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Anchimeric assistance effect on regioselective hydrolysis of branched PEGs: a mechanistic investigation

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Abstract—Branched poly(ethylene glycols) (PEG2) are nowadays widely used for protein and peptides bioconjugation, for their favourable properties (such as the ability to protect the protein surface in an 'umbrella like' fashion). The discovery that $mPEG_2$-LysMetβ AlaOEt lost one mPEG chain during standard base-catalysed ester hydrolysis conditions prompted us to investigate the hydrolytic stability of such systems and the mechanism involved in the PEG chain loss. A series of branched PEGs, substituted with different aminoacids and dipeptides, have been prepared to test the influence of steric hindrance, chain lengths, ramification and Lys-AA amide substitution on hydrolysis. Unexpected results reveal an anchimeric assistance of the Lys-AaA amide proton to the hydrolysis of the carbamoyl moiety joining mPEG to the α-amino group of lysine through the formation of an hydantoin system.

1. Introduction

Polyethylene glycol (PEG) is a polymer widely used as a covalent modifier of biological macromolecules and particulates as well as a carrier for low molecular weight drugs. Its applications in pharmaceutical chemistry are receiving in the last few years an increasing attention due to its unique properties, witnessed by the flourishing number of reviews in the field.[1–3]

It is virtually nontoxic, nonantigenic and nonimmunogenic, and therefore seems to be a good candidate for human use both in pharmaceutical and in food applications. Moreover, it is widely acknowledged that the modification of proteins by covalent attachment of poly(ethylene glycol) (PEG) can eliminate some drawbacks of native proteins and improve their physicochemical, biomedical and pharmacological properties.

Bioconjugation to macromolecules of pharmaceutical interest led to PEG-bound α-interferon,[4] α-2b-interferon (INTRON® A, Shering Plough; PEGASYS®, Hoffman-La Roche), asparaginase (ONCASPAR®, Enzon),[5] adenosine deaminase (ADAGEN®, Enzon)[6] and G-CSF (Granulocyte colony stimulating factor),[7] to mention only those into clinical trials or already in use. More recently, conjugation of PEG to small molecules like anticancer drugs (doxorubicin, taxol, camptothecin, Ara-C) has been proven to positively modify the pharmaceutical profile of such species.[1] Covalently linked to proteins and peptides, often through aminoacid side chain amines or hydroxyls, PEG acts as a protective layer, reducing in large extent the physiological enzymatic degradation of such polypeptides and avoiding their recognition by the host immune system. This in turn results in increased life span and circulation time of the active species, reducing the need for high doses and/or frequent administration.

The infancy of this science the bond between the polymer and the protein (or the drug) was as simple as an ester or an amide,[8] its role being of mere junction; in the recent years however, the chemistry of bioconjugation has received an increasing attention in the medicinal

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chemistry laboratories, and the set up of new selective linkers has been recognised as a prerequisite for drug optimisation.[1,9–11]

In this view, the presence of aminoacids or peptide spacers between PEG and the attached payloads provides several advantages, due to the variability of properties that may be introduced using a suitable aminoacid or peptide spacer.

Many prodrug polymeric systems have been developed using peptide sequences optimised for enzymatic degradation. Enzymes like catepsin B, present into the intracellularly located lysosomes,[12] as well as thermolysin,[13] plasmin[14] and other extracellular proteins, have been targeted.

The so-called branched PEG (or PEG2)[15] is one of these new entities, where two linear PEG chains are linked together through two functions of a tri-functional spacer (e.g. lysine) while the third is used to bind the protein. '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 (**1**, Fig. 2), has been synthesised in our laboratory[15] and is now commercially available (Shearwater Polymers Inc., USA).

The identification of PEGylation site within the protein sequence is another issue, which has been solved in some specific cases by careful Edman degradation[16] or tryptic partial hydrolysis of the protein. To overcome the intrinsic difficulties of these methods, in our laboratory we developed two peptide spacers, Met-Nle and Met-β Ala.[17,18] The presence of the methionine allows the removal of PEG by cyanogen bromide treatment, leaving a 'reporter group' (norleucine or β-alanine) that can be detected by protein sequencing and amino acid analysis techniques.

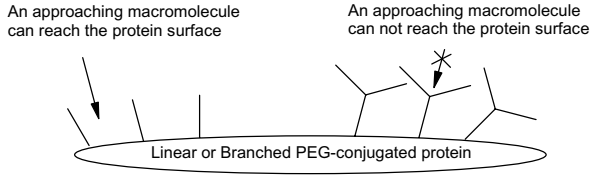


Figure 1. The 'umbrella-like' structure of branched PEG covers a larger protein surface.

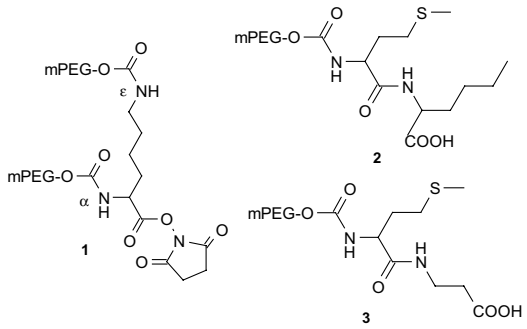


Figure 2. Branched and linear poly(ethylene glycols) (PEGs).

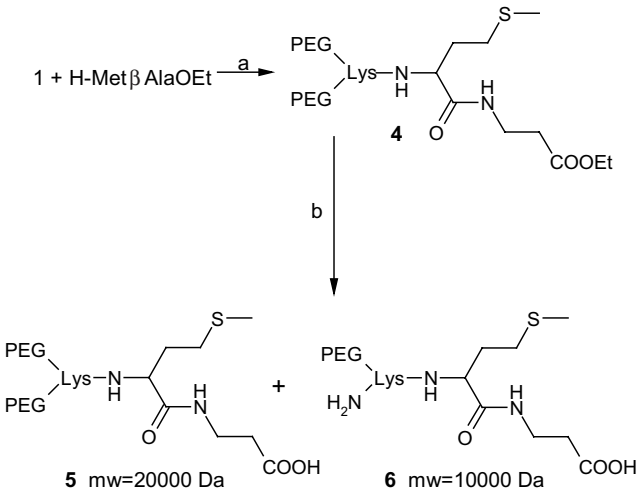
2. Chemistry

While linear PEG-Met-Nle-OH (**2**) and PEG-Met-β Ala-OH (**3**, Fig. 2) have been used in different conditions without any problem resulting from premature hydrolysis of the carbamoyl linkage between the PEG chain and the dipeptide, we encountered some difficulties during the synthesis of the branched PEG analogue PEG_2Lys-Met-β AlaOH (20,000 Da, **5**). The synthetic route (Scheme 1), involving the final mild base-catalysed hydrolysis of the ethyl ester, had been mutuated directly from the one used for the linear compounds **2** and **3**.[19]

However, during the final hydrolysis of the ethyl ester to afford the free carboxylic group we could observe, in addition to the expected product **5**, the one in which a loss in molecular weight took place. Actually this decrease in molecular weight was initially detected only once the PEG had been conjugated to a protein of 20,000 Da: instead of the expected 40,000 Da of the diPEGylated species, the MALDI-TOF mass spectroscopy returned a 30,000 Da peak, suggesting the loss of one of the two 10,000 Da PEG chains.

This peculiar behaviour prompted us to investigate the stability of the branched PEG_2Lys-Met-βAla-OEt and, possibly, find the causes for the observed degradation. We prepared a number of derivatives (Fig. 3), with different aminoacid side chains and consequently different degrees of steric hindrance.

We also prepared N-methyl aminoacids derivatives and secondary amide systems (PEG_2 LysSarOH, **11** and PEG_2Lys-ethyl iminodiacetate, **12**). Each product was synthesised according to Scheme 1 using standard



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

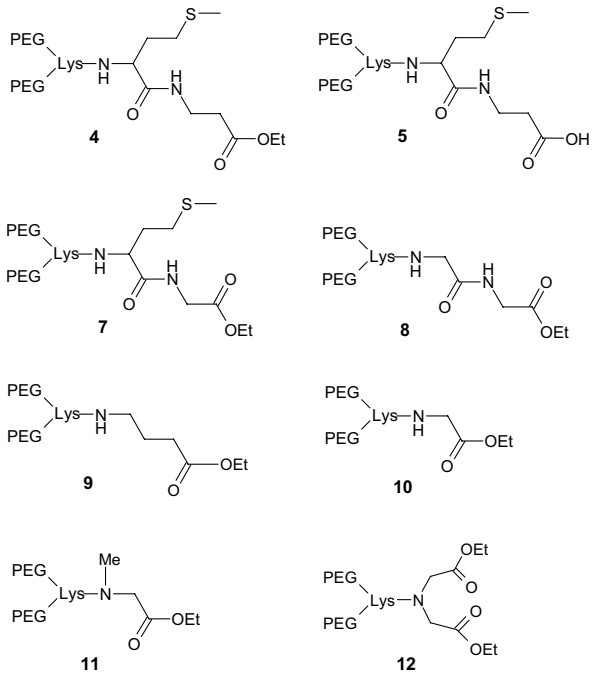
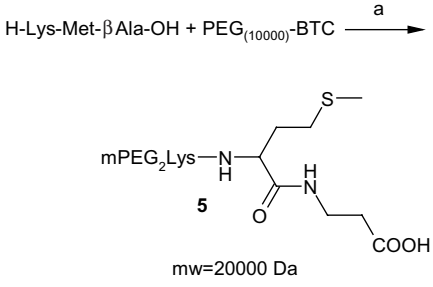


Figure 3. Synthesised branched PEG derivatives.



Scheme 2. Reagents and conditions: (a) borate buffer, pH 8, 12 h, room temperature.

peptide coupling techniques, and characterisation was achieved with ^{1}H NMR and gel-filtration HPLC. Compound **5** was instead synthesised according to Scheme 2, binding the activated species PEG$_{(10,000)}$-BTC (commercially available from Shearwater, Inc., USA) to the tripeptide H-LysMetβAla-OH in high overall yield.

3. Results and discussion

For each compound we performed the hydrolysis in three conditions, using 1 M LiOH, 1 M Na_2CO_3 and 1 M $NaHCO_3$ aqueous solutions. The hydrolysis rates (i.e., the percentage of PEG 10,000 Da forming from the starting material, measured by gel-filtration chromatography and refractive index (RI) detector) for the synthesised compounds are reported in Figure 4 for LiOH 1 M, Na_2CO_3 1 M and $NaHCO_3$ 1 M. It can be immediately appreciated that in the strongest basic conditions used (LiOH 1 M) compounds **4**–**10** rapidly hydrolyse within 6 h with loss of one PEG chain ($t_{1/2}$ spanning between 30′ to 4 h), while compounds **11** and **12** remain

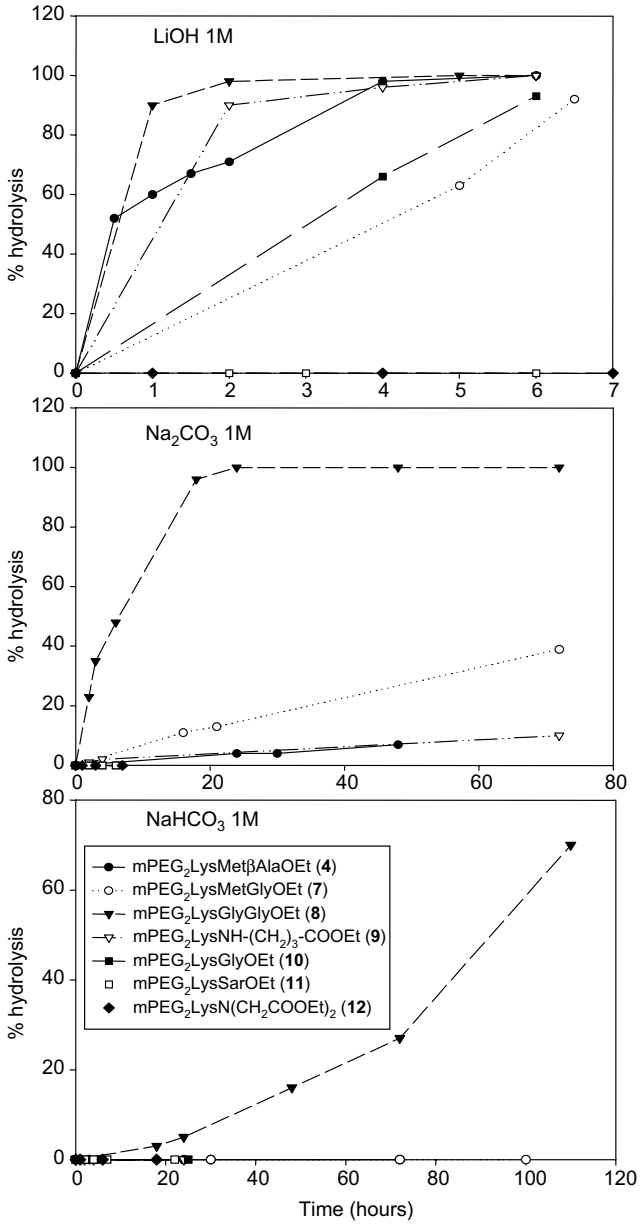


Figure 4. LiOH 1 M, Na_2CO_3 1 M and $NaHCO_3$ 1 M hydrolysis of branched PEG derivatives.

unaltered. But the strongest differences became evident in Na_2CO_3 and $NaHCO_3$ conditions.

To determine, which mechanism was involved in the hydrolysis, and also, which of the two PEG chains (the one attached to Nα or the one attached to Nε of lysine, both through a carbamate bond) was removed, we first tried to reduce the peptide chain length (PEG$_2$LysMetβAlaOEt **4**, PEG$_2$LysMetGlyOEt **7**, PEG$_2$LysGABAOEt **9**, PEG$_2$LysGlyOEt **10**).

To exclude the participation of the methionine sulfur atom to the hydrolytic process we prepared PEG$_2$LysGlyGlyOEt (**8**), where glycine took place of methionine but the total chain length from the urethane functions to the carboxylic ester was conserved. We could observe that the hydrolysis rate for this product was faster than

the hydrolysis rate of PEG_2Lys-Met-Gly-OEt (**7**) in LiOH 1 M, but strikingly higher in Na_2CO_3. In $NaHCO_3$ **8** was the only compound to show a relevant hydrolysis rate.

At this point we were not yet certain of the exact mechanism for the hydrolysis, as the variation in chain length did not significantly modify the hydrolysis rate under the three conditions tested, excluding an involvement of a terminal carboxylic group in an anchimeric assistance mechanism following the preliminary hydrolysis of the ester.

As the commercial product PEG_2LysOH is very stable to basic conditions, we felt that some other nucleophilic moiety within the peptide spacer arm could provide assistance to the hydrolysis of one of the two urethane bonds. From literature[20] we knew that Z-protected dipeptides (Z-AA-GlyOH for example) are prone to hydantoin formation under basic conditions. This is an example of a most general situation depicted in Figure 5, where a nucleophile (Nu) positioned at 3–4 atoms from a carbonyl system (ester, carbonate, carbamate) can cyclise to form a 5 or 6 membered ring.

According to this mechanism, in our systems the Lys-Met amide bond would be involved in the cyclisation and hydantoin formation process: the final proof came from the behaviour of the two secondary amide systems **11** and **12**, that did not show detectable hydrolysis products even in the harsher conditions. Further validation of the hypothesis came from the Edman degradation assay, which was carried out on the product obtained from PEG_2Lys-Met-βAla-OEt (**4**) hydrolysis. If the hydrolysed PEG chain were the one bound to the α-amino residue of lysine, the Edman degradation step would have taken place, giving a fragment dipeptide Met-β Ala. If, on the other hand, the hydrolysed chain were the ε- one, no Edman degradation would have occurred, and the entire fragment PEG_2Lys-Met-Ala-OH could have been detected. In fact, our results confirmed the formation of a fragment dipeptide Met-βAla, indicating that the PEG chain attached to the α-amino moiety of lysine was selectively hydrolysed. The proposed mechanism is depicted in Figure 6.

Still, the particular behaviour of some compounds (e.g. **7**, which hydrolyse slower than **10** in LiOH, but faster in Na_2CO_3) need further investigations, but we believe that it could be imputed to differences in structure flexibility (and therefore ease of ring closure) rather than a different reaction mechanism.

To support our hypothesis concerning the anchimeric assistance effect operating in the base-catalysed hydrolysis

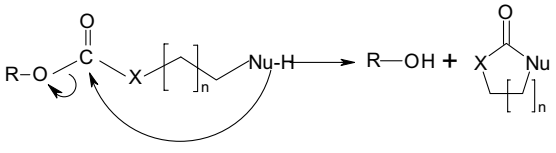


Figure 5. Intramolecular nucleophilic attack and cyclisation.

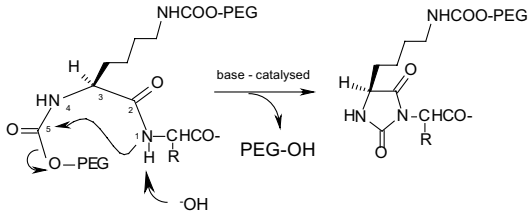


Figure 6. Proposed mechanism for PEG branched alkaline hydrolysis.

of branched PEG derivatives, a molecular modelling study was carried out on two simplified structures: R_2Lys-Met-Gly-OH and R_2Lys-Gly-Gly-OH (with R = MeOCO–). An exhaustive conformational analysis, based on a 'Stochastic Conformational Search Algorithm' was performed to sampling local minima of both the potential energy surfaces (see the Section 5 for details).

These studies put in evidence the formation of hydrogen bonding in an intramolecular ring system, stabilizing a three-aminoacids turn with the Lys-AA amide and the α-urethane moieties in PEG_2Lys-Met-βAla-OH in tight relation. These groups remained in close, but not tight, contact in PEG_2Lys-Gly-OH (Fig. 7a and b, respectively).

4. Conclusions

At the beginning of this series of experiments, we only knew that the branched PEG was degraded under basic

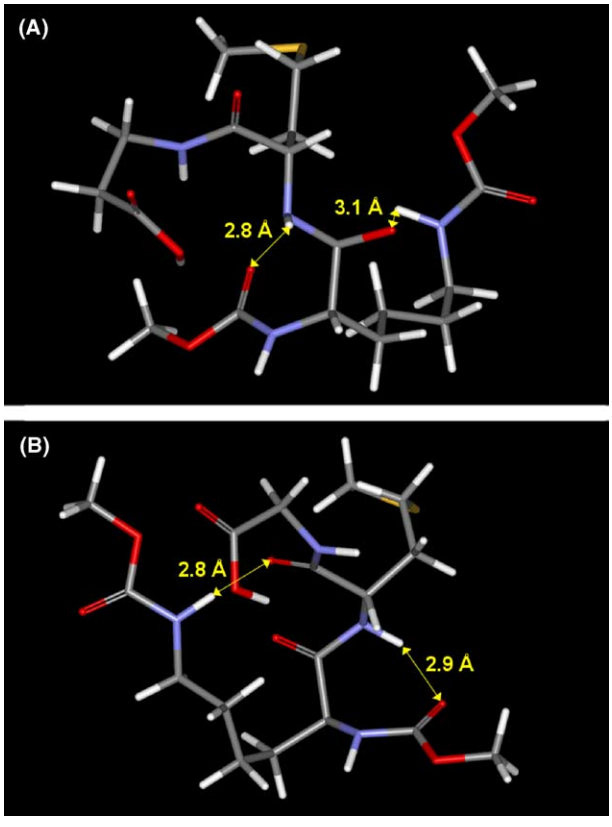


Figure 7. Molecular representation of the most stable conformation of (A) $(CH_3OCO)_2$LysMetβAlaOH (**4**) and (B) $(CH_3OCO)_2$LysMet-GlyOH (**7**).

conditions when at least one aminoacid was bound to the lysine carboxylic group. We did not know why this was not happening to the starting material PEG_2Lys-OH, nor which of the two PEG chains was eliminated.

The synthesised compounds permitted a full series of hydrolysis studies, performed at increasingly milder basic conditions (LiOH 1 M, Na_2CO_3 1 M, $NaHCO_3$ 1 M) (Fig. 4). In strong basic conditions (LiOH, 1 M), the reaction rate was almost comparable for all the compounds but **11** and **12**, as well as in $NaHCO_3$ (with the striking exception of **8**). In Na_2CO_3 we could observe a different behaviour depending on the compounds structure, and in particular: on the chain length (the hydrolysis rate is higher for **7**, with **9** and **10**, which behave as **4**); on the steric bulk (**8** is the most flexible, due to two glycine residues, compared to **4** and **7**; surprisingly, **9** and **10**, where flexibility should be even higher, are less prone to hydrolysis).

Disubstituted amides (**11** and **12**) did not undergo hydrolysis even in harsh alkaline conditions, demonstrating that the presence of a primary amide is essential and the reaction mechanism proposed, based on the deprotonation of the amide and hydantoin cyclisation, is indeed the responsible for the loss of the α PEG chain.

These findings are of particular importance in the synthesis of the branching PEGs carrying a reporter group (Nleu or β-Ala) flanking a methionine, for protein PEGylation purposes.[19] Unexpected loss of one polymer chain, when basic conditions were used for coupling reactions (e.g. DIPEA or other tertiary amines) to deprotonate amine residues and facilitate PEG conjugation, was in fact observed. Using N-methyl aminoacids flanking lysine (e.g. N-methyl methionine), we will avoid such risk and improve the PEGylation technology.

The easy cleavage of one of the two polymer chains in branched PEGs by mild base-catalysed hydrolysis may play an important role in their removal from the body when a high molecular weight PEG is used for protein or drug conjugation; it is in fact known that the renal excretion threshold is around 50 kDa. Interestingly, 20 kDa polymer material were found in urine following administration of α-interferon conjugated with 40 kDa branched PEG.[21]

We also devised a new route to compound **5**, that overcome the hydrolysis issues embedded in the previous synthetic route (Scheme 2) and provides the branched compound in good overall yield.

5. Experimental section

5.1. Materials

$mPEG_2$LysOSu, Mw 20,000, and mPEG-BTC, Mw 10,000, were purchased by Shearwater Polymers (Huntsville, AL). *N*,*N*-Dicyclohexylcarbodiimide, *N*-hydroxybenzotriazole, Boc-methionine, β-alanine methyl ester, glycine methyl ester, glycyl-glycine were purchased from Sigma Aldrich S.r.l. (Milano, Italy).

HPLC analyses were performed on a Jasco HPLC system (880-PU pump, Jasco 830-RI refractive index detector) coupled to a Shimadzu C-R4A Chromatopac integrator and a Phenomenex BIOSEP SEC S3000 Gel filtration column; mobile phase 80% aq Na_2HPO_4 0.1 M, NaCl 0.3 M, pH 7, 20% acetonitrile. Ion-exchange chromatography was performed on a Pharmacia chromatography column (resin QAE Sephadex A-50 provided by Pharmacia-Biotech) equipped with a Pharmacia P-10 pump and carousel fraction collector. Iodine assay on collected fraction was used to detect presence of PEG. Dipeptides and aminoacids esters were synthesised according to standard peptide synthesis protocols and purified by Flash chromatography. ^{1}H NMR confirmed the reported structures.

5.2. Synthesis of PEG2 derivatives (4, 7–12)

0.537 mmol of aminoacid or dipeptide were dissolved in 10 mL of anhydrous $CHCl_3$ in inert atmosphere. The solution was adjusted to pH 8 with Et_3N and 500 mg of $mPEG_2$LysOSu (mol. wt. 20,000 Da) were added portionwise. The mixture was stirred at room temperature for 12 h and then extracted with 1 M HCl (2 × 30 mL). Aqueous phases were extracted with fresh chloroform (2 × 20 mL) and the combined organic phase were dried with Na_2SO_4 and concentrated to small volume. The residue was added dropwise to 100 mL of diethyl ether under magnetic stirring. The precipitate was filtered and dried. The crude product was purified by ion exchange chromatography (Sephadex QAE A50 anion exchange resin) using mQ grade H_2O as mobile phase. When all the title compound was collected, increase of ionic strength (0.01 M NaCl) allowed recovery of PEG_2LysOH. The desired product was freeze-dried to afford a white crystalline powder. Gel filtration HPLC (Phenomenex BioSep Sec 3000 analytical column, eluent NaH_2PO_4 0.1 M, NaCl 0.3 M, pH 7, 20% acetonitrile, 1 mL/min; Refractive Index on-line detector) indicated one single peak corresponding to an apparent mol. wt. of 20,000 Da (according to calibration curve with known molecular weight PEGs).

5.3. PEG_2Lys-Met-βAla-OEt (4)

Yield = 89%; t_R = 7.70 min; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.26 (t, 3H); 1.45 (m, 2H); 1.63 (m, 2H); 1.85 (m, 2H); 1.92 (t, 2H); 2.1 (s, 3H); 2.5 (m, 4H); 2.76 (m, 2H); 3.38 (s, 3H); 3.40–3.88 (m, 2H + PEG); 3.95 (m, 1H); 4.20 (q, 2H); 4.23 (m, 1H); 5.27 (d, 1H); 6.75 (t, 1H); 7.05 (t, 1H); 7.20 (d, 1H).

5.4. PEG_2Lys-Met-Gly-OEt (7)

Yield = 72%; t_R = 7.76 min; ^{1}H NMR ($CDCl_3$) δ (ppm): 1.28 (t, 3H); 1.44 (m, 2H); 1.65 (m, 2H); 1.85 (m, 2H); 1.92 (t, 2H); 2.1 (s, 3H); 2.5 (br s, 2H); 2.77 (m, 2H); 3.36 (s, 3H); 3.40–3.88 (m, 2H + PEG); 3.95 (m, 1H); 4.22 (q, 2H); 4.24 (m, 1H); 5.35 (d, 1H); 6.70 (t, 1H); 6.95 (t, 1H); 7.15 (d, 1H).

5.5. PEG_2Lys-Gly-Gly-OEt (8)

Yield = 77%; t_R = 7.72 min; ^1H NMR ($CDCl_3$) δ (ppm): 1.27 (t, 3H); 1.45 (m, 2H); 1.64 (m, 2H); 1.81 (m, 2H); 2.5 (br s, 2H); 2.55 (br s, 2H); 2.77 (m, 2H); 3.36 (s, 3H); 3.40–3.88 (m, PEG); 3.92 (m, 1H); 4.28 (q, 2H); 5.65 (d, 1H); 5.74 (t, 1H); 6.9 (t, 1H); 7.18 (d, 1H).

5.6. PEG_2Lys-NH-$(CH_2)_3$-CO_2Et (9)

Yield = 67%; t_R = 7.98; ^1H NMR ($CDCl_3$) δ (ppm): 1.28 (t, 3H); 1.45 (m, 2H); 1.64 (m, 2H); 1.81 (m, 2H); 2.04 (m, 2H); 2.31 (m, 2H); 2.77 (m, 2H); 3.36 (s, 3H); 3.40–3.88 (m, PEG); 3.92 (m, 1H); 4.15 (m, 2H); 4.28 (q, 2H); 5.65 (d, 1H); 5.69 (t, 1H); 6.8 (t, 1H).

5.7. PEG_2Lys-Gly-OEt (10)

Yield = 70%; t_R = 8.08; ^1H NMR ($CDCl_3$) δ (ppm): 1.27 (t, 3H); 1.45 (m, 2H); 1.64 (m, 2H); 1.81 (m, 2H); 2.5 (br s, 2H); 2.77 (m, 2H); 3.36 (s, 3H); 3.40–3.88 (m, PEG); 3.92 (m, 1H); 4.28 (q, 2H); 5.65 (d, 1H); 5.74 (t, 1H); 6.9 (t, 1H).

5.8. PEG_2Lys-Sar-OEt (11)

Yield = 90%; t_R = 8.19; ^1H NMR ($CDCl_3$) δ (ppm): 1.27 (t, 3H); 1.45 (m, 2H); 1.64 (m, 2H); 1.81 (m, 2H); 2.12 (s, 3H); 2.5 (br s, 2H); 2.77 (m, 2H); 3.36 (s, 3H); 3.40–3.88 (m, PEG); 3.92 (m, 1H); 4.28 (q, 2H); 5.65 (d, 1H); 5.74 (t, 1H).

5.9. PEG_2Lys-N$(CH_2CO_2Et)_2$ (12)

Yield = 89%; t_R = 7.69; ^1H NMR ($CDCl_3$) δ (ppm): 1.27 (t, 6H); 1.45 (m, 2H); 1.64 (m, 2H); 1.81 (m, 2H); 2.5 (s, 4H); 2.76 (m, 2H); 3.36 (s, 3H); 3.41–3.84 (m, PEG); 3.9 (m, 1H); 4.28 (q, 4H); 5.65 (d, 1H); 5.74 (t, 1H).

5.10. Alternative synthesis of PEG_2Lys-Met-βAla-OH (5)[19]

1 g (0.1 mmol, 4 equiv) of PEG-BTC (10,000 Da) was added portionwise, over 30 min time, to a solution of 11.5 mg (0.027 mmol) of the tripeptide H-LysMetβAla-OH in 3 mL of borate buffer 0.1 M (pH 8). During addition of PEG the pH was controlled and adjusted with NaOH 1 M. The resulting solution was stirred at room temperature for 12 h, then it was adjusted to pH 3 (HCl 0.1 N) and extracted with $CHCl_3$ (6 × 50 mL). The organic fractions combined were dried with Na_2SO_4, concentrated and dropped into a round bottom flask containing 200 mL of diethyl ether. The precipitate was filtered with a sintered funnel and dried under vacuum, to yield 0.93 g of a crude product. The crude material was applied to a chromatography column loaded with QAE-50 ion-exchange resin and eluted with a step gradient of water and 0.01 M aq NaCl to separate unreacted PEG (uncharged) from the title compound (0.48 g, 88%).

5.11. Hydrolysis assays

Each compound was tested for hydrolysis in increasingly stronger basic conditions; 10 mg were dissolved in 1 mL of basic solution (1 M LiOH, 1 M Na_2CO_3 and 1 M $NaHCO_3$). The resulting solutions were kept at 25 °C into a thermostatic bath under agitation. At given time intervals 100 μL of reaction mixture were pooled, diluted with 100 μL of eluent (phosphate buffer pH 7, 20% CH_3CN) and injected into a BioSep SEC S3000 gel filtration analytical column. The column was calibrated with commercial standards of PEG 5, 10 and 20 KDa, and retention times were plotted to draw a curve of mol. wt. versus elution. The hydrolysis samples chromatograms gave the percentages of hydrolysed PEG (Mw = 10 KDa) (PEG%) and starting material (Mw = 20 KDa) (PEG2%).

5.12. Computational methodologies

Calculations were performed on a Silicon Graphics Octane R12000 workstation. The simplified models of branched PEGs, R_2Lys-Met-Gly-OH and R_2Lys-Gly-Gly-OH (R = MeOCO–) respectively, were built using the 'Protein Builder' module of Molecular Operating Environment (MOE 2001.01)[22] Peptide structures were minimised using Amber94 all-atom force field,[23] implemented by MOE modelling package, until the rms value of Truncated Newton method (TN)[24] was <0.0001 kcal mol^{-1} Å^{-1}. Charges for the peptide derivatives were imported from the Amber94 force field database.

To exhaustively explore the conformational space of both peptide structures, per performed a 'Stochastic Conformational Search' implemented by MOE. The Stochastic Conformational Search method generates conformations by randomly sampling local minima of the potential energy surface. This method is similar to the RIPS method described by Ferguson and Raber[25] which generates new molecular conformations by randomly perturbing the position of each coordinate of each atom in the molecule by some small amount, typically less than 2 Å, followed by energy minimisation. Each conformer was minimised using Amber94 all-atom force field until the rms value of Truncated Newton method (TN) was <0.0001 kcal mol^{-1} Å^{-1}. Charges for the peptide derivatives were imported from the Amber94 force field database. To model the effects of solvent (water) more directly, a set of electrostatic interaction corrections is used. MOE suite implemented a modified version of GB/SA contact function described by Still and co-workers.[26] These terms model the electrostatic contribution to the free energy of solvation in a continuum solvent model.

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