Bioactive PEG-Peptides

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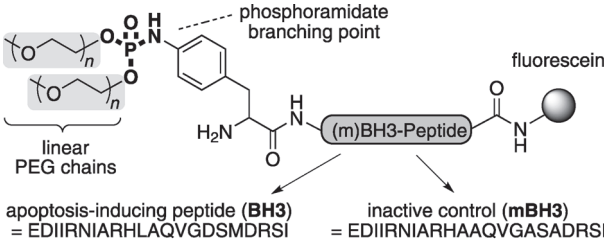
Stabilization of Peptides for Intracellular Applications by Phosphoramidate-Linked Polyethylene Glycol Chains**

*Nicole Nischan, Alokta Chakrabarti, Remigiusz A. Serwa, Petra H. M. Bovee-Geurts, Roland Brock, and Christian P. R. Hackenberger**

Dedicated to Professor Andreas Herrmann on the occasion of his 60th birthday

Peptide- and protein-based drugs have become important therapeutic agents; however, their short circulating half-lives due to clearance in the kidney, antigenicity, and proteolysis present serious obstacles to their application.[1] An established strategy to overcome these problems is the attachment of polyethylene glycol (PEG) to proteins, which shields the protein surface from proteases and prevents them from inducing an adaptive immune response.[2] In this context, it has been shown that branched PEGs protect far better than linear PEGs of the same molar mass due to the so-called "umbrella effect".[3] If the molecular weight of the PEG is about 30 kDa or higher, renal clearance is dramatically decreased.[4] Similarly, also other polymeric materials like poly(*N*-(2-hydroxypropyl)methacrylamide) (HPMA)[5] and even biopolymers themselves, for example, random coils consisting of proline, alanine, and serine residues (PASylation),[6] have been used to stabilize, shield, and solubilize biopolymers. In addition to the modification of biopolymers, the benefits of PEGylation have also been exploited in the design of various drug-delivery vehicles.[7]

Currently, many protein PEGylation strategies address the nucleophilic side chains of canonical amino acids, i.e. amine or thiol groups, which are reacted with electrophilic PEG derivatives.[8] However, as these approaches are not site-specific, the biological activity of the proteins can be decreased. Also, the identification of the PEGylation site and extent of the protein conjugates is challenging.[9] As an alternative, site-specific PEGylation strategies mostly rely on the expression of proteins containing noncanonical amino acids. These carry reactive handles that can be PEGylated chemoselectively: most commonly used are ketones,[10] alkynes,[10b,11] and azides.[10,12] In our own laboratory, we have contributed to these methods with the reaction of a *p*-azidophenylalanine (Pap)-containing protein in a Staudinger-phosphite reaction with PEG-phosphites, which proceeded in excellent yields and chemoselectivity.[13] The Staudinger-phosphite PEGylation strategy has the advantage that inexpensive linear PEG-monomethyl ethers can be used for the synthesis of symmetric PEG-phosphites, which lead after the chemoselective Staudinger reaction to a promising PEG branching point by simultaneous attachment of two PEG chains to the protein through a phosphoramidate linkage (Figure 1).



n	PEG size / Da	apoptosis inducing	inactive control
0	0	BH3 unPEGylated	mBH3 unPEGylated
4	200	BH3-PEG200	mBH3-PEG200
15 ($\bar{n}$)	750	BH3-PEG750	mBH3-PEG750
45 ($\bar{n}$)	2000	BH3-PEG2000	mBH3-PEG2000

Figure 1. Structure and nomenclature of PEGylated phosphor-amidate-linked BH3 peptide conjugates used in this study.

In the current paper we first probe the impact of the phosphoramidate-linked PEG chains on the stability of biologically active peptides in cell lysates. Furthermore, we address the rarely investigated intracellular distribution and finally the biological activity of the PEGylated peptides in an intracellular environment, proposing that the branching point ensures significantly increased peptide stability with small- to medium-sized PEG chains while maintaining peptide function in cells. Specifically, we decided to apply the Staudinger-phosphite PEGylation strategy to the modification of a synthetically challenging proapoptotic peptide. Our 22 amino acid model peptide was derived from the BH3 domain of the

[*] N. Nischan,[+] R. A. Serwa, Prof. C. P. R. Hackenberger
Leibniz-Institut für Molekulare Pharmakologie (FMP)
Robert-Rössle-Strasse 10, 13125 Berlin (Germany)
and
Humboldt Universität zu Berlin, Department Chemie
Brook-Taylor-Strasse 2, 12489 Berlin (Germany)
and
Freie Universität Berlin, Institut für Chemie und Biochemie
Takustrasse 3, 14195 Berlin (Germany)
E-mail: hackenbe@fmp-berlin.de
A. Chakrabarti,[+] P. H. M. Bovee-Geurts, Prof. R. Brock
Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre
P.O. Box 1901, 6500 HB Nijmegen (The Netherlands)
[+] These authors contributed equally to this work.

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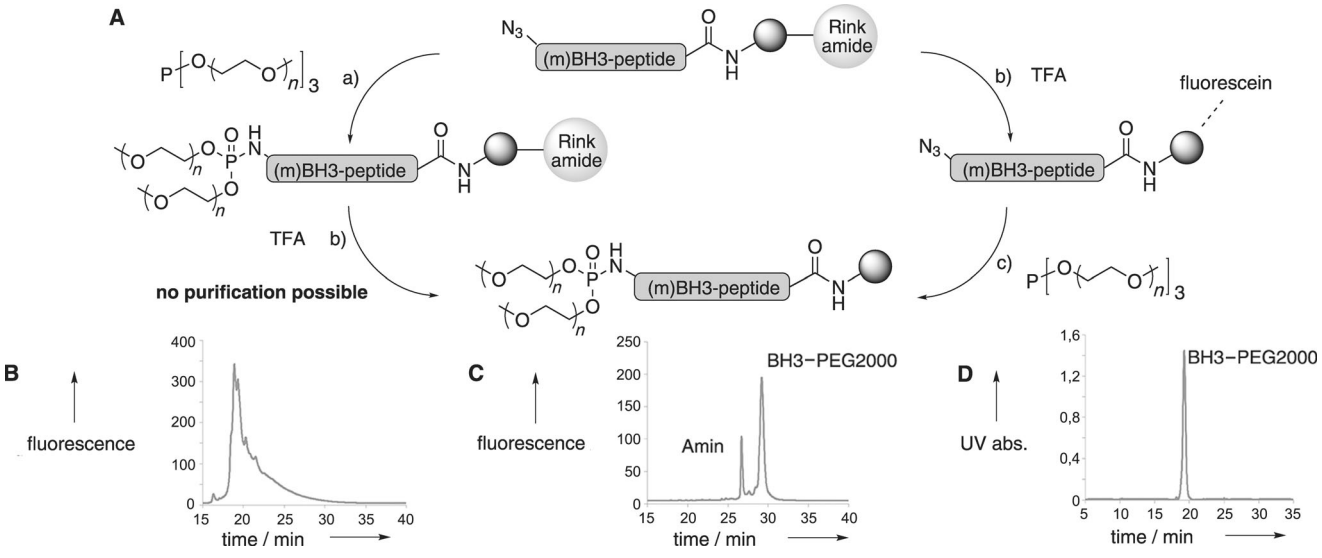
proapoptotic protein BID, for which the measurement of caspase-3 activity serves as the functional readout for induction of controlled cell death.[14] Caspase-3 is an effector caspase, activated during the early phase of apoptosis.[15] BID, the "activator" proapoptotic BH3-only Bcl-2 protein, can directly interact with BAX, a multidomain proapoptotic Bcl-2 effector protein, to form BAX homooligomers in the outer mitochondrial membrane inducing release of cytochrome C and subsequent activation of caspases.[16] We have previously used BID-BH3 peptides to investigate the impact of conjugated linear HPMA scaffolds[14] and multivalency[17] on intracellular activity. Both studies demonstrated that inside the cell, the free peptide does not show activity. Other studies employed conformational constraints[18] or membrane anchoring[19] as principles to yield activity. In addition, linear PEGylated BH3 peptides have previously been used in combination with cancer-cell-directed hormones and drugs to induce apoptosis in cancer cells.[20] Given the general requirement for activity-enhancing peptide modifications, this peptide therefore provides a highly interesting test case for assessing the impact of PEGylation on intracellular peptide activity.

The BH3 peptide as well as a mutated nonactive analogue (mBH3) carried a carboxyfluorescein fluorophore at the C-terminus (Figure 1) to allow the analysis of peptide degradation by fluorescence correlation spectroscopy (FCS) and intracellular peptide distribution by confocal microscopy. The phosphoramidate-branched PEG chains were positioned at the N-terminus of the peptides, which contained two PEG monomethyl ether chains consisting of either 4 (in the BH3- or mBH3-PEG200 peptide; for nomenclature see Figure 1), 15 (in (m)BH3-PEG750) or 45 (in (m)BH3-PEG2000) ethylene glycol units.[21] A dimethylphosphoramidate unit served as an unPEGylated control peptide ((m)BH3 unPEGylated), to which no stabilizing polymer was coupled.

Fluorescent peptides with an N-terminal Pap residue were synthesized on a carboxyfluorescein-lysine containing Rink amide resin[22] by standard Fmoc-based solid-phase peptide synthesis (SPPS; Scheme S1). Subsequent Staudinger-phosphite PEGylation with readily available PEG-phosphites (see the Supporting Information) is possible by employing as substrates either a protected arylazido-BH3 peptide on the solid support or an unprotected peptide in solution. First, we focused on the Staudinger-phosphite reaction on the solid support (Scheme 1 A left), because an excess of the PEG-phosphites as well as the PEG-alcohol that is formed during the reaction can be washed away directly.[23] Unfortunately, despite various efforts of attempts at optimization, the occurrence of several truncated peptidic side products during SPPS led to an mixture of modified peptides after the Staudinger-phosphite PEGylation on the solid support, which was inseparable by HPLC owing to the broad retention time of the PEGylated molecules (Scheme 1 B). To overcome this problem, we decided to take advantage of the chemoselectivity of the Staudinger-phosphite reaction and performed the PEGylation after the azido-containing peptides had been cleaved from the resin and purified by HPLC (Scheme 1 A right). To our delight we found that the reaction proceeded very smoothly in 100 mM TRIS Buffer at pH 8.4 with 30 equivalents of phosphite added in three portions, delivering a crude product which could easily be purified by preparative HPLC (Scheme 1 C). Although this synthetic route still requires an additional final purification step, we were able to obtain pure PEG-phosphoramidate-modified peptides as verified by LC-HRMS (see the Supporting Information).



Scheme 1. A) Staudinger-phosphite PEGylation on the solid support (left) and in solution (right). a) 10 equiv of PEG-phosphite, DMF, 30 °C, 16 h. b) 95 % TFA, RT, 2 h c) 10 equiv of PEG-phosphite, 100 mM TRIS buffer pH 8.2, 30 °C, 16 to 72 h. With: $n = 0$ for (m)BH3 unPEGylated, $n = 4$ for (m)BH3-PEG200, $n = 15$ (avg.) for (m)BH3-PEG750, $n = 45$ (avg.) for (m)BH3-PEG2000 and BH3 = EDIIRNIARHLAQVGDSMDRSI, mBH3 = E-DIIRNIARHAAQVGASADRSI. B) Test cleavage (HPLC, C18, water/acetonitrile gradient) after PEGylation on the solid support. C) HPLC of crude product after PEGylation of unprotected azido-BH3 peptide in solution. D) Isolated BH3-PEG2000 after HPLC. For characterization of PEGylated BH3 peptide products as well as for applied gradients see the Supporting Information.

Highly PEGylated probes have previously been reported to form aggregates or micelle-like structures[24] which may strongly influence the in vivo activity of any biologically active molecule. In light of the subsequent cellular experiments, we first investigated the behavior of the PEG-peptide conjugates in solution. For this purpose, we employed dynamic light scattering (DLS) to measure the hydrodynamic radius and determine whether the conjugates formed aggregates. At a concentration of 1 mM in PBS, all conjugates were present as monomers, demonstrating that all tested BH3 peptides have no tendency to aggregate (see the Supporting Information).

Next we probed the stability of the PEGylated and unPEGylated peptides against proteolytic cleavage upon incubation in crude cell lysate, which was determined by fluorescence correlation spectroscopy (FCS).[25] FCS derives information on the concentration and diffusion behavior of molecules from the analysis of temporal fluctuations of fluorescence resulting from diffusion of particles through a confocal detection volume of less than a femtoliter.

PEGylation by the Staudinger-phosphite reaction was found to dramatically increase the proteolytic half-life of the peptide (Figure 2a,b). There was a positive correlation

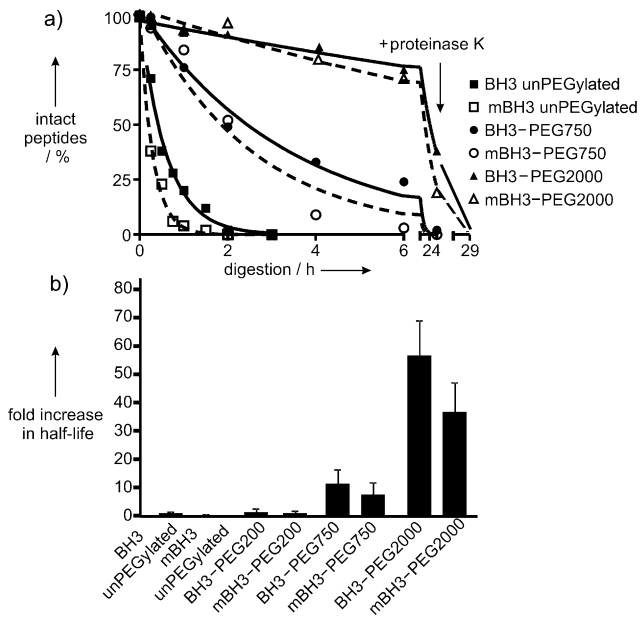


Figure 2. Proteolytic stability of the unPEGylated and PEGylated peptides. a) Representative proteolytic degradation experiment using 2 μM peptides incubated with crude cell lysates of Jurkat E6.1 cells and analysis using FCS. b) Fold increase in average relative half-life and standard deviation of the unPEGylated BH3 peptide and the PEGylated conjugates of three independent experiments.

between the PEG chain length and the increase in stability (Figure 2b). In comparison to the unPEGylated control peptide, BH3-PEG200, BH3-PEG750, and BH3-PEG2000 showed increases in half-life by factors of 1.5, 11, and 57. Addition of a high concentration of proteinase K resulted in a complete degradation of the PEGylated BH3 conjugates (Figure 2a and Figure S1) demonstrating that PEGylation inhibits, but not completely impedes accessibility of the peptide to proteases. Differences observed between the wild-type and the mutant conjugates were within the limits of significance (Tables S1 and S2).

The next step in our study addressed the intracellular distribution of phosphoramidate PEGylated peptides, especially, since long-chain PEGs are known to induce membrane fusion.[26] Based on the increased stability in cell lysate, we focused on the BH3-PEG750 and BH3-PEG2000 conjugates. Cellular delivery was facilitated by electroporation, in which a short electric pulse leads to the transient formation of pores in the plasma membrane that act as a size filter. To investigate the time-dependent intracellular peptide distribution, Jurkat cells were electroporated with 100 μM of BH3-PEG750, BH3-PEG2000, unPEGylated BH3, or just fluorescein as a control and monitored periodically for 6.5 h using live-cell confocal microscopy (Figure 3). To our delight, 30 min after electro-

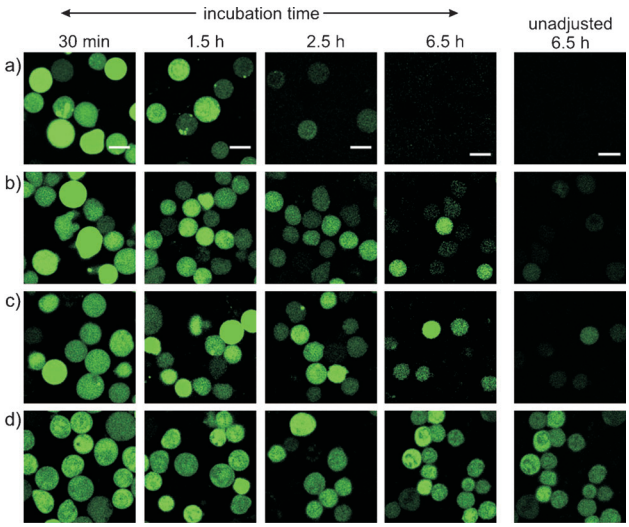


Figure 3. Time-dependent intracellular distribution of unPEGylated BH3 and conjugates. Jurkat E6.1 cells were electroporated with 100 μM of a) fluorescein and b) unPEGylated BH3, c) BH3-PEG750, or d) BH3-PEG2000 and confocal microscopy images were recorded over 6.5 h at the indicated time points. Scale bars correspond to 10 μm. All images recorded for each time point have the same scale bar. Brightness and contrast parameters were adjusted to visualize the localization of the peptides for 6.5 h. The panel labeld "unadjusted" refers to images showing cell-associated intensity without brightness/contrast adjustments.

poration all of the probed peptide conjugates showed a homogenous distribution throughout the cytoplasm and nucleus comparable to free fluorescein. As observed previously[14] fluorescein exited the cells in less than an hour. In contrast, both the unPEGylated BH3 and the PEGylated counterparts showed a homogenous distribution throughout the first 5 h and also, after 6.5 h the major part of the fluorescence was still homogenously distributed (Figure 3); only with BH3-PEG2000 there was some heterogeneity in the nuclear distribution after 6.5 h. We also observed considerably higher intracellular retention of BH3-PEG2000 compared to that of unPEGylated or BH3-PEG750 peptides;

fluorescence of the latter two peptides showed similar intracellular residence times (Figure 3, unadjusted 6.5 h; Figure S3). Previously, we have observed for BH3 peptides coupled to an HPMA polymer, that an increase in proteolytic stability was not reflected by a prolonged intracellular residence time of fluorescence.[14] Since we observed such a correlation for several different peptides, this finding indicates that for the unPEGylated BH3 peptide a proteolytic fragment is formed that does not readily cross the plasma membrane.[25]

Next, we addressed the effect of a stabilizing PEG-phosphoramidate modification on the intracellular activity of the BH3 peptide. For this experiment, we focused on the BH3 peptides with the smallest PEG chains that showed sufficient stability, since PEG modifications can be associated with loss of activity of the conjugated proteins.[27] Consequently, we chose the BH3-PEG750 peptide, which displayed considerable stabilization in cell lysates.

After electroporation with BH3-PEG750 as well as the mutated counterpart (mBH3), cells were incubated for 6 h, and apoptosis-inducing activity was measured by a caspase-3 assay. Electroporation experiments were performed with 100 as well as 300 μM peptide solutions. Additionally, 0.5 μM staurosporine acted as a positive control for caspase-3 activation in Jurkat cells. Our studies demonstrated that only BH3-PEG750 showed a concentration-dependent significant and specific increase of caspase-3 activity in comparison to cells that had been electroporated without peptide (mock electroporation); (Figure 4a, Tables S3–S5). In contrast, introduction of the unPEGylated BH3 peptide led only to a slight increase in caspase-3 activation relative to the mock electroporated cells; however, this increase was nonspecific at the lower concentration, since the same level of activity was also observed for unPEGylated mBH3. Specific activity could only be seen for the unPEGylated BH3 peptide at concentrations as high as 300 μM (Figure 4a). In light of the high peptide concentrations one should note explicitly that for molecules of the size used in this study, only a minor fraction is delivered into the cytoplasm. Concentrations inside the cell will therefore be much lower than in the electroporation cuvette.[28]

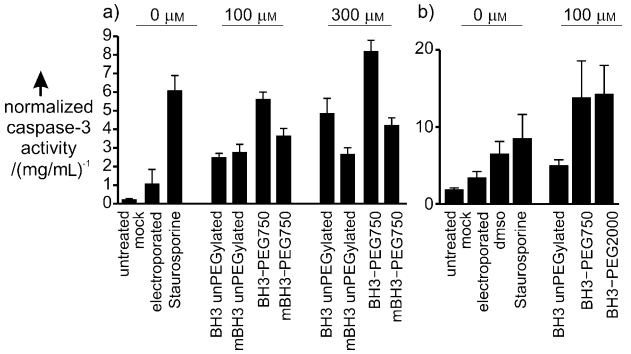


Figure 4. Activation of caspase-3 in electroporated Jurkat E6.1 cells after 6 h of incubation. For the individual experiments the fold increase in caspase-3 activity was normalized relative to the total activity and to the respective protein content. Activity scale for the *y* axis corresponds to value $\times 10^{-2}$. a) Caspase-3 activation by BH3 peptide, (m)BH3 unPEGylated, and (m)BH3-PEG750. Results represent the mean ± standard error for three independent experiments. b) Comparative caspase-3 activity for BH3 unPEGylated versus BH3-PEG750 and BH3-PEG2000 of two independent experiments. Results represent mean ± standard error.

Finally, we compared intracellular activity induced by BH3-PEG750 and BH3-PEG2000 delivered at a concentration of 100 μM. Interestingly, in spite of the longer intracellular retention time of fluorescence, PEG2000 did not confer a higher activity (Figure 4b).

In summary, we have successfully probed the impact of small- to medium-sized oligo- and polyethylene glycol chains on the stabilization against proteolysis in cell lysates and the intracellular activity of apoptosis-inducing BH3 peptides. Phosphoramidate-linked PEGylated BH3-peptides were obtained by the chemoselective Staudinger-phosphite reaction in solution resulting in a more than 57-fold increase in the half-life of the peptide in Jurkat cell lysate when two only medium-sized PEG 2000 chains ($n = 45$) were attached. Subsequently, we probed the intracellular proapoptotic activity of the PEGylated BH3 peptides. For BH3-PEG750 it could be demonstrated that N-terminal phosphoramidate PEGylation leads to a significant concentration-dependent increase of intracellular activity, while increasing the molecular weight of the peptide only by a factor of 1.5 compared to the unPEGylated peptide. Moreover, medium-sized branched phosphoramidate PEG chains retained the homogenous cytoplasmic distribution of the peptide. Taken together, these results demonstrate the potential of phosphoramidate-linked PEGylated biopolymers in the selective targeting of intracellular biological pathways of pharmaceutical and clinical importance. Further studies combining the Staudinger-phosphite PEGylation strategy with direct cellular uptake is currently underway in our laboratories.

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[28] For example, for peptides of 20 amino acids in length, intracellular peptide concentrations were less than a fifth of those in the electroporation cuvette; see Ref. [25].

