

## New technologies to prolong life-time of peptide, protein and low-molecular-weight drugs in vivo

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### Abbreviations

*Fmoc*, 9-fluorenylmethoxycarbonyl;  
*FMS-OSu*, 2-sulfo-9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide; *hGH*, human growth hormone;  
*HPLC*, high-performance liquid chromatography;  
*HSA*, human serum albumin; *LMW*, low-molecular-weight; *MAL*, *Fmoc-OSu* 9-hydroxymethyl-2-(amino-3-maleimidopropionate)-fluorene-*N*-hydroxysuccinimide;  
*MAL-FMS-OSu*, 2-sulfo-9-hydroxymethyl-7-(amino-3-maleimidopropionate)-fluorene-*N*-hydroxysuccinimide -  
*PEG* polyethyleneglycol; *STZ*, streptozocin

### Introduction

Most peptide and protein drugs are short-lived species *in vivo* having a circulatory half-life of several minutes. This is particularly valid for non-glycosylated proteins with a molecular mass of less than 50 kDa. Since peptide/protein drugs are not absorbed orally, prolonged maintenance of therapeutically active polypeptide drugs in the circulatory system is of primary clinical importance. Another major shortcoming of injected polypeptide drugs is their elevated concentration in the circulatory system shortly after administration, which may amount to 100–1000 times above the therapeutical level. Such overdosing may lead to undesirable side effects such as over-stimulation or down-regulation of receptor sites.

In this review we describe two new strategies that overcome the aforementioned problems of systemically injected peptide/protein drugs. The first strategy includes *Fmoc* or *FMS* derivatization of peptides, proteins and low-molecular-weight drugs, converting them into inactive prodrugs that undergo reactivation with desirable pharmacokinetic patterns in body fluids. This *Fmoc/FMS*-technology led to development of a second strategy, reversible pegylation. Accordingly, under physiological conditions inactive pegylated peptide/protein drugs release the native active parental molecule at slow rates, and in homogeneous fashion, thus facilitating prolonged therapeutic effects following a single administration.

### Results and Discussion

In a series of studies we have covalently linked *Fmoc* or *FMS* ((7-sulfo)-9-fluorenylmethoxycarbonyl) moieties to the amino side chains of peptides and proteins. We found that upon incubation in aqueous buffer, simulating normal human serum conditions, *FMS* or *Fmoc* moieties undergo slow, spontaneous and homogenous hydrolysis, releasing

the peptide-protein in a non-modified form. Rates of hydrolysis at physiological conditions were manifested with  $t_{1/2}$  value of  $6 \pm 2$  h for *FMS*-protein conjugated and with  $t_{1/2} = 24 \pm 3$  h for *Fmoc*-protein conjugates. *Fmoc/FMS* is detached from peptides and proteins non-enzymatically at rates which are exclusively dependent on the pH, temperature and the protein composition in the serum. These three parameters are maintained in mammals in strict homeostasis.

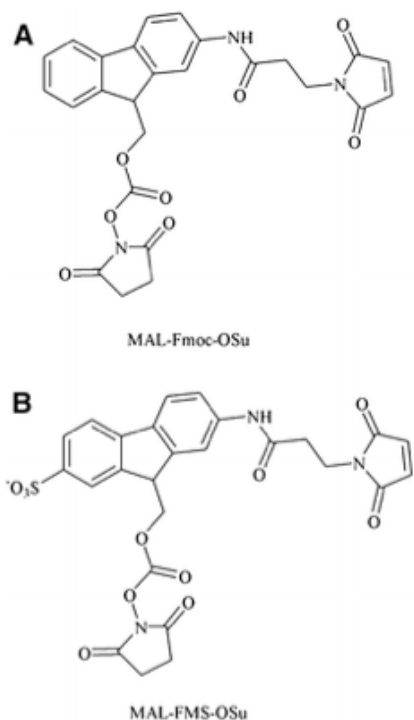
This line of research also revealed that all peptides and proteins containing two *FMS*/mol or more are long-lived species *in vivo*, with no exception. We next found that the *FMS*-technology can also be applied to elongate the life-time of nonpeptidic, low-molecular-weight drugs such as aminoglycosides. Low-molecular-weight drugs containing two *FMS* moieties/mol associate with albumin with sufficient affinity to lower rates of clearance from the circulation [1-5].

*Applying our FMS/Fmoc technology for solving the major drawback of conventional pegylation* - PEGylation of therapeutic peptides/proteins creates molecules that exhibit superior pharmacokinetic stability compared to their corresponding unmodified parent molecules. However, this approach becomes unproductive if conjugates lose their biological activity upon PEGylation [6-9]. This major deficiency can be overcome if PEG chains are linked to the peptide/protein drugs through a chemical bond that undergoes slow hydrolysis under physiological conditions. Our contributions in this direction are presented below.

*Reversible Pegylation* - Based on our *Fmoc/FMS* technology we have designed and synthesized two hetero-bifunctional agents of the structures shown in Figure 1.

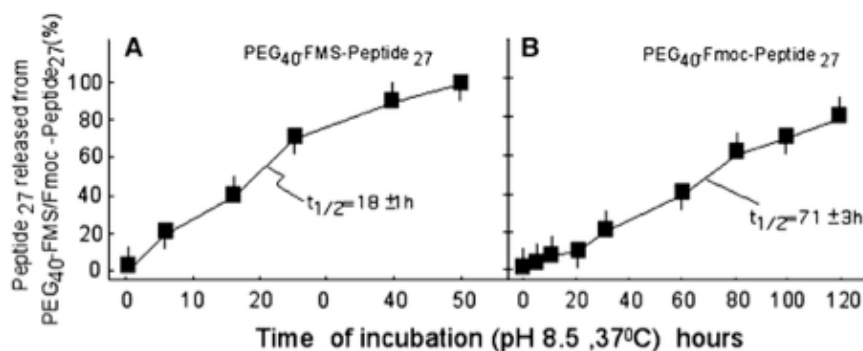
These agents allow the linkage of any peptide or protein containing amino function(s) through a slowly hydrolysable bond. The *MAL* (3-maleimidopropionic acid)-moiety of this compound allows the attachment of sulfhydryl containing polyethylene glycol (i.e. PEG<sub>40</sub>-SH, a 40 kDa branched polyethyleneglycol containing a sulfhydryl moiety). We have developed the experimental conditions to allow covalent linkage of a single PEG-chain to peptide/protein drugs through either *MAL-FMS-OSu* (9-hydroxymethyl-2-(amino-3-maleimidopropionate)-7-sulfofluorene *N*-hydroxysuccinimide;) or *MAL-Fmoc-OSu*. (9-hydroxymethyl-2-(amino-3-maleimidopropionate)-fluorene-*N*-hydroxysuccinimide). A PEG-chain of 40 kDa (PEG<sub>40</sub>) was used exclusively. The attachment of a single PEG<sub>40</sub>-chain to any peptide and/or protein was documented

to prolong the life-time of the corresponding conjugates 7-10 times in rodents. A molecular mass of 60-70 kDa is normally taken as the molecular mass cut-off for kidney filtration. A PEG chain of 40 kDa, however, possesses an enlarged Stoke's radius. Its effective molecular mass therefore significantly exceeds the molecular mass cut-off for kidney filtration.



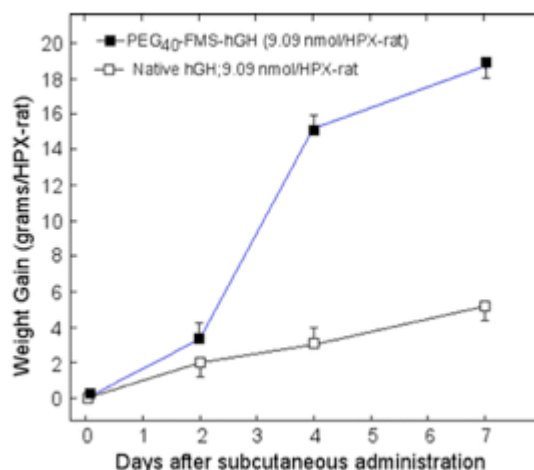
**Fig. 1.** Structures of MAL-Fmoc-OSu and MAL-FMS-OSu

PEG<sub>40</sub>-Fmoc/FMS conjugates release the linked peptide upon incubation in an aqueous buffer simulating normal human serum conditions. Figure 2 shows the rate of release of peptide<sub>27</sub> (Peptide<sub>27</sub>: a nonlysine-containing synthetic peptide of 27 amino acids) upon incubation in 0.1 M phosphate buffer (pH 8.5, 37°C). Both conjugates release the covalently linked peptide at a slow rate and in a homogenous fashion with a  $t_{1/2}$  value of  $18 \pm 1$  h for PEG<sub>40</sub>-FMS-peptide<sub>27</sub> and a  $t_{1/2}$  value of  $71 \pm 3$  h for PEG<sub>40</sub>-Fmoc-peptide<sub>27</sub>. (Figure 2A,B).



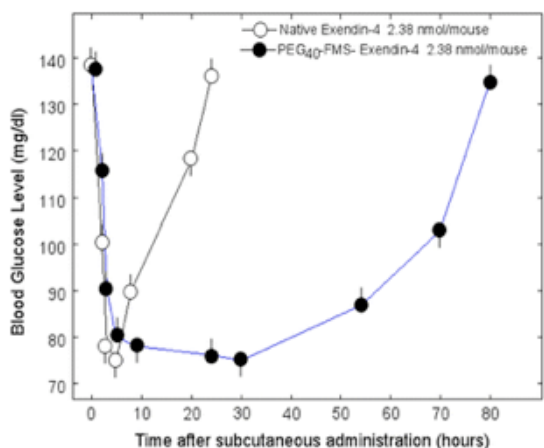
**Fig. 2.** Rates of release of peptide<sub>27</sub> from PEG<sub>40</sub> FMS- and PEG<sub>40</sub>-Fmoc-peptide<sub>27</sub> at pH 8.5, 37°C

The strategy of reversible pegylation has been applied to several peptide and protein drugs, all of which undergo inactivation by conventional pegylation. The list includes extendin-4, peptide YY<sub>3-36</sub>, interferon  $\alpha$ 2, atrial natriuretic peptide and human growth hormone [hGH, references 10-15]. The protracted actions of PEG<sub>40</sub>-FMS-hGH in hypophysectomized rats and of PEG<sub>40</sub>-FMS-extendin-4 *in vivo* are illustrated in Figure 3 and Figure 4 respectively.



**Fig. 3.** Weight gain by hypophysectomized rats given a single subcutaneous injection of hGH or PEG<sub>40</sub>-FMS-hGH. Hypophysectomized rats ( $n = 12$  per group) received a single subcutaneous administration of native growth hormone (9.09 nmol/HPX-rat □) or of PEG<sub>40</sub>-FMS-hGH (9.09 nmol/HPX-rat ■) on day 0. Daily weight gains were recorded over a period of seven days. Each point is the arithmetic mean of 12 HPX-rats  $\pm$  SEM.

We also applied our strategy to link insulin to the single cysteinyl moiety of human-serum albumin using MAL-Fmoc-OSu. The conjugate thus obtained released the covalently-linked insulin at a slow rate under physiological conditions and facilitated prolonged glucose-lowering effect following administration to streptozocin-treated hyperglycemic rats [14].



**Fig. 4.** Glucose-lowering patterns of native exendin-4 and PEG<sub>40</sub>-FMS-exendin-4 following a single subcutaneous administration into CD1-mice. CD1 mice (n = 6 per group) were administered a single dose of native exendin-4 (□, 2.39 nmol/mouse) or PEG<sub>40</sub>-FMS-exendin-4 (■, 2.39 nmol/mouse). At the time points indicated, circulating glucose levels were determined. Each point is the arithmetic mean of six mice ± SEM.

Recently, we applied our approach to low-molecular-weight substances such as Aminoglycosides. For example, PEG<sub>40</sub>-FMS-gentamicin released low concentration of this aminoglycoside to the circulatory system in a continuous fashion over a period exceeding 15 h following intravenous administration into rats (manuscript in preparation).

Thus, the major problem of inactivating short-lived therapeutic peptide and protein drugs by conventional pegylation has been ameliorated, enabling now lifetime extension, bioavailability and efficacy of existing peptide drugs as well as those to be discovered by genomics and proteomics.

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