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N-terminal specificity of PEGylation of human bone morphogenetic protein-2 at acidic pH

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ABSTRACT

Site-specific PEGylation offers the possibility to modify a therapeutic protein without interfering with its biological activity. Previously, a preferential N-terminal PEGylation has been reported for several proteins when the reaction was performed at acidic pH. In the present study it was explored if acidic pH favors N-terminal PEGylation of bone morphogenetic protein-2 (BMP-2). PEGylation by poly(ethylene glycol) aldehyde (PEG-AL) or poly(ethylene glycol) carboxymethyl succinimidyl ester (PEG-NHS) was performed at moderate acidic pH of 4. Comparing with PEG-NHS, PEG-AL converted more BMP-2 mainly to mono- or di-PEGylated derivatives at much less molar excess and shorter duration. Analysis of Tryptic fragments of the PEGylated derivatives indicated a partial N-terminal PEGylation specificity. PEG-AL exhibited higher specificity than PEG-NHS. UV spectrometry proved that PEGylation improved the solubility of BMP-2 in PBS. Surface plasmon resonance showed that PEGylation decreased the binding of BMP-2 proteins to a type II receptor. Remarkably, mono-PEGylated BMP-2 with PEG-AL showed higher cellular bioactivity than unmodified protein. Higher N-terminal PEGylation specificity correlates with higher receptor binding affinity and cellular activity. In summary, PEGylation of BMP-2 by PEG-AL and PEG-NHS at acidic pH exhibits a partial N-terminal specificity which however might be sufficient for an efficient site-specific PEGylation process.

1. Introduction

Bone morphogenetic protein-2 (BMP-2), as a member of the large transforming growth factor-β (TGF-β) superfamily of multifunctional cytokines, can induce ectopic bone and cartilage formation in adult vertebrates (Reddi, 1997, 1998) and plays important roles in early embryonal development in animals (Hogan, 1996). It has received large interest during the past decade due to its therapeutic use in regenerative medicine. BMP-2 is applied together with a carrier during surgery (Seeherman and Wozney, 2005). Its poor solubility under physiological conditions, the presence of a heparin-binding site, and a short circulation half life promote a desirable localized action of BMP-2 for indications as spinal fusion, repair of non-union fractures, and sinus lift augmentation (Robinson et al., 2008; Kwong and Harris, 2008).

Protein PEGylation (attachment with PEG) has been a successful approach to slow down ultrafiltration in the kidney, to alter tissue distribution, and to reduce phagocytosis, proteolysis and immunogenicity (Veronese and Pasut, 2005; Caliceti and Veronese, 2003). BMP-2 and its analogues TGF-β1 and TGF-β2 were PEGylated by poly(ethylene glycol) carboxymethyl succinimidyl ester (PEG-NHS) under slightly alkaline conditions to immobilize protein in tissue engineering scaffolds in order to improve local bone regeneration (Bentz et al., 1998), lengthen local fibroblastic response (Liu et al., 2007) or increase muscle matrix production (Brebda et al., 2001). This work mostly focused on the immobilization and did not analyze the attachment site of PEG and the subsequent effect on the bioactivity of proteins.

PEGylation protocols frequently employ amine reactive PEGs. This however, can lead to differing attachments and loss of biological activity, since lysine residues are abundant in proteins and are often part of functionally important sites. Thus, site-specific PEGylation is an attractive possibility offering the advantages to retain bioactivity of protein drugs and to generate chemically identical entities with predictable pharmaceutical behavior. Different routes were employed for this purpose. Mutagenesis technique has been the only general route currently employed to delete unde-

Abbreviations: BMP, bone morphogenetic protein; PEGylation, conjugation with poly(ethylene glycol); TGF, transforming growth factor; PEG-NHS, poly(ethylene glycol) carboxymethyl succinimidyl ester; G-CSF, granulocyte-colony stimulating factor; GLP-1, glucagon-like peptide-1; EGF, epidermal growth factor; PEG-AL, PEG aldehyde; DMSO, dimethylsulfoxide; $NaBH_3CN$, sodium cyanoborohydride; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; HCl, hydrogen chloride; NaCl, sodium chloride; PBS, phosphate buffered saline; ALP, alkaline phosphatase; BMP-2-monoPEG-AL, mono-PEGylated BMP-2 by PEG-AL; BMP-2-diPEG-AL, di-PEGylated BMP-2 by PEG-AL; BMP-2-monoPEG-NHS, mono-PEGylated BMP-2 by PEG-NHS; BMP-2-diPEG-NHS, di-PEGylated BMP-2 by PEG-NHS.

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sired potential attachment site or generate single attachment site, and subsequently realize site-specific PEGylation (Rosendahl et al., 2009; Zappe et al., 2008; Veronese et al., 2007). In our previous work, based on the fact that BMP-2 does not contain free cysteine in its homodimer (Scheufler et al., 1999), BMP-2 cysteine analogues were generated by site-directed mutagenesis at amino acid positions Ala 2, Asn 56, and Glu 96 (Hu et al., 2010). PEGylation at engineered Cys 56 and 96 located near the receptor binding epitopes exhibited significant reduction in BMP-2 activity. PEGylation at Cys 2 in the N-terminal segment far away from binding epitopes showed a bioactivity comparable to BMP-2 wild type. However, cysteine specific PEGylation is demanding both with respect to work and time. Often PEGylation products are obtained in low yields, since mutants have to be generated and SH chemistry requires special precautions. Therefore, it would be desirable to develop easier methods to achieve site-specific PEGylation with retention of the bioactivity of proteins.

Kinstler et al. (2002) developed an easy and specific PEGylation method for the N-terminal amino group by using PEG aldehyde or PEG-NHS at moderate acidic pH. The selective reaction of the N-terminal α-amine in the presence of lysine ε-amines was attributed to their different pK_a values (7.6–8.0 for the former and 10.0–10.2 for the latter). Granulocyte-colony stimulating factor (G-CSF) (Kinstler et al., 1997), glucagon-like peptide-1 (GLP-1) (Lee et al., 2005), epidermal growth factor (EGF) (Lee et al., 2003), and endostatin (Nie et al., 2006) were reported to be PEGylated specifically at the N-terminus employing this approach.

The present experiments explore the specificity of PEGylation of BMP-2 at acidic pH. Mature BMP-2 exists as a disulfide-bonded homodimer consisting of nine lysine residues in each monomer. Four lysine residues occur in a flexible N-terminal segment and five in the cysteine knot domain, which is crucial for receptor binding (Scheufler et al., 1999). Six of the lysines are recovered in a large Tryptic fragment of BMP-2. Now, PEG-AL and PEG-NHS were reacted with wild type BMP-2 in a simple one-step reaction. The two PEGs were compared with respect to reactivity, yield and site-specificity. The solubility, the receptor binding activity, and cellular activity of PEGylated proteins were investigated.

2. Materials and methods

2.1. Materials

Recombinant human BMP-2 was expressed in *Escherichia coli* and purified as described previously (Ruppert et al., 1996; Groppe et al., 1998).

Poly(ethylene glycol) aldehyde (PEG-AL, Mn = 5008 Da, SUNBRIGHT ME-050AL), and poly(ethylene glycol) carboxymethyl succinimidyl ester (PEG-NHS, Mn = 5383 Da, SUNBRIGHT ME-050AS) were purchased from NOF, Inc. (Kyoto, Japan). Functional PEGs were dissolved in analytical grade dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, U.S.A.) and stored at −20 °C. Sodium cyanoborohydride ($NaBH_3CN$) was purchased from Sigma (St. Louis, MO, U.S.A.). Buffer chemicals and SDS-PAGE chemicals were purchased from Carl Roth (Karlsruhe, Germany). Phenylmethylsulfonyl fluoride (PMSF) and Trypsin (excision grade) were purchased from Merck (Darmstadt, Germany).

2.2. PEGylation at analytical scale

BMP-2 (0.1 mg, 1 mg/mL) and different amounts of PEG-AL or PEG-NHS were incubated in pH 4.0 acetic acid buffer (50 mM sodium acetate-acetic acid, pH 4.0) and 10% (v/v) DMSO. 40 mM $NaBH_3CN$ was added during reaction with PEG-AL. The PEGylation mixture was shaken at 700 rpm at 4 °C for up to 10 h for PEG-AL and 48 h for PEG-NHS.

Samples were taken at different time intervals and diluted 4 folds with non-reducing loading buffer for SDS-PAGE.

2.3. PEGylation at preparative scale

2.3.1. With PEG-AL

BMP-2 (5 mg, 1 mg/mL) and PEG-AL (2 mg/mL, molar ratio of PEG to protein of 10:1) reacted in pH 4.0 acetic acid buffer plus 40 mM $NaBH_3CN$ and 10% (v/v) DMSO. The reaction was shaken at 700 rpm at 4 °C for 10 h.

2.3.2. With PEG-NHS

BMP-2 (2 mg, 1 mg/mL) and PEG-NHS (50 mg/mL, molar ratio of PEG to protein of 250:1) reacted in pH 4.0 acetic acid buffer plus 10% (v/v) DMSO. The mixture was shaken at 700 rpm at 4 °C for 48 h.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a 12% polyacrylamide as described previously (Laemmli, 1970). 2–5 folds dilution by non-reducing sample buffer was used for sample preparation. The gels were stained with Coomassie Blue.

2.5. Evaluation of stained gels with Image J

Image J was downloaded from the website of the National Institutes of Health (http://rsbweb.nih.gov/ij/). The intensity distribution of the stained protein bands after SDS-PAGE was determined with Image J at 2-digit accuracy.

2.6. Reversed phase HPLC (RP-HPLC)

RP-HPLC was performed on a liquid chromatography system equipped with a GRACE VYDAC 214TP54 C4 column (250 mm × 4.6 mm), a Merck Hitachi L-6200A intelligent pump, a Merck Hitachi L-4000 UV detector and a Gilson FC203B Fraction Collector. The wavelength in the detector was set at 280 nm.

The PEGylation mixture (2–5 mL) was concentrated 4–10 folds with a centrifugal filter device Centricon® (MwCO 5000) to 0.5 mL. Then buffer was changed to 0.1% trifluoroacetic acid (TFA) by 3 times dilution and concentration to remove most of DMSO. The solution was then loaded on the 0.1% TFA equilibrated RP-HPLC column. For elution, 0.1% TFA (eluent A) and acetonitrile (eluent B) were used. Gradient starts from 0% to 25% eluent B in 10 min, 25–80% B in 55 min, and then 80–100% B in 5 min.

2.7. Trypsin digestion

0.1 mg PEGylated BMP-2 dissolved in 70 μL 1 mM HCl was mixed with 10 μL 0.1 mg/mL Trypsin solution in 1 mM HCl. A 20 μL solution of 2.5 M Tris and 250 mM NaCl, pH 8.5 was then added, and the whole mixtures were kept at 37 °C for 4 h. 1 mM PMSF was added to stop the reaction before sample preparations for SDS-PAGE.

2.8. Solubility analysis in phosphate buffered saline (PBS)

The UV spectra of PEGylated BMP-2 in PBS (150 mM NaCl, 10 mM sodium phosphate salt, pH 7.4) were recorded with an UV–Visible Spectrophotometer (CARY 50 BIO, Varian). Unmodified BMP-2 was used as the control. Stock aqueous solutions containing 4 μM of free or PEGylated BMP-2 were diluted with the same volume of double concentrated PBS, mixed thoroughly and kept at room temperature for 2 h. The spectra of the solution were recorded between 250 nm and 320 nm.

2.9. Biosensor interaction analysis

Biosensor experiments were carried out on a BIAcore 2000 system (Pharmacia Biosensor) at 25 °C at a flow rate of 10 μL/min in running buffer (10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 7.4, 500 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid, 0.005% surfactant polyoxyethylene sorbitan P20) with a data collection rate of 2.5/s. A CM5 biosensor chip was first coated with streptavidin in all four flow cells, and subsequently biotinylated Activin receptor IIB (ActR-IIB) was immobilized on the streptavidin matrix in flow cells 2 and 3 (Kirsch et al., 2000).

2.10. C2C12 alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was determined in serum-starved mouse C2C12 cells. The promyoblast C2C12 cells (ATCC-CRL1772) in Eagle's minimal essential medium containing 10% fetal calf serum were incubated at 1×10^5 cells/mL in 100 μL aliquots in a 96-well plate for 24 h at 37 °C and 5% carbon dioxide. Afterwards, the medium was substituted with 100 μL fresh medium containing 2% fetal calf serum containing various concentrations of the BMP proteins. After a further cultivation for three days, cells were lysed in 100 μL lysis buffer (0.1 M glycine, 1% nonyl phenoxylpolyethoxylethanol, 1 mM Magnesium chloride, 1 mM Zinc chloride, pH 9.6) and alkaline phosphatase activity was determined in 100 μL aliquots of the cleared lysate using 100 μL 0.3 mM p-nitrophenylphosphate in the color buffer (0.1 M glycine, 1% nonyl phenoxylpolyethoxylethanol, 1 mM Magnesium chloride, 1 mM Zinc chloride, pH 9.6) as substrate. Absorbance at 405 nm was recorded after incubation of 50 min.

3. Results

3.1. PEGylation at pH 4

The reactivity of BMP-2 under acidic conditions with two amine-specific PEG-derivatives was compared. (i) PEG-NHS carries N-hydroxysuccinimide as reactive group, which is usually applied at pH 8. (ii) PEG-AL forms a Schiff base with an amino group which is subsequently transformed into a stable secondary amine by mild reduction with $NaBH_3CN$. PEGylation of BMP-2 at different concentrations was analyzed by SDS-PAGE, as shown in Fig. 1.

Both activated PEGs yielded predominantly mono- and di-PEGylated proteins, which can be recognized after SDS-PAGE as the first and second band above unmodified BMP-2 migrating as a protein with an apparent molecular weight of 26,000 Da. Multiple PEGylated forms are present in only low amount. PEG-AL showed a higher reactivity than the NHS-activated PEG (Fig. 1). With 10 h reaction time and a 10-fold molar excess of PEG-AL, 42% mono-PEGylated and 28% di-PEGylated BMP-2 were observed (Table 1). In contrast, PEG-NHS of 250-fold molar excess yielded only 36% mono- and 18% di-PEGylated BMP-2 in 48 h. 42% of the BMP-2 protein still remained unmodified.

Remarkably, no BMP-2 monomers were observed after incubating with $NaBH_3CN$ for 10 h (lane 2 in Fig. 1a), indicating that at least the inter-monomer disulfide bond of BMP-2 is stable under this condition. As mentioned above, native BMP-2 is a disulfide bonded dimer with a crucial cysteine knot in each of its monomers (Scheufler et al., 1999).

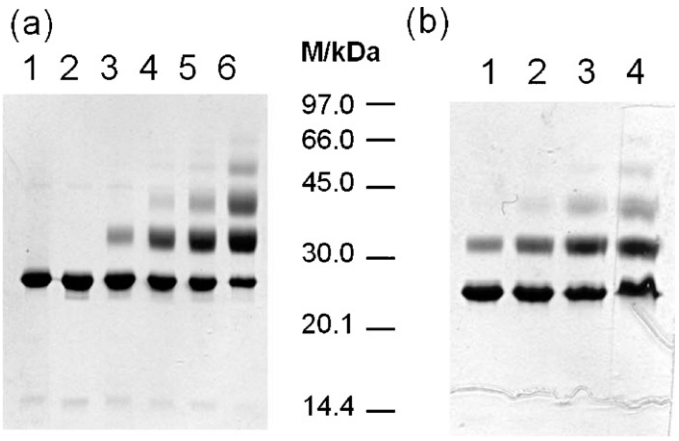

Fig. 1. SDS-PAGE analysis of modified BMP-2 proteins after PEGylation by PEG-AL for 10 h (a) and PEG-NHS for 48 h (b). Lanes 1 and 2 in (a) correspond to unmodified BMP-2 and BMP-2 incubated with 40 mM $NaCNBH_3$. Lanes 3–6 in (a) correspond to PEG-AL/protein ratios of 1:1, 3:1, 5:1, 10:1, respectively. Lanes 1–4 in (b) correspond to PEG-NHS/protein ratios of 1:1, 30:1, 100:1, 250:1, respectively.

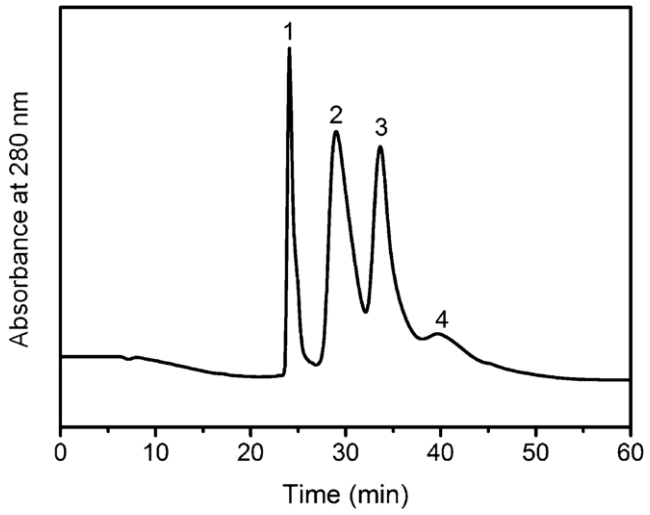

Fig. 2. RP-HPLC separation of modified BMP-2 proteins PEGylated at a 10-fold molar excess with PEG-AL for 10 h. Peak 1: unmodified BMP-2; 2: mono-PEGylated BMP-2; 3: di-PEGylated BMP-2; 4: multi-PEGylated BMP-2.

3.2. Preparation of mono- and di-PEGylated BMP-2 proteins

The modification reaction at preparative scale was performed at a large excess of PEG-AL (10-fold molar excess) and PEG-NHS (250-fold molar excess) in order to generate also some di-PEGylated BMP-2 (see Section 2). As shown in Fig. 2, unmodified, mono- and di-PEGylated BMP-2 could be readily separated by RP-HPLC. The relative amounts of the different forms, as measured by the peak areas, correspond well to those determined by densitometry of the stained protein bands after SDS-PAGE (Table 1). The separated mono- and di-PEGylated BMP-2 proteins have purity better than 90% (Fig. 3, lanes 2–6).

Table 1
Relative PEGylation of BMP-2 obtained after labeling with PEG-AL and PEG-NHS. Stained protein gels as shown in Fig. 1 were evaluated with Image J at 2-digit.

PEG	PEG/protein molar ratio	Time (h)	Unmodified BMP-2 (%)	Mono-PEGylated BMP-2 (%)	Di-PEGylated BMP-2 (%)	Multi-PEGylated BMP-2 (%)
PEG-AL	10:1	10	22	42	28	8
PEG-NHS	250:1	48	42	36	18	4

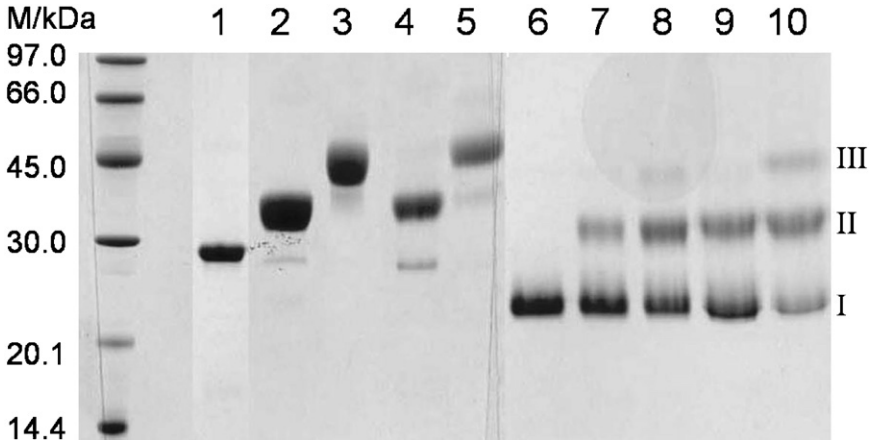

Fig. 3. SDS-PAGE analysis of unmodified and PEGylated BMP-2 proteins, and their Trypsin digests. Lanes 1–5 represent unmodified BMP-2, BMP-2-monoPEG-AL, BMP-2-diPEG-AL, BMP-2-monoPEG-NHS, and BMP-2-diPEG-NHS, respectively. Lanes 6–10 correspond to the Trypsin digests of the proteins shown in lanes 1–5. The band labeled "I" corresponds to large BMP-2 fragment missing the N-terminal part. Bands II and III correspond to the large BMP-2 fragment with one and two attached PEG, respectively.

3.3. Trypsin digestion

The BMP-2 dimer contains 2×9 lysine ε-amino groups and 2×1 N-terminal α-amino groups, which potentially can react with the PEG derivatives. 2×4 of the lysines occur in the flexible N-terminal segment and 2×5 in the cysteine knot domain. In order to get a first estimate on the site-specificity of the PEGylation at pH 4, the BMP-2 proteins were digested with Trypsin which cleaves the unmodified BMP-2 predominantly after Lys 8 and Arg 9 (Hu et al., 2010). The resultant large fragments containing 6 lysine side chains can be detected by SDS-PAGE (Fig. 3, lanes 6–10). The N-terminal fragment comprising the N-terminus, Lys 3, Lys 5 and Lys 8 was not further analyzed.

In Fig. 3 the protein bands labeled "I" correspond to the unmodified large fragment of BMP-2, protein bands labeled "II" and "III" correspond to the mono- and di-PEGlated large BMP-2 fragments. For a schematic representation see Fig. 4. The mono-PEG-AL derivative yields mainly (74%) an unmodified large fragment (Table 2), and also in the BMP-2-diPEG-AL the majority (60%) of the large fragment remains unmodified. This indicates that the PEGylation with the AL-derivative occurred predominantly at the N-terminal peptide.

PEGylation specificity of PEG-NHS was less pronounced. Here 66% of the unmodified large fragment from BMP-2-monoPEG-NHS and only 36% of the unmodified large fragment from BMP-2-diPEG-NHS were observed.

In summary, PEG-AL and PEG-NHS exhibit a partial specificity during labeling of BMP-2. A random reaction of all amino groups would yield 60% of the label in the large fragment. PEG-AL exhibited higher specificity for the N-terminal segment of BMP-2 than PEG-NHS under the applied acidic condition. It remained undetermined in the present experiments if this site specificity is caused by a preferential reaction of the N-terminal amino group. However, based on the assumption that all lysine ε-amino groups become equally labeled one can calculate that 61% $[100 - (100 - 74) \times 9/6]$ of the label is bound to the N-terminal amino group in BMP-2-monoPEG-AL and 49% $[100 - (100 - 66) \times 9/6]$ in BMP-2-monoPEG-NHS.

3.4. Solubility of PEGylated BMP-2 proteins

BMP-2 is poorly soluble under physiological condition. In PBS the solubility limit is around 200 nM. In the organism this might prevent systemic activity of BMP-2. In order to see whether PEGylation improves solubility in PBS, the spectral properties of 2 μM solutions of free and PEGylated BMP-2 were determined, as shown in Fig. 5. Rather than showing a typical spectrum with a broad peak between 300 nm and 250 nm for protein clear solution, free BMP-2 at 2 μM concentration in PBS showed a spectrum with a

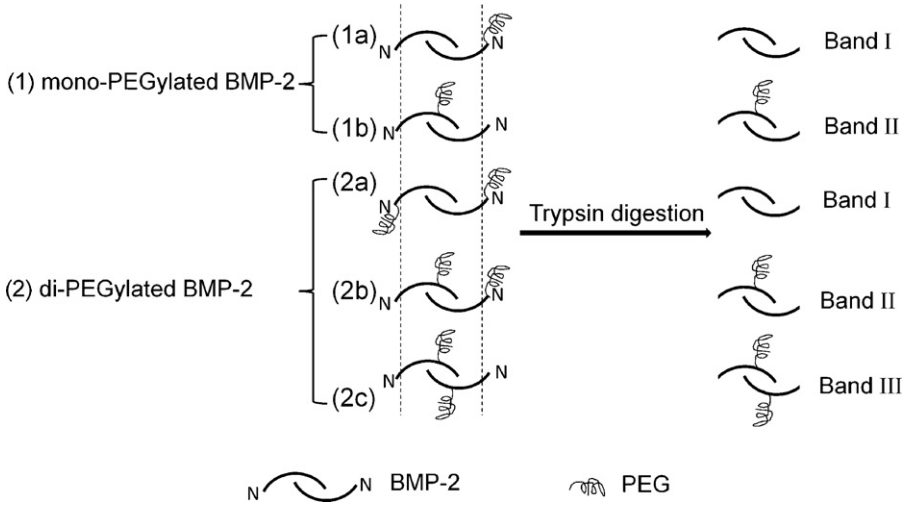

Fig. 4. Illustration of the possible position isomers of mono- and di-PEGylated BMP-2 and their corresponding large Tryptic fragments. N indicates the N-terminal segment.

Table 2
Distribution of PEG label in the large Tryptic fragment of mono- and di-PEGylated BMP-2. Stained gels as presented in Fig. 3, lanes 7–10 were evaluated with Image J at 2-digit.

	I (%)	II (%)	III (%)		I (%)	II (%)	III (%)
BMP-2-monoPEG-AL	74	26	–	BMP-2-diPEG-AL	60	34	6
BMP-2-monoPEG-NHS	66	34	–	BMP-2-diPEG-NHS	36	46	18

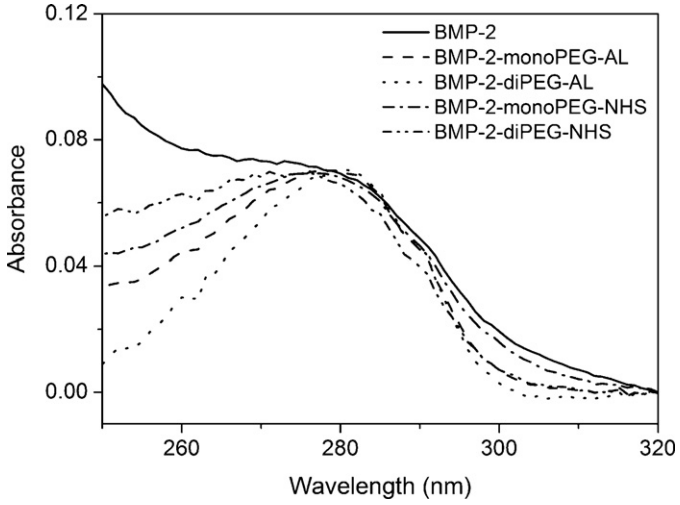

Fig. 5. UV spectra of 2 μM solutions of free BMP-2 and PEGylated BMP-2 proteins in PBS.

continuous increase of absorbance from 320 nm to 250 nm, which is generally observed due to light scattering when protein aggregates are present, since according to the Rayleigh scattering theory the light with shorter wavelength scatters more intensely. Similar-shaped spectra were reported for TGF-β at pH 4.8–10 when TGF-β aggregates were presented (Pellaud et al., 1999). Although possibly the variation around 250 nm can be partially attributed to differences in the molecule, in comparison with the irregular spectrum of 2 μM free BMP-2, the spectra of 2 μM mono- or di-PEGylated BMP-2 proteins with much more regular protein absorption peaks could rather signify more molecularly dispersed BMP-2 protein solutions. This indicates that the attached PEG enhances indeed the solubility of BMP-2 under physiological pH and ionic strength. The improved solubility due to the PEGylation probably broadens the application dose, which is very significant for the therapeutic use of BMP-2.

3.5. Binding of PEGylated BMP proteins to the BMP receptor

In order to explore how the PEGylation alters receptor binding, BMP-2 proteins modified with one or two PEG molecules according to the AL- or the NHS-method were submitted to Surface Plasmon Resonance (Biacore) interaction analysis with immobilized ectodomain of BMP type II receptor ActR-IIB. The recorded sensorgrams (Fig. 6) show that the PEGylation generally lowers the binding affinity of the mono-PEGylated, and even more so for the di-PEGylated BMP proteins.

However, in addition it can be recognized that the BMP-2 proteins modified by the PEG-AL bound somewhat stronger than the BMP-2 proteins modified with PEG-NHS. This suggests that AL-PEGylation at pH 4 has a less severe effect on receptor affinity than the NHS-PEGylation. Thus, in comparison to the NHS-PEGylation lower modification of the large Tryptic fragment after AL-PEGylation (see Table 2) correlates with a higher biological activity as measured by receptor binding (Fig. 6).

3.6. Bioactivity evaluated by alkaline phosphatase cell assay

The biological activity of the PEGylated BMP-2 proteins was analyzed with C2C12 cells using unmodified BMP-2 as a reference, as shown in Fig. 7. Remarkably, although 26% of the large Tryptic fragment with crucial receptor binding domain was attached with PEG, overall BMP-2-monoPEG-AL exhibited higher cellular bioactivity than unmodified BMP-2 protein. The other three PEGylated BMP-2s showed lower bioactivity than unmodified BMP-2. Correlating with receptor binding activity, the BMP-2 proteins modified by the PEG-AL induced more alkaline phosphatase than BMP-2 proteins modified with PEG-NHS. In addition, mono-PEGylated proteins remained higher cellular bioactivity than di-PEGylated proteins.

4. Discussion

The PEGylation of amino groups takes place primarily via a nucleophilic substitution reaction: an unprotonated amino group attacks the carbonyl group of an aldehyde (PEG-AL) or ester (PEG-

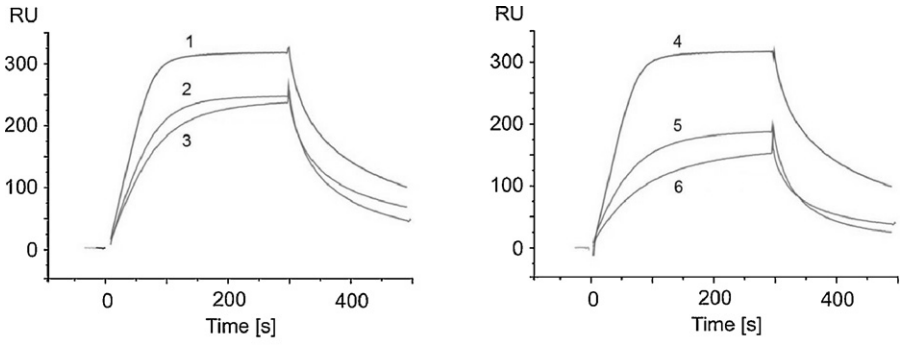

Fig. 6. Sensorgrams of the interaction of PEGylated BMP-2 proteins with the ectodomain of BMP receptor ActR-IIB (Biacore). The ectodomain of ActR-IIB was immobilized at the flow cell 2 of a streptavidin-coated CM-5 chip (Hu et al., 2010). Purified BMP-2 proteins at 20 nM concentration were perfused for 300 s (see Section 2). 1 and 4, BMP-2; 2, BMP-2-monoPEG-AL; 3, BMP-2-monoPEG-NHS; 5, BMP-2-diPEG-AL; 6, BMP-2-diPEG-NHS.

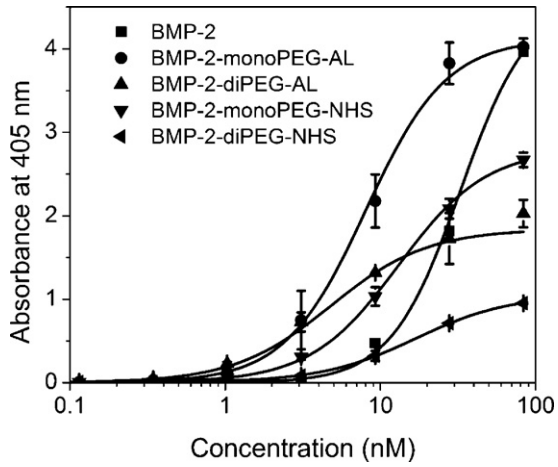

Fig. 7. Biological activity of mono- or di-PEGylated BMP-2 with PEG-AL and PEG-NHS. Dose-dependent induction of alkaline phosphatase in C2C12 cells was determined for BMP-2-monoPEG-AL (●), BMP-2-diPEG-AL (▲), BMP-2-monoPEG-NHS (▼) and BMP-2-diPEG-NHS (◄). Unmodified BMP-2 (■) analyzed in parallel served as a reference.

NHS). In a first approximation, all primary amines present in a protein exhibit a similar reactivity at basic condition. Therefore, a partial PEGylation with PEG-NHS under basic condition produces a heterogeneous mixture of isomers PEGylated at different sites (Lee et al., 2005; Wylie et al., 2001). The N-terminal α-amine has a pK_a of 7.6–8.0 which differs from the more basic pK_a of 10.0–10.2 of the ε-amino group of lysine (Wong, 1991). Therefore, under acidic conditions a higher percentage of the N-terminal amino group exists in the unprotonated state making it more reactive than the ε-amine of lysine. In comparison, the carbonyl group in aldehyde is more electron deficient than that in ester. This correlates with a higher reactivity of the PEG aldehyde compared to the PEG ester in the present experiments.

Peptide mapping data reported for G-CSF (Kinstler et al., 1997) and GLP-1 (Lee et al., 2005) indicate a specific N-terminal PEGylation at moderate acidic pH values (4–5) with aldehyde functionalized PEG and slightly acidic pH value (6.5) with NHS functionalized PEG. These proteins contain only a few lysines (4 and 2, respectively). The BMP-2 homodimer contains 18 lysine residues. The majority of them (12 lysines) can be recovered in the large Tryptic fragment. The PEG label identified in this fragment therefore indicates that in BMP-2 PEGylation at pH 4 is not completely specific for the N-terminal amino group. It is still undetermined in how far lysine PEGylation is influenced by steric factors and by the ionic environment in the protein. The relative small percentage of lysine-attached PEG label recovered in the large Tryptic fragment strongly suggests that more than half of the PEGylation exists in the N-terminal amino group. PEG-AL showed higher N-terminal specificity under the applied condition than PEG-NHS. A possible superior N-terminal specificity of the AL PEG was not analyzed in detail, for instance at different concentrations of the PEG aldehyde. It remains therefore unknown if the experimental conditions or the chemistry of the reaction are responsible for this observation.

Receptor binding of BMP-2 is mediated by the cysteine knot domain which is contained in the large Tryptic fragment (Sebald et al., 2004). The reduced binding affinity for the receptor of the PEGylated BMP-2 proteins can be possibly attributed to the interaction of the attached PEG with the binding epitopes (Mittl et al., 1996). A similar reduction was observed for all the PEGylated BMP-2 cysteine analogues analyzed previously (Hu et al., 2010). Remarkably, PEGylation of a cysteine at position 2 near the N-terminus showed an improved biological activity in a cell-based assay. The N-terminal segment of BMP-2 contains a heparin binding site which reduces the specific activity of BMP-2 in cellular assays probably by interacting with heparinic sites in the extracellular matrix (Ruppert et al., 1996). The attached PEG might shield the heparin binding site and prevent a competitive interaction with the extracellular matrix, and thus improve the bioactivity of the PEGylated A2C analogues. The extent of BMP-2 PEGylation appears to be critical for N-terminal specificity and bioactivity. Mono AL-PEGylated BMP-2 exhibited the best N-terminal specificity and the most pronounced enhancement of bioactivity. At higher PEGylation level lysine side chains near or in the receptor binding epitopes become modified leading to an inhibition of bioactivity. Nevertheless, higher N-terminal PEGylation specificity correlates with both stronger receptor binding and higher cellular activity of the BMP-2 protein.

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