimer by oxime ligation.

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Table 1 List of constructs with calculated and measured masses

Molecule	Calculated mass (Da)	Measured mass by MS (Da)	Difference
AoA-EMP (monomer)	2247.65	2247.48	0.17
EMPK-AoA (monomer)	2375.82	2377.24	1.42
N-EMP short dimer	7210.60	7209.80	0.8
N-EMP medium dimer	8420.10	8420.47	0.37
N-EMP long dimer	9629.52	9629.50	0.02
C-EMPK-AoA short dimer	7466.90	7466.94	0.04
C-EMPK-AoA medium dimer	8676.40	8676.79	0.39
C-EMPK-AoA long dimer	9885.80	9886.71	0.91
N-EMP-ERP heterodimer	6603.75	6603.79	0.04
C-EMPK-AoA-ERP heterodimer	6731.92	6731.94	0.02

linker either by their *N*- or *C*-terminus (Figure 1). By designing monodisperse linkers of different lengths, we synthesized a panel of new compounds expected to be agonists of EPOR. The peptides were synthesized using Boc chemistry on MBHA resin and had an identical sequence, except that an additional Boc-Lys(Fmoc) was introduced at the *C*-terminal EMP derivative (EMPK-AoA) to attach the aminooxy group. Following chain extension, selective deprotection by piperidine was followed by AoA-OSu coupling on resin, which gave the desired peptide. After resin cleavage and HPLC purification, the peptides containing two cysteines were oxidized with hydrogen peroxide to the disulfide. This rather strong oxidation method was preferred to air oxidation, since the latter was not efficient enough. After purification, both peptides gave a single peak on the analytical RP-HPLC and possessed the expected masses (Table 1, Figure 2). The PEG-like linkers were synthesized on the hydroxyl resin following the protocol described in Ref [17]. This allowed us to prepare monodisperse linkers of desired lengths and with a chosen functionality at both ends. We decided to synthesize three different linker lengths composed of various numbers of 'PEG-succ' groups. After the successive and controlled addition of a selected number of 'PEG' and 'succ' units, a Boc-protected serine residue was coupled for later transformation to an aldehyde functionality. To avoid the presence of linkers with missing PEG-succ units, we used large excesses of reagents. These Boc-Ser(Bzl)-$[PEG\text{-}succ]_{4,6,8}$-OH polyamide fragments were then used to acylate both amino groups of the PEG diamine to obtain symmetrical linkers composed of either 8, 12 or 16 PEG-succ units (referred to as *short*, *medium* and *long*). After deprotection, terminal serine residues were oxidized with periodate to obtain the dialdehyde linker [23,25]. We found it important not to dry the dialdehydes because they became less soluble and less reactive.

Finally, the purified aminooxy peptides (either *N*- or *C*-terminal derivative) were reacted with the dialdehyde linkers to form oxime dimers in mild conditions (acetate buffer pH 4.5). A 1.5-fold excess of peptide over the aldehyde group was used. This chemoselective reaction allowed formation of exclusively *N*- or *C*-terminally linked dimers of precise lengths (*N*-EMP dimer or *C*-EMPK dimer) without any side reaction between the linker and peptide side chains, in contrast to general acylation, which may react with either the α- or ε-amino groups [6,21]. All constructs gave a single peak on

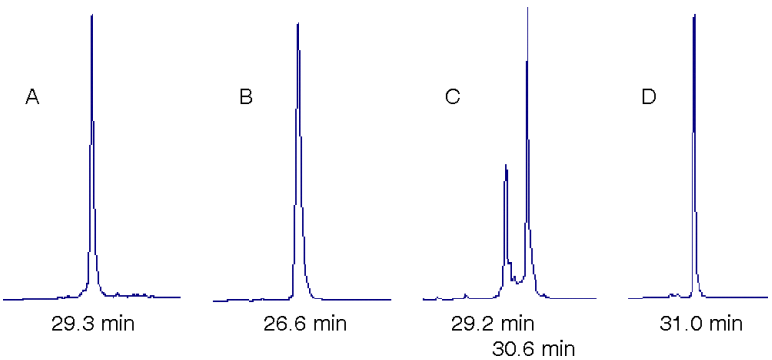

Figure 2 HPLC traces of *N*-EMP long dimer synthesis. Pure AoA-EMP (A) was reacted with pure long dialdehyde (B) by oxime chemistry. After a 60-min reaction (C), two species were observed and the expected product was purified by HPLC (D).

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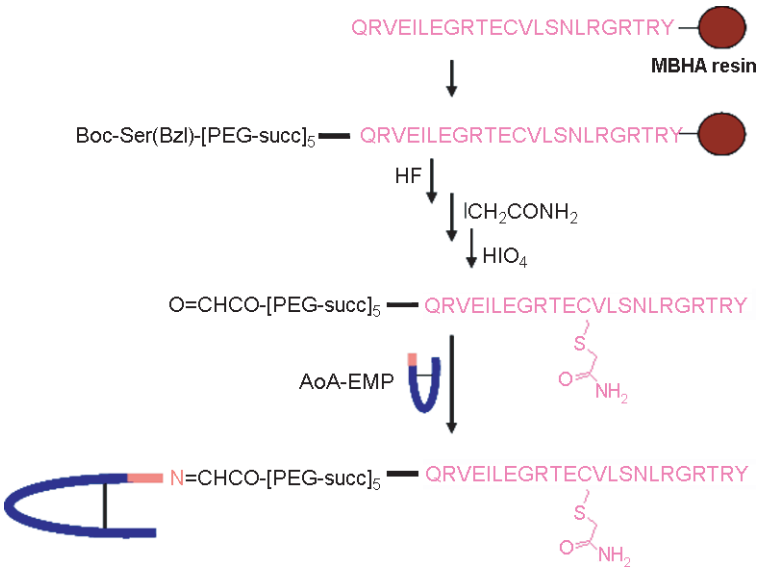

Figure 3 Strategy for the synthesis of *N*-EMP-ERP heterodimer. ERP was synthesized by SPPS on MBHA resin and five 'PEG-succ' units were added on-resin to the chain. After acid cleavage, Cys protection with iodoacetamide and Ser oxidation to glyoxylyl function, the ERP construct with an aldehyde terminal linker was obtained. Reaction with purified AoA-EMP gave the expected product.

RP-HPLC and possessed the expected masses (Table 1, Figure 2). Traces of a linker missing the 'PEG-succ' unit were observable by MS, but these were insignificant compared to the inhomogeneity of the commercially available PEGs [17]. MALDI analysis of monomeric AoA-EMP derivatives showed an additional mass with 164 Da adduct that could not be identified. Since the pure EMP dimer molecules had one single mass, no further purification was performed. The biological activity of the different compounds is currently under investigation.

Synthesis of ERP-EMP dimers

A new type of EPO analog, a heterodimer, was synthesized in an attempt to profit from multivalency (Figure 3). Besides EMP, the peptide ERP was synthesized and used. ERP was found to activate EPOR by interacting with a site distant from the hormone binding site [13,26], whereas EMP was shown to bind EPOR at the same site as EPO [27]. Moreover, ERP and EPO were shown to have synergic action *in vitro* [13]. Hence, a heterodimer EMP-ERP is expected to bind more strongly to EPOR than an EMP dimer or an ERP dimer.

For this heterodimeric construct, the idea was to synthesize a heterodimer composed of one ERP and one EMP linked by a flexible linker. The synthesis strategy was different from that for the EMP homodimers, although a similar monodisperse polyamide linker was used; for the heterodimer we synthesized the ERP construct by SPPS and added on-resin five 'PEG-succ' units on its *N*-terminus. By finally coupling a serine residue, we obtained pure Ser-[PEG-succ]$_5$-ERP after resin cleavage and HPLC purification. As there were many impurities in the crude material, we tried coupling the prepurified Boc-Ser(Bzl)-[PEG-succ]$_6$-OH on-resin to the chemically synthesized ERP. Using PyBOP as a coupling agent with 3 equivalents of DIEA, the expected product was synthesized, but unfortunately over-acylation was observed. Solution-phase coupling was also attempted but no reaction occurred. This was probably due to the remaining water content of the linker, even after 15 h of exposure to high vacuum on the lyophilizer. To oxidize the terminal serine to glyoxylyl, we first needed to protect the free cysteine of the ERP to avoid sulfonic acid formation. This was performed by alkylating the molecule with 20 equivalents of iodoacetamide. Alkylation was a reasonable approach because ERP was reported to be active under reducing conditions, showing that a preformed disulfide was probably not necessary for activity [26]. The serine was then oxidized with periodate to obtain the necessary aldehyde for oxime ligation. Reaction with pure AoA-EMP or EMPK-AoA gave the expected heterodimer products (*N*-EMP-ERP and *C*-EMPK-ERP heterodimers). Analytical RP-HPLC gave one single peak and the MS data were as expected (Table 1, Figure 4).

CONCLUSIONS

New synthetic EPO analog dimers have been synthesized following a protocol that allows great flexibility in linker length and attachment point. Starting from known EMP sequences that were discovered by phage display [5] and from ERP (a peptide active on

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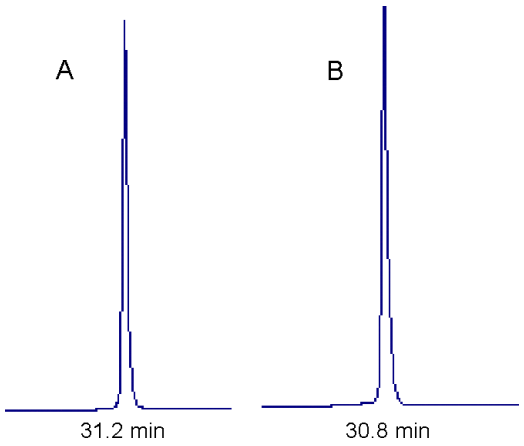

Figure 4 HPLC traces of *N*-EMP-ERP (A) and *C*-EMPK-ERP (B) constructs.

EPOR through an alternative binding site), new multivalent constructs were designed. Using monodisperse polyamide linkers of desired lengths, monomeric peptides [6] were separated by chosen distances. Attaching the peptides to the linkers by oxime bonds allowed the synthesis of homogeneous constructs compared to previous EPO analogs [6,15], with the possibility to attach the peptides through their *N*- or *C*-terminus. With a choice of linkers of different lengths and EMPs with functional aminooxy group at different positions, the possibilities for new compounds are large. The synthesis strategy used in this work for the synthesis of multivalent compounds is applicable to any active peptide for a corresponding receptor. The oxime bond is compatible with human use [28] and is stable under physiological conditions [29], so the molecules lend themselves to biological tests.

The heterodimeric constructs also combine the advantages of monodispersity of the linker with construct homogeneity, and open a new approach for EPOR targeting. By targeting the receptor on two distinct sites with one single molecule, moreover with active peptides that have synergic activity, the improvement for biological activity is promising.

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