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An Improved Procedure for the Synthesis of Branched Polyethylene Glycols (PEGs) with the Reporter Dipeptide Met-βAla for Protein Conjugation

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Abstract—A new and more efficient route to the synthesis of branched PEG for protein conjugation, bearing a reporter dipeptide Met-βAla, is described, which allows better purification of the final product by ion exchange chromatography. The product has the combined advantages of an 'umbrella-like' branched structure, which allows a better coverage of the protein surface, and the presence of the dipeptide Met-βAla which has been used to detect the position of PEGylation within the peptide sequence.

Conjugation of proteins and peptides with biocompatible polymers is a novel technology which is receiving increasing attention for its pharmacological and pharmaceutical implications,[1–3] as well as for its applications in the immobilisation and solubilisation of enzymes for organic phase reactions.[4,5]

Many proteins have been linked to one or more polymeric chains, in order to protect them, once administered, from rapid enzymatic degradation and to increase their half life. Examples of such conjugates are PEG-Asparaginase, PEG-Superoxide dismutase,[6] PEG-α-2a interferon,[7] to name only a few.

Polyethylene glycol (PEG) is a widely used polymer in this field, because of its unique physical and chemical properties: it is totally biocompatible, being non-antigenic, non-immunogenic and non-toxic.[8] Such properties are also transferred to its ballast, which is therefore protected from immune system degradation and from adhesion to other proteins (serum albumin, etc.).

One of the main problems embedded into the conjugation of PEG chains to a protein is the determination of the exact site of binding. Many different chemistries have been so far developed in order to address specific aminoacidic residues within a primary sequence,[9] and therefore reduce the number of possible isomers, but either the reaction efficiency is weak, or the target residues are uncommon, leading in the first case to low reaction yields, in the second case to procedures of excessively narrow scope.

Amine residues are common reactive moieties present in protein side chains, and this, together with the wide variety of *N*-alkylation or *N*-acylation techniques available, makes them a very attractive target for PEGylation.[6–9] But the widespread availability of amino groups within a given protein is also a drawback, in some cases resulting in multiple attachment of several PEG molecules, which often causes a complete loss of bioactivity.

In such cases, either no attempt is made to characterise the mixture of mono- and poly-PEGylated products, or poorly reliable techniques can be envisaged, for example partial enzymatic digestion of the protein, which gives an approximate idea of the distance of the PEGylation site from a specific and known site of enzymatic degradation (the PEG molecule with its steric bulk prevents the enzymatic digestion of the peptide sequence in the surroundings of the PEGylation site, and no digestion products will form).

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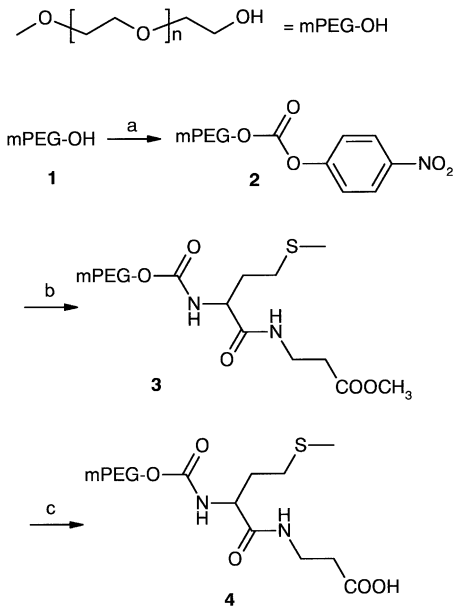
In our laboratory, we had already prepared[10,11] a single chain monomethoxy PEG polymer (mPEG-OH) carrying a dipeptidic spacer methionine-norleucine (Met-Nle) or methionine-βalanine (Met-βAla). The presence of the methionine allows the removal of the polymer chains from the protein molecule by cyanogen bromide treatment following a well known reaction in protein chemistry. After cleavage at the methionine level, Nle or β-Ala remain linked to the protein as a reporter group in the position formerly occupied by the PEG chains. The protein, devoid of its polymeric burden, may be more easily fractionated into the position isomers than the PEGylated form, and the different components can be analysed by any sequencing procedure.

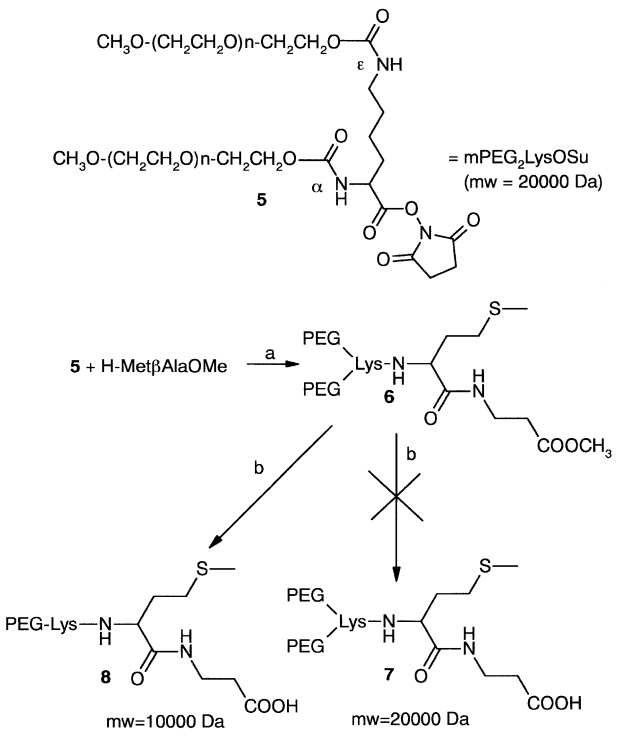
The full protein, or the peptides obtained by digestion, carrying the unnatural aminoacids norleucine or β-alanine, may be analysed by MALDI or electrospray mass spectrometry, revealing the weight gain due to the new aminoacid. The digestion products can also be characterised by amino acid analysis after exhaustive acid hydrolysis, or sequenced by Edmann degradation.[12] Aminoacid analysis of the fully digested protein indicates the number of Nle or β-Ala residues, and therefore the number of PEG chains attached.

In a previous paper, we described the syntheses of a linear mPEG chain bearing the dipeptides Met-Nle and Met-βAla as the reporter spacers.[10] In those syntheses, the conjugation of the dipeptide methyl ester to a mPEG–OH activated as *p*-nitrophenyl carbonate (**2**) proceeded smoothly to afford the ester **3** (Scheme 1).

Hydrolysis of the ester moiety in NaOH 1 N provided the final product **4**, which could be purified from the unreacted mPEG–OH by ion exchange chromatography (QAE Sephadex A50 anion exchange resin).



Scheme 1. Reagents and conditions: (a) *p*-nitrophenyl chloroformate, Et_3N, dry dichloromethane; (b) H-MetβAlaOMe, dry dichloromethane; (c) 1 N aq NaOH.



Scheme 2. Reagents and conditions: (a) Et_3N, dry dichloromethane; (b) 1 N aq NaOH.

'Branched' PEG analogues are known to be superior with respect to the linear ones in creating an 'umbrella-like' surface coverage of the protein (Fig. 1), thus protecting it from proteolysis and reducing its inactivation during conjugation. An example, $PEG_2LysOSu$ (**5**, Scheme 2), has been synthesised in our laboratory[13] and is now commercially available (Shearwater Polymers Inc., USA).

In order to prepare a PEG derivative with both the branched structure and the reporter dipeptide Met-βAla, we at first decided to adapt the synthetic procedure already used for the linear compound **4**, but starting from the commercially available $PEG_2LysOSu$ active ester **5** (Scheme 2).

To our surprise, MALDI-TOF spectrometry and size-exclusion HPLC of the hydrolysed product returned a single peak of half the expected molecular weight, suggesting the loss of one of the two mPEG chains (**8**).

We investigated such a singular behaviour, which did not occur with the single branched derivative **4**, using different pH conditions for hydrolysis, different

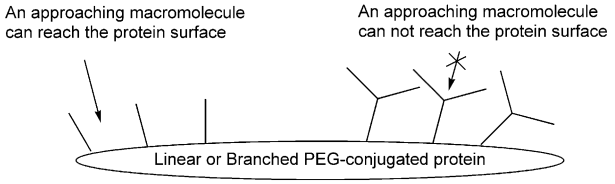
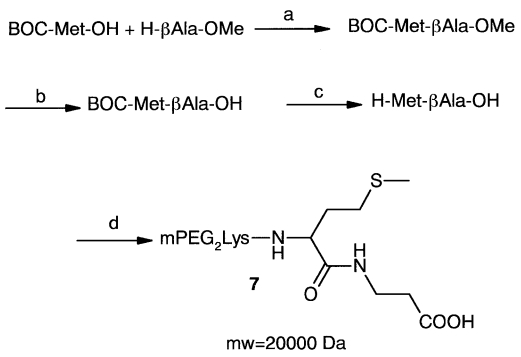
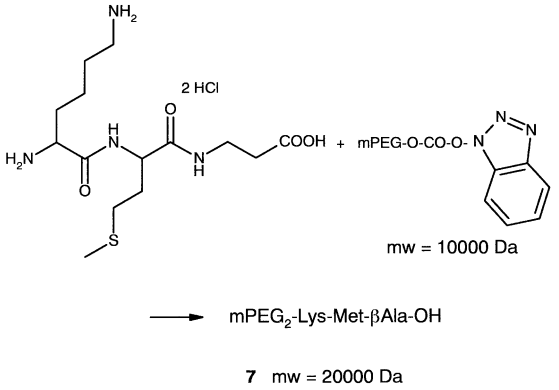


Figure 1. The 'umbrella-like' structure of branched PEG covers a larger protein surface (from ref 10).



Scheme 3. Reagents and conditions: (a) DCCI, HOBt, Et_3N, dry dichloromethane; (b) 1 N aq NaOH; (c) TFA, 2% H_2O; (d) **5**, Et_3N, dry dichloromethane.



Scheme 4. Reagents and conditions: borate buffer 0.1 M, pH 8, rt, 12 h.

aminoacidic sequences and Molecular Modelling tools, and the results, which suggest an anchimeric assistance of the carboxylic moiety to the Nα-carbamoyl function hydrolysis, will be presented in a future communication. As several attempts with different standard basic hydrolysis procedures failed to afford the branched product from the ester **6**, we felt that the simplest way to overcome the problem was to conjugate the branched PEG_2LysOSu with the dipeptide H-Met-βAla-OH, prepared from the fully protected BOC-Met-βAla-OMe by basic hydrolysis of the methyl ester and removal of BOC with TFA/2% H_2O.

This 'second generation' procedure (Scheme 3) finally gave the desired product, although a severe limitation was in this case the lack of purification techniques which could separate the unreacted branched PEG_2Lys-OH from the product **7**: both the reagent and the product were negatively charged, bearing a terminal carboxylic group, and exhibited similar molecular weights, thus precluding preparative size-exclusion or ion-exchange chromatography purification.

The unreacted PEG_2Lys-OH could also compete with the final product **7** in the protein conjugation step, leading to mixtures of differently substituted products.

This fact prompted us to investigate a completely different and somewhat more elegant approach, in which the tripeptide H-Lys-Met-βAla-OH was first prepared by standard solid-phase synthesis, and mPEG-OH, activated as its benzotriazolyl carbonate (mPEG-OBt),[14] was coupled to the two amine residues of lysine to afford the branched derivative in one step (Scheme 4).

The starting material mPEG-OH, being uncharged, could be easily removed by ion exchange chromatography, and pure PEG_2Lys-Met-βAla-OH (**7**) was recovered and characterised by size-exclusion HPLC (Phenomenex® Biosep SEC S3000 column, RI detector), MALDI-TOF mass spectroscopy, ^{1}H NMR and titration of the carboxylic groups.[15]

In conclusion, we reported an improved synthesis for the branched PEG derivative PEG_2Lys-Met-βAla-OH, which is a useful tool for PEGylation reactions of proteins and easy detection of the PEGylation sites within the protein amino residues. The synthesis has been designed bearing in mind the need for an easy purification of the unreacted PEG-OH from the product, in view of a possible industrialisation of the process.

Acknowledgements

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15. Compound **7**: Crystalline, off white solid. ^{1}H NMR [$CDCl_3$] δ 1.5 (m, 2H), 1.72 (m, 2H), 1.78 (m, 2H), 2.01 (m, 2H), 2.2 (s, 3H), 2.5 (t, 2H), 2.6 (m, 2H), 2.9 (m, 2H), 3.2–3.8 (m, PEG), 4.0 (m, 1H), 4.45 (m, 1H), 8.3 (t, 1H), 8.9 (d, 1H). HPLC (Phenomenex® Biosep SEC S3000 gel-filtration column, Jasco 830 RI refractive index detector, mobile phase $NaHPO_4$ 0.1 M, NaCl 0.3 M, 20% CH_3CN): single peak at 7.6 min, apparent M_m 20,000 Da (according to calibration curve with PEG of M_w 5000–40,000). Mass (MALDI-TOF): 20,000 ± 100 Da, titration (NaOH 0.01 N) of carboxylic groups: 0.99 equivalents.