Chemical instability of pharmaceutical peptides in polymeric controlled release systems

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(met een samenvatting in het Nederlands)

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“But time is short, and science is infinite…”

Thomas Hardy (1840-1928)

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Chapter 1

General introduction

1. Therapeutic peptides

Peptides are an important group of biopharmaceuticals, situated between the classic low-molecular-weight drugs and the macromolecular pharmaceutical proteins. Peptides are typically classed as molecules comprising between two and fifty amino acids residues. Therapeutic peptides are generally less immunogenic and are more cost-efficient in comparison with recombinant proteins and antibodies. Peptides also offer several advantages over small organic molecules, as they have a greater efficacy, selectivity and specificity [1-3]. The majority of the presently marketed peptide products are peptide hormones or peptide derivatives that simulate the action of hormones (i.e. oxytocin is a peptide hormone and desmopressin is an analogue of the natural pituitary hormone). Actually, peptides offer immense medicinal potential [1,4] as agonists or antagonists for receptors in oncology. As examples, goserelin and leuprolide, two gonadotropin releasing hormone agonists, are used to treat hormone-sensitive breast and prostate cancer, and octreotide (a somatostatin mimic) is used against various tumors. Peptides also acts as enzyme inhibitors (i.e. ritonavir a protease inhibitor peptide)[5]. Current peptides in development include antimicrobial peptides, such as cathelicidins and defensins [6]. Recently, synthetic peptides have also emerged as vaccines, such as human papillomavirus (HPV) peptide [7-9].

2. Chemical instability of peptides

Peptides contain multiple functional groups, many of which can undergo chemical changes [10]. During formulation, processing and storage of a peptide drug product, the peptide is exposed to conditions that can have significant effects on its integrity. Several degradation pathways that peptides may undergo during formulation and administration lead to chemical instability [10,11]. Chemical instability can be defined as any process involving modification of the peptide by covalent bond formation or covalent bond cleavage, generating new chemical entities [12]. Most frequently described reactions leading to chemical instability of peptides and protein are described in the following paragraphs.

2.1. Deamidation and isomerization

Deamidation is one of the most common hydrolytic reactions of peptides and proteins which is subject to both acid and base catalysis. The hydrolysis of the side chain amide bond of asparagine (Asn) or glutamine (Gln) residues leads to the

formation of a free carboxylic acid thus forming aspartic acid (Asp) and glutamic acid (Glu) residues, mutating the amino acid sequence through chemical reaction [13,14]. When deamidation occurs at Asn sites, it modifies the Asn side chain to produce Asp and/or isoaspartic acid (isoAsp) [15] with loss of ammonia (Figure 1). At neutral to basic pH, a structural isomer of Asp, isoAsp, is also produced and is generally the major product (Figure1). The formation of isoAsp not only changes the amino acid sequence but also alters the peptide backbone by introducing an extra methylene group (i.e., a β-amino acid).

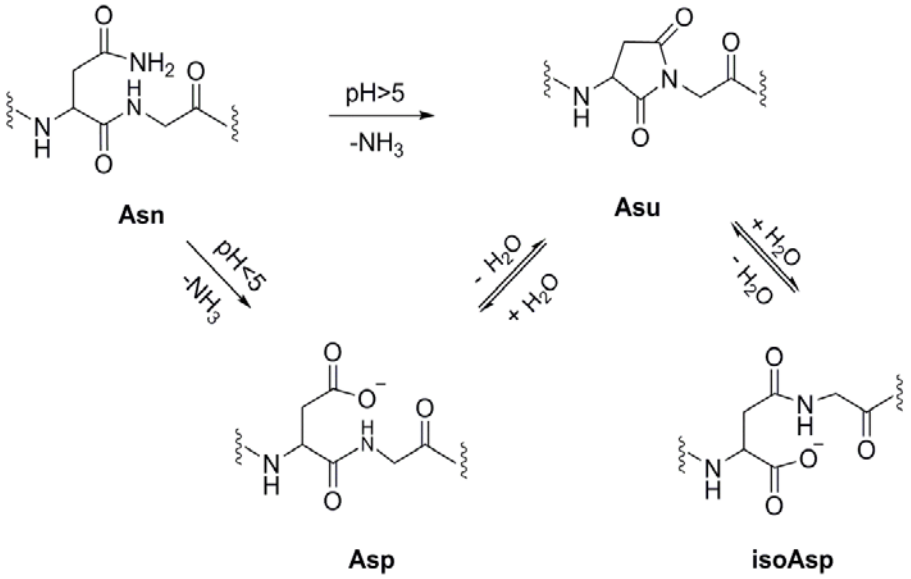


Figure 1. Asparagine (Asn) deamidation and aspartate (Asp) isomerization. At pH>5, deamidation of Asn proceeds through a cyclic imide (succinimide, Asu) intermediate with loss of ammonia to yield the Asp- and isoasparate (isoAsp)-containing variants. At acidic pH, the Asn side chain undergoes direct hydrolysis, giving the Asp residue exclusively. Isomerization of Asp to isoAsp also occurs through the Asu intermediate [11].

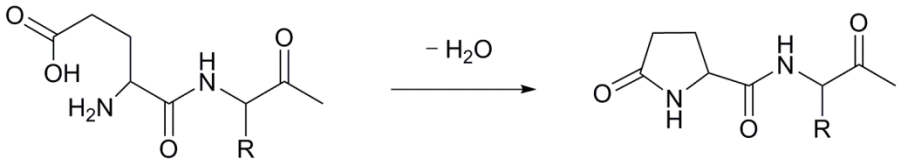
2.2. Oxidation

Oxidation is another primary chemical degradation pathway that can occur in a peptide. Peptides that contain cysteine (Cys), methionine (Met), histidine (His), tyrosine (Tyr) and tryptophan (Trp) residues can potentially be chemically changed due to their high reactivity with various reactive oxygen species. Cys and Met are

susceptible for oxidation because of their sulfur atom, and His, Tyr and Trp because of their (hetero)aromatic ring [16].

2.3. N- terminal cyclization

N-terminal glutamic acid (Glu) and glutamine (Gln) residues can spontaneously cyclize to result in the formation of pyroglutamic acid (pyroGlu) (Figure 2). The mechanism is a nucleophylic attack of an N-terminal amine to the carbonyl group of Glu or Gln followed by loss of water or ammonia, respectively [17].



N-terminal glutamic acid Pyroglutamic acid

Figure 2. Conversion of *N*-terminal Glu to pyroGlu. Attack of the *N*-terminal amine on the Glu side chain produces the five-membered pyroglutamyl ring with loss of water.

2.4. β-elimination

Peptides with disulfide bonds might undergo destruction to form free thiol groups through β-elimination. This reaction is commonly observed when peptides are incubated at higher pH values or elevated temperature. When cysteine-containing proteins are heated at 100 °C, even at a neutral pH, they undergo destruction to form free thiols [18,19].

3. Delivery challenges for pharmaceutical peptides and proteins

For successful delivery of pharmaceutical peptides one should preserve the chemical integrity of bioactives so that they function properly [20,21]. However, peptide and protein pharmaceuticals (or drugs) suffer from short half-lives, chemical and physical instability [22]. Therefore, rational therapies with peptides and protein often require the use of suitable delivery systems [23], but formulating biopharmaceuticals with the optimal therapeutic efficacy as well as optimal stability is highly challenging [24]. Controlled release systems aim to increase the time over which an bioactive's concentration is maintained at the appropriate therapeutic levels, to tailor delivery to a specific site, or to overcome

the short half-lives or instability of peptide and protein therapeutics [25,26]. Polymeric delivery systems are a promising tool for the controlled release of biopharmaceutical peptides and proteins. Yet, the biggest issue with such formulations intended for long-term controlled release (days to months), is the instability of peptides and proteins [20]. Many manufacturing methods to prepare polymeric controlled release formulation may expose peptides/proteins to potentially damaging conditions, such as aqueous/organic interfaces, elevated temperatures, vigorous agitation, and polymerization initiators [20]. Moreover, upon administration for long periods of time, the microenvironment of the (degrading) polymer matrix and the products of polymer degradation can cause peptide instability. Thus, proteins/peptides are exposed to a variety of stress factors over the entire life of a controlled-release system, including formulation, long-term storage, and administration [27].

Among the various polymeric delivery systems, hydrogels and polyesters are polymeric system that are highlighted here, because these are frequently investigated systems for the design of long acting controlled release systems for biotherapeutics, such as proteins and peptides [28,29].

3.1. Hydrogels

A hydrogel consists of a three dimensional polymer network that is able to retain large amounts of water with conservation of the network structure [30]. As a result of the high water content, hydrogels are generally considered as biocompatible materials, which makes them particularly suitable for biomedical and pharmaceutical applications [29,31]. The structure of hydrogels is preserved due to the presence of crosslinks between polymer chains [32]. Depending on the type of crosslinker, the crosslink density and the equilibrium water content, hydrogels can have different physical properties. Release of peptides and proteins from a hydrogel can occur by one or a combination of three different mechanisms, namely diffusion controlled, swelling controlled, and degradation controlled release [33].

Hydrogels are also used as matrices for molecular imprinting of peptides and proteins [34,35]. Molecular imprinting is a technique to create cavities in polymer matrices shaped by a template molecule and having shape memory of this template molecule. Molecularly imprinted polymers (MIPs) are used in

applications based on molecular recognition, e.g. biomolecular separation and analysis, and even controlled drug release [36-39].

Both chemical and physical crosslinking methods are used for the design of biodegradable hydrogels. Chemical crosslinking leads to covalent bonds and can be accomplished by *e.g.* radical, high-energy or enzyme-mediated polymerization. Physical crosslinking involves the introduction of non-permanent, reversible crosslinks via *e.g.* hydrophobic interactions, hydrogen bonding, stereo complexes or ionic interactions [32]. Whereas chemical crosslinking leads to networks with relatively high mechanical strength, physical crosslinking generally results in weaker hydrogels. On the other hand, the physical crosslinks can be reversibly broken by *e.g.* shear forces, which may allow injectability of the gels [40]. Additionally, the crosslinking conditions during physical crosslinking are mild, which may preserve the integrity of loaded/entrapped bioactives. Generally, chemical crosslinking is a highly versatile method to create hydrogels with good mechanical stability, but the crosslinking agents used are often toxic compounds. Moreover, crosslinking agents can give unwanted reactions with the bioactive substances present in the hydrogel matrix. For example, free radical polymerization is often used to prepare protein- and peptide-loaded hydrogels for controlled release systems and molecular imprinting [34]. However, exposure to these reactive chemical reagents may cause unwanted chemical modification of the therapeutic payload and/or grafting of the payload to the network [41]. For example, with the aim to obtain dextran hydrogels that release recombinant human interleukin (rhIL-2) in a controlled manner, Cadée et al. initiated the crosslinking reaction by the addition of potassium peroxodisulfates (KPS) and N,N,N,N-tetramethylethylenediamine (TEMED) to an aqueous solution of methacrylated dextran containing the protein. It was found that the methionine residues in rhIL-2 are susceptible to oxidation by KPS [42]. Likewise, Schillemans et al. observed oxidation of cytochrome C when they added the protein to the hydrogel precursors and subsequently initiated network formation by the addition of ammonium peroxodisulfates (APS) and TEMED. On the other hand, post-loading of preformed gels with protein did not result in oxidation of protein [43].

3.2. Polyesters

Aliphatic polyesters, particularly poly(D,L-lactic-co-glycolic acid) (PLGA), are used to stabilize peptides and proteins and control their release kinetics [44]. Over the past two decades, PLGA controlled systems have generated tremendous interest because of their excellent biocompatibility and biodegradability [45,46]. Most importantly, PLGA drug formulations have been approved by the United States Food and Drug Administration (FDA) as biodegradable controlled release systems. Additionally, it is possible to tune the overall physical and chemical properties of the delivery device by parameters such as polymer molecular weight, ratio of lactide to glycolide of the polymer and drug concentration, to achieve a desired dosage and release interval depending upon the drug type [47]. Several commercially available therapeutic peptides, including leuprolide acetate (Lupron Depot®), octreotide acetate (Sandostatin LAR®), and goserelin acetate (Zoladex® implant), are formulated in a PLGA polymer [26,28]. PLGA, however, has some limitations and drawbacks which limit their extensive use in drug formulations. The polyester undergoes hydrolysis to yield lactic and glycolic acid as well as their oligomers as degradation products. Yet, the same characteristics that make PLGA amenable to controlled release formulations can lead to peptide and protein instability because of the accumulation of carboxylic acid oligomers and monomers formed upon hydrolysis produce an acidic microclimate within the degrading polymer matrix, initiating acid catalyzed peptide degradation and derivatization. The hydrolyzable PLGA ester bonds are also subject to nucleophilic interactions with incorporated drugs [27,48]. Although peptides are often assumed to be stable during the manufacturing process [49], interaction between the loaded peptide and PLGA may cause unwanted modification of peptides such as deamidation, proteolysis and acylation [50-53].

3.2.1. Peptide acylation in PLGA matrices

Acylation involves the nucleophilic attack of peptide residues such as the N-terminus or lysine side chains to the electrophilic carbonyl groups of the lactate or glycolate ester of PLGA. This results in the formation of a peptide PLGA adduct. After hydrolysis of the attached polyester chain, the resulting peptide is eventually modified with remaining glycolic or lactic acid units through a hydrolytically stable amide bond (Figure 3).

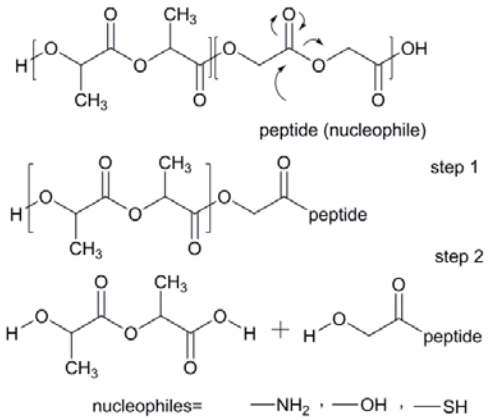


Figure 3. Proposed mechanism of reaction between peptide and PLGA [51].

The extent and kinetics of this reaction depends on the reactivity of the polymer such as lactide:glycolide ratio, the nucleophilicity of the drug, and the hydrophobicity of the polymer [48,54-56]. Murty et al. found that acylation of octreotide decreased with increasing lactide composition (PLGA 50:50 > PLGA 85:15 > PLA 100) [48]. Likely, due to steric hindrance of the methyl group on the alpha-carbon of lactide units, the nucleophilic attack of amines of the peptide more readily occurred on glycolic acid rather than on lactic acid units. Later, they investigated the effect of water uptake on octreotide acylation in PLGA 85:15 and PLA microspheres. Octreotide in microspheres that were stored under anhydrous conditions did not undergo acylation. The authors proposed that because of water uptake, the hydrolytic cleavage of the polymeric backbone created an acidic microenvironment to facilitate the covalent coupling of peptide with polymer [49].

3.2.2. Strategies to inhibit peptides acylation in PLGA

Several methods to minimize peptide acylation within PLGA matrices have been proposed. pH-modifying excipients [57] and water-soluble divalent cationic salts [58] have been investigated as excipients to prevent acylation and stabilize peptides [59,60]. Recently, Feng et al. evaluated the inhibitory effect of dications on the acylation of an acidic peptide (exenatide) outside or inside PLGA microspheres. They showed that Ca^{2+} did not prevent acylation, Mn^{2+}resulted in some inhibition of acylation, whereas Zn^{2+} possessed the greatest inhibitory effect [61]. However, the effects of excipients on inhibition of acylation varied depending on the properties of the individual peptides [62]. Lucke et al. investigated whether a blockcopolymer of poly(ethylene glycol) (PEG) and

poly(D,L-lactic acid) (PLA) would reduce peptide acylation since PEG can in principle reduce the accumulation of acid degradation products and peptide adsorption to the PLGA surface. However, this approach did not show a favorable effect concerning peptide acylation inside degrading polymer microspheres [63]. Na et al. presented a PEGylation strategy to prevent acylation of octreotide. However, PEGylation of the lysine residue resulted in significant loss of its biological activity [64,65]. Therefore, reversibly or temporarily blocking the amine groups of peptides could be an interesting approach to prevent acylation in degrading PLGA matrices. Ahn et al. investigated reversible blocking of the amine groups of octreotide with maleic anhydride (MA) to prevent acylation in PLGA. Importantly, the hydrolysis of maleiated amines is acid catalyzed [66]. And because the hydrolytic degradation of PLGA results in the formation of acid degradation products which accumulate in the polymer matrix resulting in pH decrease [67,68], intact peptide is supposed to be formed from MA conjugates and subsequently released into the surrounding. Indeed a substantially inhibition of the formation of acylated octreotide was observed. However, the maleiated octreotide was released faster than its conversion into intact octreotide inside a PLGA film. Since de-blocking after release is rather slow at physiological conditions the possible toxicity of maleiated octreotide should be evaluated [69]. Vaishya et al. reported minimizing octreotide acylation by masking the reactive nucleophile amine of octreotide with reversible hydrophobic ion-pairing complex which is stable in acidic pH inside the PLGA particles and can dissociate to native octreotide at physiological pH. Dextran sulfate A-octreotide and dextran sulfate B-octreotide complexes that were encapsulated in PLGA microparticles showed <7 % acylation during *in vitro* release from a gel matrix based on pentablock polymer PLA250-PCL1250-PEG1500-PCL1250-PLA250. However, they did not show the extent of acylation of unprotected octreotide as a reference in their gel matrix [70] which means that the effect of the tested excipients on prevention of peptide acylation is not convincingly shown.

4. Aim and outline of this thesis

The chemical stability of a peptide drug is of great importance since it becomes less effective as it undergoes unwanted modification or degradation. The objective of this work is to increase the understanding of chemical instability of pharmaceutical peptides in polymeric controlled release formulation, specifically hydrogels and PLGA microspheres. In addition, we tried to provide a generic

solution for stabilizing peptides in terms of prevention of acylation and alternative catalyst in radical polymerization.

In an attempt to prepare peptide (thymopentin) imprinted in acrylamide hydrogel, we observed modification of thymopentin during the polymerization. This triggered us to further investigate the type of modification. So, in **Chapter 2** the effect of initiator and catalyst (ammonium peroxodisulfate (APS) and N,N,N,N-tetramethylethylenediamine (TEMED), that are frequently used for hydrogel formation, on the integrity of peptides that contain lysine is explored. Thymopentin, a decapeptide and 15 dipeptides of a library with C-terminal lysine and different N-terminal amino acids were incubated with APS/TEMED. Structural characterization of the peptide and its degradation products was carried out with liquid chromatography–ion-trap mass spectrometry (LC–ITMS), employing ion fragment analysis by both MS/MS and MS^3 techniques.

As explained above, acylation of peptides occurs in polyester matrices. It has been shown that acylation takes place to a lesser extent in more hydrophilic matrices [54]. Therefore, in **Chapter 3** the acylation of the model peptide octreotide in PLGA microspheres is compared with that in microspheres of polyesters with hydrophilic comonomer, i.e., poly(D,L-lactic-co-glycolic-co-hydroxymethyl glycolic acid) (PLGHMGA) and a multiblock copolymer of poly(ε-caprolactone)-PEG-poly(ε-caprolactone) and poly(L-lactide) ((PC-PEG-PC)-(PL)). The acylation products were characterized in great detail using LC-MS/MS.

From chapter 3 and previous work by others, it is known that lysine residues and the free N-terminus are the major sites of acylation of peptides. However, in order to investigate other possible sites of acylation, **Chapter 4** investigates the acylation of goserelin peptide that lacks lysine and a free N-terminus yet contains other nucleophilic residues, i.e. serine, tyrosine and arginine, which potentially can be acylated.

Based on the knowledge about the major sites and mechanisms of acylation, **Chapter 5** presents a new method to inhibit acylation of peptides in PLGA formulations by reversely blocking the amino groups of a model peptide (octreotide) with a novel self immolative protecting group.

Chapter 6 summarizes the findings and conclusions of this thesis. In addition, perspectives and suggestions for future research are given.

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Chapter 2

Methyleneation of peptides by N,N,N,N-tetramethylethylenediamine (TEMED) under conditions used for free radical polymerization: a mechanistic study

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Abstract

Free radical polymerization is often used to prepare protein and peptide-loaded hydrogels for the design of controlled release systems and molecular imprinting materials. Peroxodisulfates (ammonium peroxodisulfates (APS) or potassium peroxodisulfates (KPS)) with N,N,N,N-tetramethylethylenediamine (TEMED) are frequently used as initiator and catalyst. However, exposure to these free radical polymerization reagents may lead to modification of the protein and peptide. In this work, we show the modification of lysine residues by ammonium peroxodisulfate (APS)/TEMED of the immunostimulant thymopentin (TP5). Parallel studies on a decapeptide and a library of 15 dipeptides were performed to reveal the mechanism of modification. LC–MS of APS/TEMED-exposed TP5 revealed a major reaction product with an increased mass (+12 Da) with respect to TP5. LC–MS^2 and LC–MS^3 were performed to obtain structural information on the modified peptide and localize the actual modification site. Interpretation of the obtained data demonstrates the formation of a methylene bridge between the lysine and arginine residue in the presence of TEMED, while replacing TEMED with a sodium bisulfite catalyst did not show this modification. Studies with the other peptides showed that the TEMED radical can induce methyleneation on peptides when lysine is next to arginine, proline, cysteine, aspargine, glutamine, histidine, tyrosine, tryptophan, and aspartic acid residues. Stability of peptides and protein needs to be considered when using APS/TEMED in in situ

polymerization systems. The use of an alternative catalyst such as sodium bisulfite may preserve the chemical integrity of peptides during in situ polymerization.

1. Introduction

Peptide and protein bioactives (biopharmaceuticals) are presently an important class of pharmaceuticals due their favorable properties as compared to small molecule drugs. They possess high and selective activity, which opens their potential application for the treatment of a variety of diseases. Advances in biotechnology have now created the possibility to produce therapeutically active peptides and proteins on a commercial scale [1]. However, formulating biopharmaceuticals with the optimal therapeutic efficacy as well as optimal stability is highly challenging [2]. Recently, there has been a growing interest in polymeric delivery systems for peptides and proteins to achieve sustained therapeutic concentrations of biopharmaceuticals in the circulation. Polymeric matrices may also protect peptide and protein drugs from degradation and consequently enhance their efficacy [3]. Moreover, in the field of biomolecular analysis there is an increasing interest in peptide and protein imprinted polymers, due to their ability to selectively recognize and bind their targets in competitive media [4-7].

Free radical polymerization is often used to prepare protein- and peptide-loaded hydrogels for controlled release systems and molecular imprinting [8-10]. For example, Verheyen et al. used free radical polymerization to co-polymerize methacrylamide-modified lysozyme molecules with methacrylated dextran for covalent incorporation of the protein in the resulting hydrogel network and intracellular triggered release of this enzyme [11]. In a recent study, it was reported that human serum albumin (HSA) and immunoglobulin G (IgG) could be separated by molecularly imprinted hydrogels [12].

Potassium peroxodisulfate (KPS) and ammonium peroxodisulfate (APS) with tetramethylenediamine (TEMED) are frequently used as initiator/catalyst for the preparation of hydrogels from hydrophilic building blocks. However, these reagents may cause unwanted modifications of the protein/peptide drugs during gel formation, which could result in structural changes and loss of biological activity [13]. Several studies investigated possible chemical modification of

protein and peptide biopharmaceuticals that occur during the formulation [14,15]. For example, with the aim to obtain hydrogels that release recombinant human interleukin (rhIL-2) in a controlled manner, Cadée et al. initiated the cross-linking reaction of an aqueous solution of methacrylated dextrans containing the protein by the addition of KPS and TEMED. It was found that the methionine residues in rhIL-2 are susceptible to oxidation by KPS [16]. Schillemans et al. also found oxidation of cytochrome C when they added the protein to the hydrogel precursors and subsequently initiated network formation by the addition of APS/TEMED. On the other hand, post-loading of preformed gels with protein did not result in oxidation of the protein [9]. Kafka et al. prepared poly(ethylcyanoacrylate) (PECA) nanoparticles loaded with the bioactive D-Lys6-GnRH peptide by an *in situ* interfacial polymerization process using a w/o-microemulsion containing the peptide in the dispersed aqueous phase. The peptide D-Lys6-GnRH was reactive with the ethylcyanoacrylate (ECA) monomer, resulting in copolymerization of the peptide with the monomer. MALDI TOF/TOF (tandem) MS analysis revealed that the histidine residue of D-Lys6-GnRH interacts covalently with the ECA monomer during the polymerization process. The reaction mechanism suggested a nucleophilic attack of the histidine side chain of the peptide to the ECA monomer to initiate the reaction resulting in the covalent attachment of the peptide to the polymer [17]. In a more recent study, they showed that the C-terminal glutamic acid residue of the fragments of insulin-like growth factor 1 (IGF-1) covalently binds to poly(alkylcyanoacrylate) (PECA) during polymerization [18].

The aim of the present study was to investigate the possible modification of the bioactive peptide thymopentin (TP5) under conditions used for preparation of imprinted hydrogels. Thymopentin is a well-known immunomodulatory drug that is the active segment 32-36 of thymopoietin (human thymus hormone which contains 49 amino acids). TP5 is a synthetic pentapeptide with the following sequence: Arg–Lys–Asp–Val–Tyr [19]. This peptide is used for the treatment of autoimmune diseases such as chronic lymphatic leukemia, rheumatoid arthritis, and atopic dermatitis. Moreover the peptide causes the induction of T cell differentiation [20-22]. Potential therapeutic effects of TP5 as well as its sequence that contains a variety of functional groups that are present in other therapeutic peptides and proteins, brought us to use it as a model for preparing peptide imprinted polymeric networks. In the present work the effect of the

initiator/catalyst (APS)/TEMED on the integrity of thymopentin under conditions used for preparation of imprinted hydrogels was investigated [23].

Structural characterization of the peptide was carried out with LC–ion-trap mass spectrometry (ITMS) employing electrospray ionization (ESI). Using ion fragmentation by collision-induced dissociation (CID), ITMS is able to perform both MS/MS and MS^3 providing detailed structural information. Low energy CID yields predominantly b-ions and y-ions of peptides in MS/MS that can be further fragmented in MS^3 to reveal the nature and site of peptide modifications[24,25]. To gain more insight into the specificity and mechanism of peptide modification, similar experiments were carried out with other peptides, including one decapeptide and a library of 15 dipeptides.

2. Results

2.1. Analysis of thymopentin exposed to APS/TEMED

In an attempt to prepare a thymopentin (TP5) imprinted hydrogel using APS/TEMED as the initiator/catalyst [23], after washing the hydrogel to extract the peptide template, the washing fractions showed new satellite peaks in the UPLC chromatogram (supporting information, Figure S1). However preparing the imprinted TP5 hydrogel by KPS/$NaHSO_3$ did not show these extra chromatogram peaks in the extracts. To gain insight into this phenomenon we decided to investigate the effect of the APS/TEMED on the integrity of TP5. Therefore, TP5 was incubated with APS with or without TEMED and subsequently analyzed by LC-MS. The LC-MS chromatograms of incubated TP5 with APS/TEMED showed two peaks, corresponding to TP5 and a degradation product, respectively, similarly to the observation of the washing fractions of the imprinted hydrogel. Figure 1 shows the base peak chromatogram of TP5 incubated with APS/TEMED. The mass spectrum of the first main peak (retention time 10.7 min, indicated in Figure 1 as A) showed intense signals at m/z 680.3 and 340.6, corresponding to singly and doubly protonated native TP5 (MW = 679.4 Da), respectively. The second main peak (degradation product, retention time 11.2 min; indicated in Figure 1 as B) exhibited intense signals at m/z 692.3 and 346,6, indicating that thymopentin modification encompassed a mass increase of 12 Da. Based on the exact mass measurement (table S1), the modified compound could be identified as thymopentin with an extra carbon atom. The extent of modification was temperature dependent: incubation of TP5 with APS/TEMED at room temperature

for 16 h also resulted in modification of TP5, but the degradation (based on peak integrals) was 20% compared with 56 % at 50˚C. In contrast, TP5 incubated only with APS (and thus in the absence of TEMED) at 50˚C did not show the extra peak at 11.2 min.

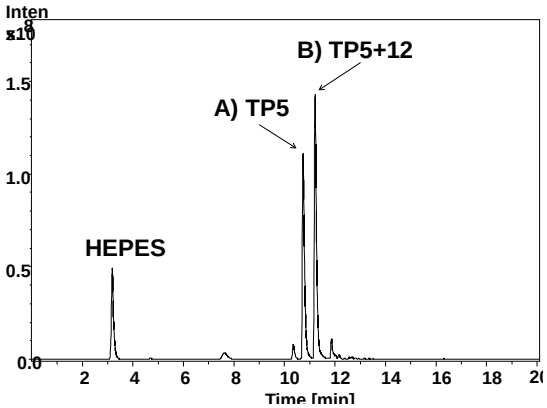

Figure 1. Base peak chromatogram obtained by LC–MS of TP5 incubated with APS/TEMED at 50˚C for 16 h. (A) TP5 $[M+H]^+$=680.3, (B) main degradation product $[M+H]^+$=692.3.

In order to investigate the identity of the main degradation product and obtain structural information about the nature of the observed peptide modification, the sample incubated with APS/ TEMED was analyzed by LC–MS^2. MS^2 detection was performed on precursor ions with m/z 680.3 (TP5) and 692.3 (modified TP5). Figure 2 shows the resulting tandem mass spectra. The spectrum of TP5 (Figure 2A) nicely shows the expected b_2, b_3, b_4 ions ascribed to cleavage of the peptide bonds that contain the N-terminus and y_1 ions that contain the C-terminus. The peak at m/z 517.4 is attributed to the formation of (b_4+H_2O) [26]. Figure 2B shows the tandem mass spectrum of the modified TP5 (precursor ion, m/z 692.3). In line with the mass of the protonated molecular ion, the m/z of all observed b ions (b2-b4) for the modified TP5 also increased with 12 Da as compared to the native peptide. Also m/z 517.4 ascribed to (b_4+H_2O) shows this mass increase of 12 Da. However, the m/z of the observed y_1 and z_1 ions which contains the tyrosine residue are unmodified. From these data it is concluded that the modification that leads to the mass increase of 12 Da occurs either on the arginine or the lysine residue of TP5.

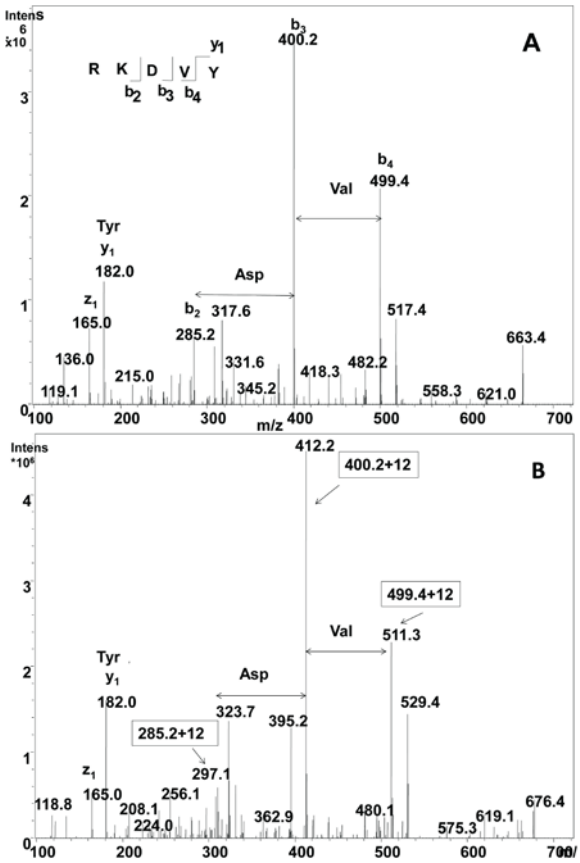

Figure 2. Tandem mass spectrum of (A) TP5 (precursor ion, $[M+H]^+$=680.4), (B) modified TP5 (precursor ion, $[M+H]^+$=692.3).

To find out the site of modification (lysine or arginine) in the APS/TEMED exposed TP5, LC–MS^3 was performed, using the b_2 ions observed with tandem MS detection as precursor ions (m/z 285.2 and 297.2 for the native and modified peptide, respectively). Figure 3A shows the MS^3 spectrum of the Arg-Lys residue of native TP5. The peak at m/z 157.1 is due to the protonated arginine residue fragment. The peak at m/z 129.1 can be ascribed to either a fragment of Lys [27] or the Arg-immonium ion, whereas the main peak at m/z 268 is the Arg-Lys residue that lost ammonia. Figure 3B shows the MS^3 result on m/z 297.2 (b_2 ion) of the modified peptide. The overall spectral pattern is quite different from that of the corresponding fragment of the native peptide. The only similarity is a peak corresponding to the loss of ammonia at m/z 280 (Δm=-17). The peak at m/z

157.1 and m/z 169.1 are ascribed to the protonated arginine residue and increment of +12 on arginine residue, respectively. On the other hand, the peak at m/z 113 is ascribed to the modified immonium ion of Lys [28]. In other words, the +12 Da mass extra can be tagged both on Arg and Lys residue, which suggests that the modification is not the result of simple modification of either of the two amino acids by a group of 12 mass units. The mass at m/z 252.2 (Δm=-45) is related to loss of ammonia and the terminal carbonyl group (fragmentations 1 and 2, see inset of Figure 3B). The mass at m/z 224.1 can be explained by cleavage of the peptide bond between Arg and Lys and subsequent loss of the carbonyl group of that peptide bond (fragmentations 3, Figure 3B), while the Arg and Lys remained connected to each other with the extra mass of 12. We therefore conclude that a methylene bridge is formed between the two residues, as shown in the inset of Figure 3B. This will be further explained in the Discussion section (*vide infra*). Moreover, based on the exact mass measurement (Table S1), the modified compound could be identified as thymopentin with an extra carbon atom.

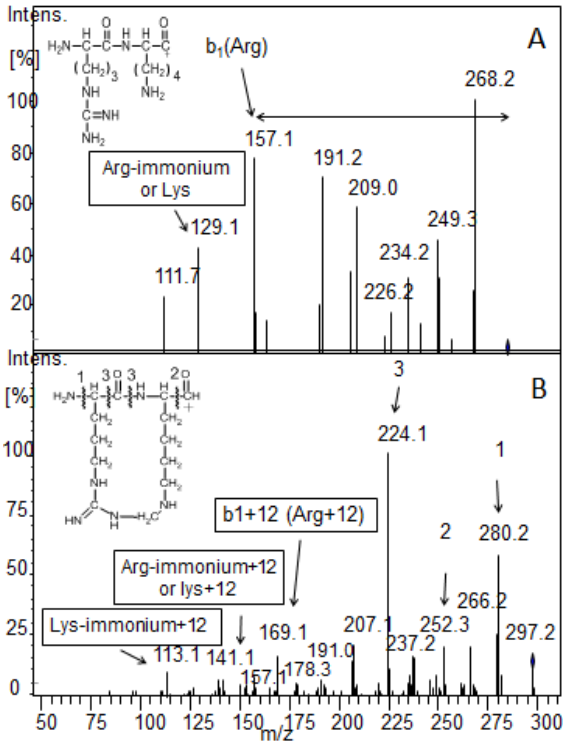

Figure 3. MS^3 spectrum of (A) TP5 (precursor ions, 680.3 → 285.2), (B) modified TP5 (precursor ions, 692.3 → 297.2); peak 1 at m/z 280.2, Δm -17, corresponds to loss of ammonia; peak 2 at m/z 252.2, Δm -45, loss of ammonia & carbonyl; peak 3 at m/z 224.1, Δm -73, loss of ammonia & 2x carbonyl.

2.2. Analysis of the decapeptide exposed to APS/TEMED

To further investigate whether the peptide modification occurs either on the lysine or the arginine residue, or on both, another peptide (a decapeptide with the sequence QKSLSLSPGK) containing two lysines but lacking an arginine residue, was incubated with APS/TEMED at 50˚C overnight and analyzed with LC–MS. Figure 4A shows the LC–MS results of the native decapeptide. Actually, two peaks are observed in the chromatogram showing the presence of an impurity with a mass that is 17 Da less than that of the original decapeptide, indicating elimination of ammonia. Indeed it is known that the N-terminal glutamine of peptides undergoes NH_3 elimination in solutions to form a cyclic pyroglutamyl residue [29].

Figure 4B and Table 1 shows the LC–MS result of the decapeptide incubated with APS/TEMED. Again, a peak was observed with additional mass of +12 Da (peak 2, appearing as a shoulder of the native peptide) and a peak that could be attributed to the pyroglutamyl impurity with an additional mass of +12 Da (peak 5). Even a peak with an extra mass of +24 was observed (peak 4).

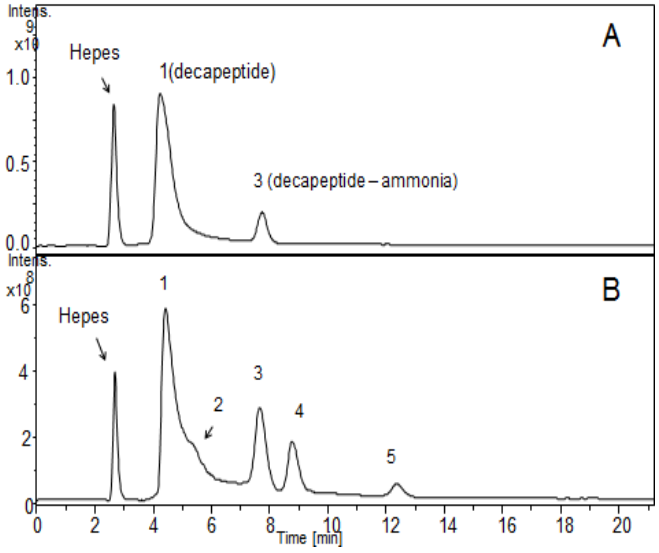

Figure 4. Base peak chromatogram of (A) native decapeptide obtained by LC–MS. (B) the decapeptide incubated with APS/TEMED at 50˚C overnight obtained by LC–MS.

Table 1. Identification of the major peaks shown in Figure 4.

Peak	$[M+H]^+$ m/z	$[M+2H]^{2+}$ m/z	Δm (Da)
1	1044.7	523.0	Native decapeptide
2	1068.8	529.0	+12
3	1027.8	514.5	-17(NH_3)
4	1068.8	535.0	+24
5	1039.8	520.5	-17(NH_3)+12

Similar as for TP5, MS^2 was performed for revealing the site of modification of the decapeptide incubated with APS/TEMED. Figures 5A, 5B and 5C show the MS^2 of native decapeptide, decapeptide+12 and decapeptide+24, respectively. The fragments that were obtained from the peaks of the decapeptide impurity (peaks 3 and 5, loss of ammonia) were the same as that of the native one and are therefore not discussed further. Figure 5A provides information about the sequence of the native decapeptide. Most of the y-ions can be seen in the spectrum and the peak at m/z 240 is ascribed to the residue of the amino acids glutamine and lysine minus ammonia (ion b2-17). Comparing the spectrum of the modified peptide (Figure 5B) with that of the native decapeptide reveals a striking mass at m/z 252.1, which indicates that the +12 adduct occurred on fragment b_2-17. In Figure 5C the mass at m/z 264.1 suggests that the +24 adduct occurred on the same residues of lysine and glutamine. There are no indications that the C-terminal lysine has been modified due to the fact that all original y ions until y_8 remained in all spectra of both the unmodified and modified peptide. These observations strongly suggests that the lysine modification is dependent on the nature of the neighboring amino acid.

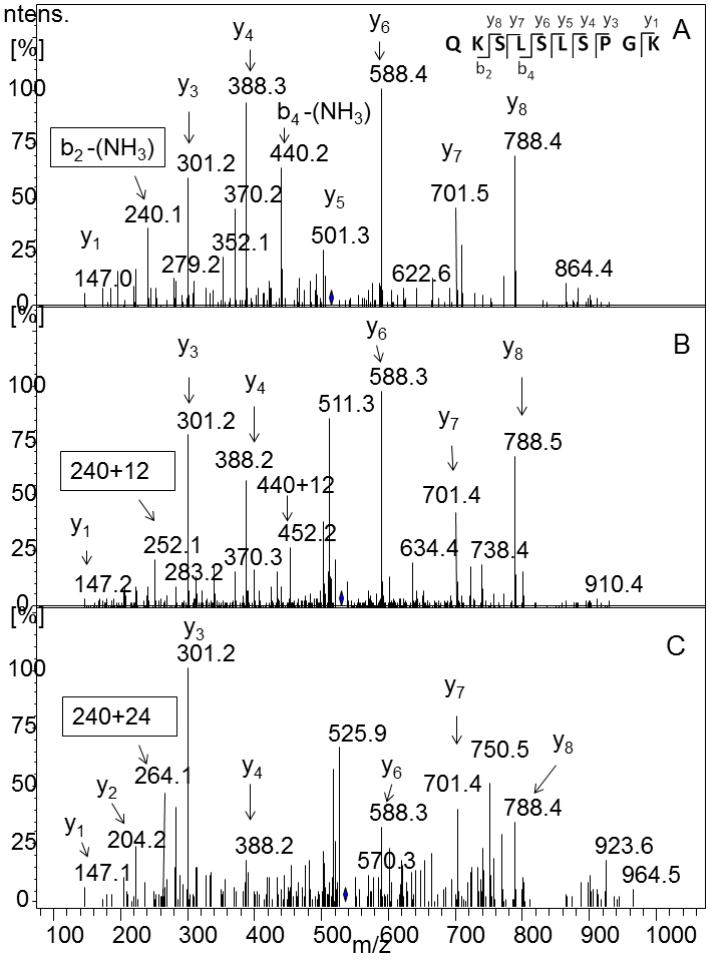

Figure 5. Tandem mass spectrum of (A) native decapeptide (precursor ion, $[M+2H]^{2+}$= 523.0), (B) modified decapeptide (precursor ion, $[M+2H]^{2+}$= 529.0), (C) modified decapeptide (precursor ion, $[M+2H]^{2+}$= 535.0).

2.3. Incubation of dipeptides from a library with APS/TEMED

In order to gain more insight into the neighboring effect of lysine modification when exposed to APS/TEMED, 15 dipeptides of a library with C-terminal lysine and different N-terminal amino acids were incubated with APS/TEMED at 50˚C overnight.

Table 2. Extent of modification of dipeptides from a library that contains C-terminal lysines, when exposed to APS/TEMED.

Dipeptide no.	Dipeptide sequence	+12 modification	relative intensity % of +12	+24 modification
1	Lys-Lys	No	—	No
2	Arg-Lys	Yes	10	Yes
3	Pro-Lys	Yes	43	No
4	Thr-Lys	No	—	No
5	Cys-Lys	Yes	68	No
6	Asn-Lys	Yes	75	No
7	Gln-Lys	Yes	29 for native & 14 for -NH3	No
8	Glu-Lys	Yes	5	No
9	His-Lys	Yes	9	No
10	Phe-Lys	No	—	No
11	Tyr-Lys	Yes	5	No
12	Trp-Lys	Yes	90	No
13	Ser-Lys	No	—	No
14	Asp-Lys	Yes	1.5	No
15	Met-Lys	No	—	No

Table 2 shows that +12 Da adducts were detected to different extents when lysine was next to arginine, glutamine, proline, cysteine, aspargine, glutamine, histidine, tyrosine, tryptophane and aspartic acid. For a detailed comparison, MS/MS experiments were carried out on the modified dipeptides to determine the position of modification

Figures 6A and 6B show the tandem mass spectra of native Arg-Lys (precursor ion m/z 303.0) and modified Arg-Lys (precursor ion m/z 315.0), respectively. Close inspection of these MS^2 spectra shows that the native Arg-Lys b_2 ion (m/z 285) underwent neutral loss of ammonia and water, whereas the modified b_2 ion (m/z 297) did not undergo loss of ammonia. Interestingly, further fragmentation of the modified one lead to production of an ion at m/z 224.0 which was also observed

in modified TP5 (*vide supra*), but was absent in the native dipeptide. This again suggests that modified Arg-Lys remains connected after full fragmentation of the peptide backbone.

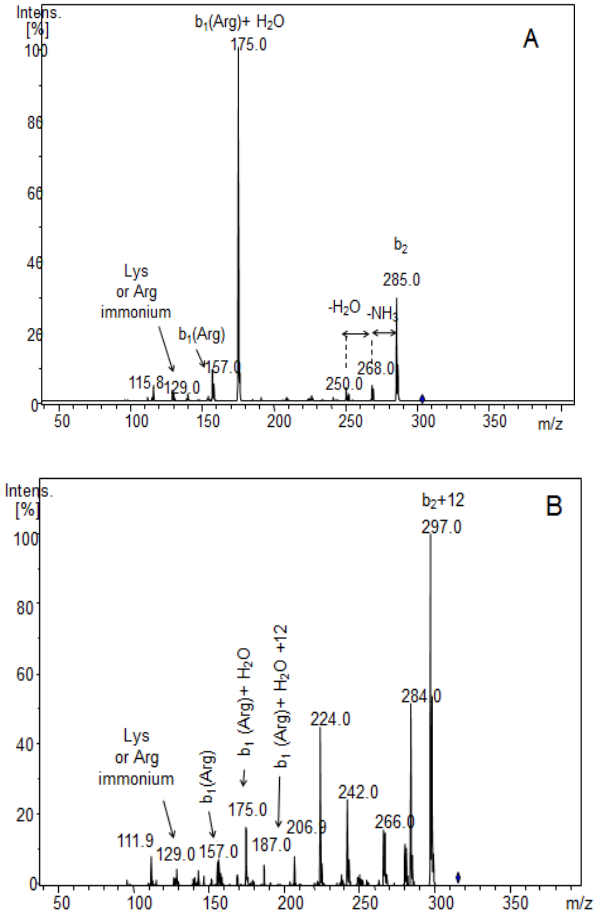

Figure 6. Tandem mass spectra of dipeptide no.2 (Arg-Lys): (A) native, precursor ion m/z 303.0, (B) modified, precursor ion m/z 315.1.

The tandem mass spectra of native and modified dipeptides 3, 5, 6, 7, 8, 9, 11, 12 and 14 from Table 2 are shown in the supporting information (Figures S2-S10). The MS/MS data indicated +12 addition on the lysine residue of the Pro-Lys, Asn-Lys, Gln-Lys, Glu-Lys, Tyr-Lys, Asp-Lys dipeptides and not on the lysine residues of Cys-Lys, His-Lys and Trp-Lys.

2.4. ^{1}H NMR spectroscopic analysis modified Trp-Lys

The dipeptide that had the highest degree of modification, i.e., Trp-Lys (see Table 2, peptide no. 12), was isolated by HPLC in sufficient quantity for NMR analysis . The ^{1}H NMR spectrum of this compound was compared with that of native Trp-Lys (Figure 7).

The most striking observation that emerges from the ^{1}H NMR spectra is peak 5, which is ascribed to the chemical shift of the CH in the pyrrole ring (δ: 7.25 ppm, 1 proton singlet) of the native dipeptide. This peak was not observed in the modified dipeptide spectrum, which showed a new peak with a chemical shift of 4.3 ppm (peak 13; 2 protons) that overlapped with the chemical shift of the α CH of the backbone lysine (peak 8). Moreover, peak 6 (δ: 3.05 ppm) of the CH_2 of native tryptophan shifted slightly upfield in modified Trp-Lys (δ: 2.91 ppm) and peak 7 (δ: 4.02 ppm) of the α CH of the backbone of native tryptophan shifted slightly downfield in modified tryptophan (δ: 4.48ppm). It is remarkable that peaks assigned to the lysine residue did not change position in the modified peptide.

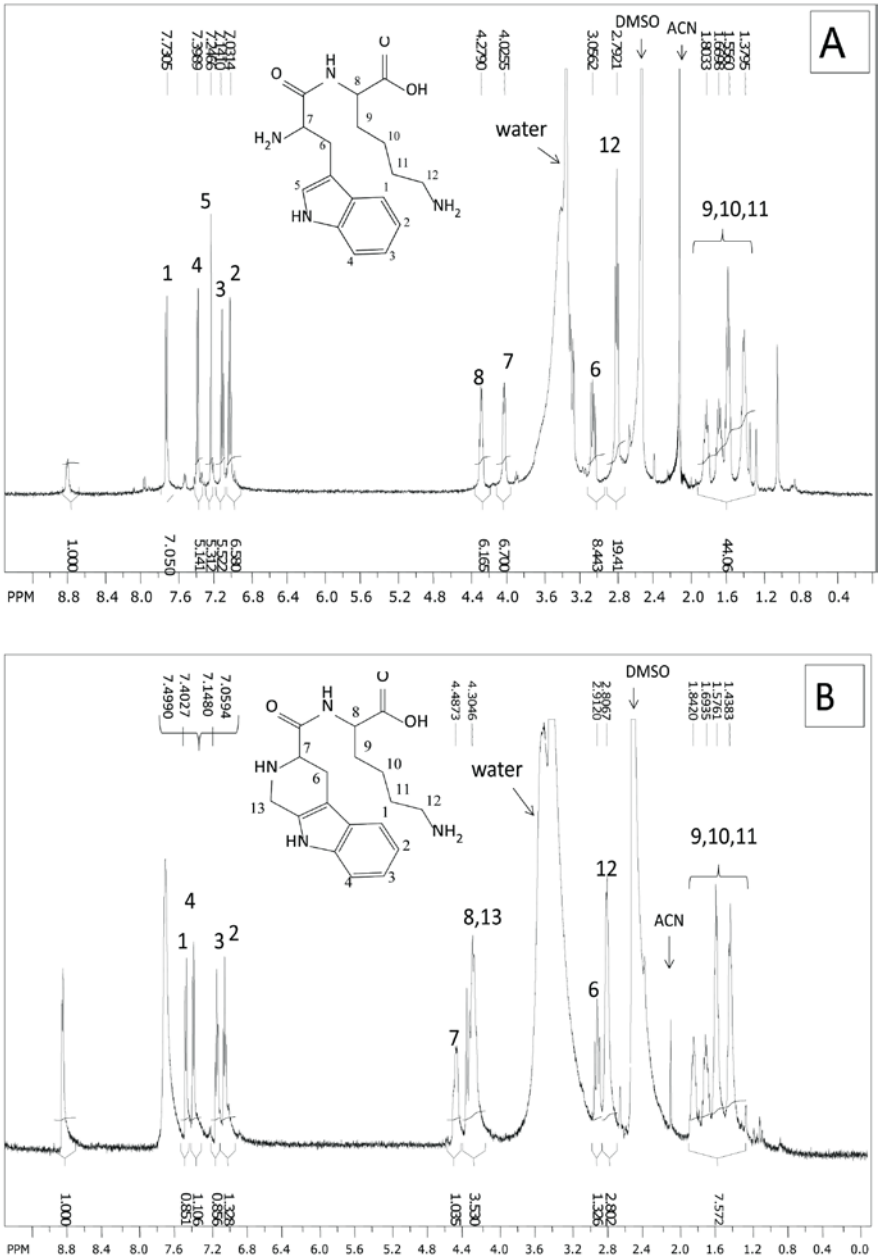

Figure 7. ^{1}H NMR spectrum of native (A) and modified (B) Trp-Lys (dipeptide no. 12, see Table 2) in deuterated DMSO.

3. Discussion

It is clear from the presented results that the 12 Da mass increase happened for TP5 on the Arg /Lys sequence. We observed 56 % of TP5 being modified at the site of Arg-Lys when incubated overnight by APS/TEMED at 50˚C. Importantly, the same modification (although to a somewhat lower extent) was also observed in the Arg-Lys dipeptide of the peptide library (Table 2) when incubated under the same conditions.

The major peak observed in the LC–MS^3 spectrum (Figure 3B) of the modified TP5 fragment at m/z 224.1 can be explained by breaking of the peptide bond with loss of ammonia and a carbonyl group, while the Arg and Lys still remain covalently connected involving a gain of mass of 12 Da. Interestingly, this only happened when the catalyst TEMED was present, and not when TEMED was absent or replaced by $NaHSO_3$, which convincingly demonstrates the involvement of TEMED in the modification of the peptide. We propose that the TEMED acts as a carbon source providing the extra mass difference, by the formation of a methylene bridge between Arg-Lys caused by TEMED radicals. The proposed mechanism of this reaction will be discussed further on (*vide infra*).

Our observations that TP5 and the decapeptide are methylated at the lysine residues led us to investigate a library of dipeptides each containing at least one lysine. For the different dipeptides studies in this paper (Table 2), under the same conditions the relative intensity of +12 modification varied from 1.5% (Asp-lys) up to 90 % (Trp-Lys), which suggests that the reactivity of the amino acid residues for methyleneation is different. For example, we observed that the presence of amide group of asparagine or glutamine next to lysine gives higher reactivity than with the carboxylic acid group of aspartate or glutamate. The guanidine group of Arg is also a reactive site in the dipeptide, although to a lesser extent than in TP5 (approx. 10% and 50% of Arg-Lys sequence were modified, respectively). On the other hand, the Gln-Lys sequence, which was present both in the dipeptide library as well as in the decapeptide, showed a higher degree of modification in the dipeptide than in the decapeptide. Apparently, there is an effect of the total sequence and/or the accessibility of residues in the peptides. It is important to note that the second (C-terminal) lysine of the decapeptide was not modified, which also indicates that methyleneation of a lysine residue depends on the nature of the neighboring amino acids. Primary aliphatic alcohols (serine and

threonine), primary amine and the thioether of methionine next to lysine are not reactive.

3.1. Mechanism of methyleneation

Interestingly, methylene bridge formation between amino acids has been reported before to occur from the reaction of peptides with formaldehyde, where the latter acts as a carbon source for additional masses of 12 Da [28,30]. It was demonstrated that lysine is methylated by formaldehyde to form $–N=CH_2$ units, followed by the nucleophilic addition of a second amino acid to the N=C double bond. However, as discussed above, in our case the methylene source must be TEMED, and also here we propose that methyleneation of lysine is the first step in the reaction sequence. According to the known formation of radicals from peroxodisulfate and TEMED (Figure 8)[31], the most probable modification is the addition of a methylene group from the TEMED radical, by the mechanism that we propose in Figure 9. First, attack of a TEMED radical on the primary amine of lysine occurs, followed by a hydrogen atom shift from the amine of the lysine to the amine of TEMED. Importantly, this hydrogen atom shift, which is followed (or preceded) by a hydrogen radical shift, is essential to cause the liberation of a methylated lysine. In principle, these reactions are all reversible and may be independent of the neighboring amino acids. The question now is, why the extent of methyleneation is dependent on the type of neighboring amino acid. Two explanations can be given: (1) the shift of the H-atom is catalyzed by appropriate neighboring functional groups, or (2) the driving force that leads to completion of the reaction, rather than reversing to the original situation, could be the subsequent methylene bridge formation with the neighboring amino acid (if the neighboring amino acid is prone to that).

Metz et al. [28-30] reported that no bridges were formed between two primary amino groups, which is in accordance with the lack of modification of Lys-Lys (peptide no. 1, Table 2) from our dipeptide library. We observed methyleneation of the peptide when the following functional groups were present next to the lysine: (1) guanidine (from Arg); (2) amide (from Gln and Asn); (3) carboxylic acid (from Glu and Asp); (4) phenol (from Tyr); (5) thiol (from Cys); or (6) secondary amine in a ring structure (from Pro, His, Trp). Therefore, if catalyzing the H-atom shift is the driving force for methyleneation, it seems that most protic functionalities that are able to add and at the same time release protons might be

responsible for the catalytic effect, except for non-conjugated alcohols (Thr, Ser) and primary amines (Lys). Interestingly, for all the amino acids such as Arg, GLn, Asn, Glu, Asp and tyr, the H-atom shift can be explained by a concerted mechanism with the formation of an intermediate 8-membered ring transition state according to Figure 10 and is thus thermodynamically quite favorable. The fact that no methyleneation with Thr-Lys, Ser-Lys or Lys-Lys was observed could be due to insufficient catalytic effect of primary aliphatic alcohols or primary amine in the proton transfer reaction that would otherwise drive the methyleneation of lysine to completion. Although it should be stressed that the proposed mechanism of Fig. 10 is hypothetical and not yet experimentally proven (e.g. by stable isotope experiments for H shifts or by radical trapping), it can very well explain the observed neighboring amino acid selectivity of the reaction.

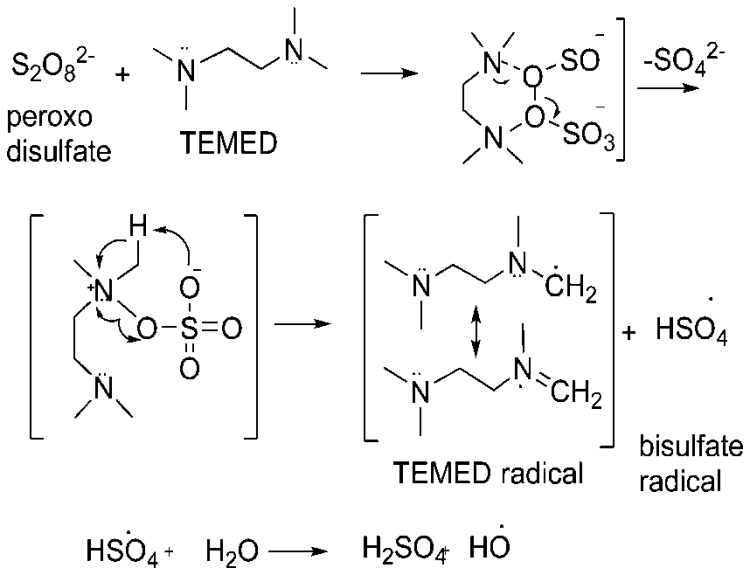

Figure 8**.** Formation of radicals from peroxodisulfate and TEMED[31]

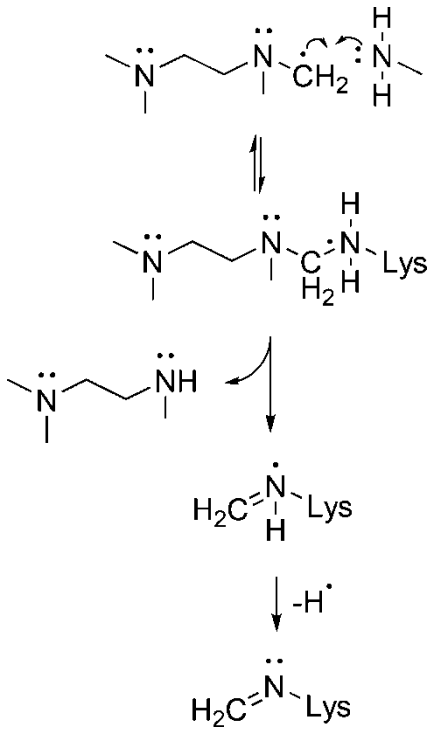

Figure 9**.** Proposed mechanism of methyleneation of Lys by the TEMED radical.

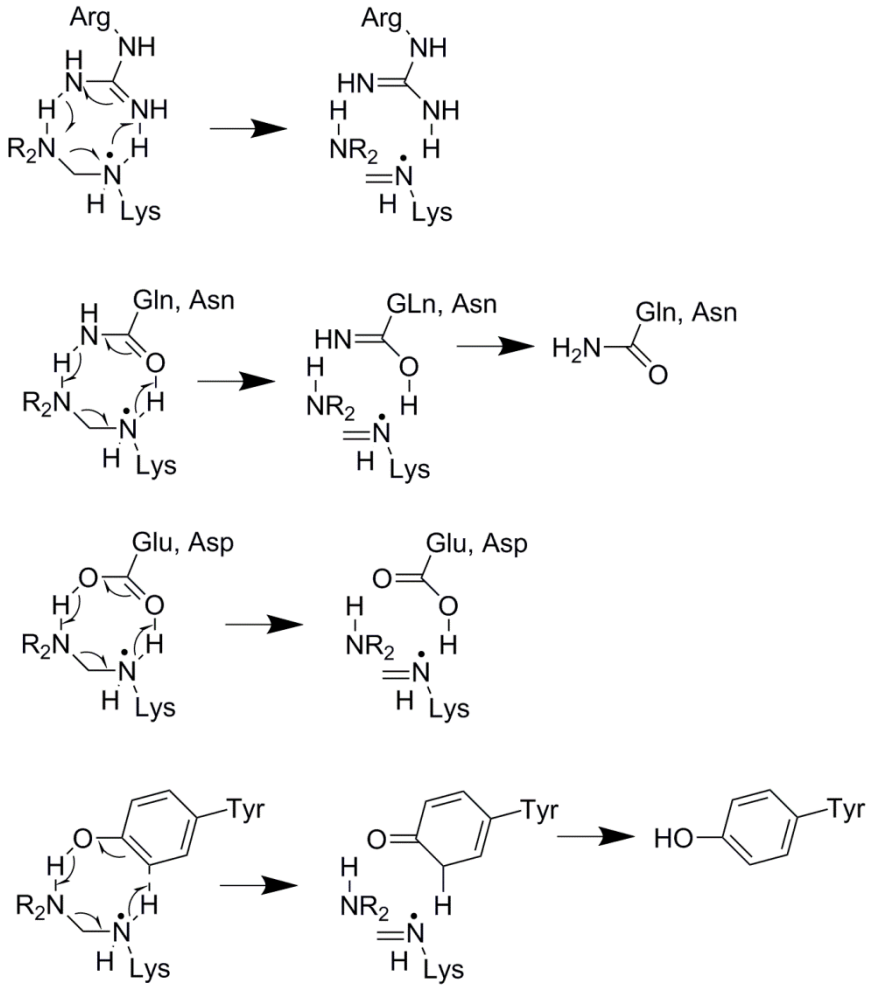

Figure 10**.** Proposed mechanism of H-atom shift in TEMED-Lys radical adduct, induced by different neighboring amino acid residues, with the liberation of a methylated lysine radical.

In principle, the resulting methylene-substituted lysine units (with mass increment of 12 Da as compared to the unmodified lysine) could exist as such, however, as explained above, the LC–MS^3 results of TP5 suggest that a methylene bridge had been formed between Arg and Lys. Interestingly, methylene bridge formation between methylated lysine and neighboring Arg has been reported previously by Metz et al. (methylated lysine being a reaction product with formaldehyde in that case) [28]. Indeed, methylene bridge formation can be easily explained by

addition of Arg to the C=N double bond of the methylated lysine according to the mechanism proposed in Figure 11. Likewise, the study of Metz et al. showed that in the presence of formaldehyde and glycine, coupling of glycine to peptides via methylene bridge occurred with the following amino acids: His, Arg, Gln, Asn, Tyr, and Trp. Interestingly, we observed methyleneation in all dipeptides containing these amino acids as well.

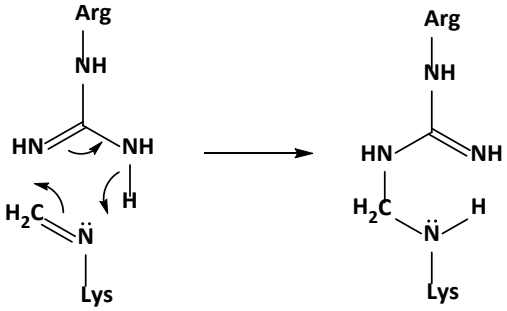

Figure 11**.** Arg-Lys bridging

With the proposed mechanism of methyleneation and possible bridge formation, we can now analyze the mass spectra of the individual dipeptides. Generally, fragmentation of lysine-containing peptides in MS/MS produces a peak with m/z 147 (C-terminal y_1 as the result of the splitting of the peptide bond) and two immonium ions, a major one at 84 Da (which lacks the ε-NH_2 group) and a minor peak at 101 Da (Figure 12) [28]. Several dipeptides after incubation with APS and TEMED showed fragmentation peaks in the MS/MS spectra that are indicative of methyleneation of lysine, i.e., at m/z 159 and at m/z 113, which are related to Lys+12 (y_1+12) and the immonium ion of Lys +12 (101+12) respectively. These fragment ions with increment of 12 Da can be found for the methylated dipeptides that contain proline (peptide 3, Figure S2B), aspargine (peptide 6, Figure S4B), glutamine (peptide 7, Figure S5B), glutamic acid (peptide 8, Figure S6B), tyrosine (peptide 11, Figure S8B), and aspartic acid (peptide 14, Figure S10B). However, we cannot conclude whether either single methyleneation happened on lysine without bridge formation, or the increment of 12 Da on lysine fragment ions is a result of cleavage in CID of a possible methylene bridge. It should be noted that the covalent bond of methylene bridge can break during MS/MS analysis, resulting in a 12 Da mass shift tag on one of the two bridged residues, depending on the stability of the ions produced in CID[32]. For example, in dipeptide 14, +12 adduction can be found both on lysine and on aspartic acid

(Asp+12, b_1 at m/z 127.9), which supports the methylene bridge formation between these two amino acids (Figure S10B).

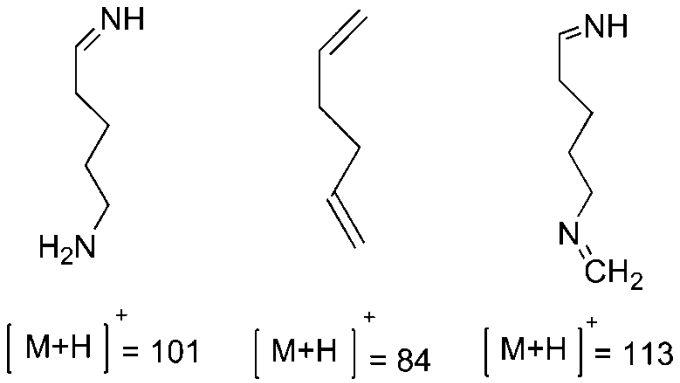

Figure 12. Structure of lysine immonium ion at m/z 84, 101 & methylated lysine immonium ion at m/z 113 [28].

In dipeptide no.3 (Pro-Lys) methyleneation occurred to a relatively large extent (43 %), but tandem mass data (Figure S2B) shows that the +12 can be tagged on lysine only. We therefore conclude that the sequence of Pro-Lys is prone to methyleneation. Proline was present in the middle of the decapeptide, but this residue was not methylated. Methylene bridge formation was not reported by Metz et al. for formaldehyde and glycine treated peptides that contain proline. However, Toews et al. pointed to single modification of proline in a peptide with the sequence of PGHDPPISYYETN-NH_2 after treating with formaldehyde. They observed a fragment ion of b_2+12 which localizes on Pro-Gly. They concluded that, as glycine is not susceptible to formaldehyde modification, the 12 Da increment was exclusively present on the amino-terminal proline [33]. However, our study does not support single methyleneation on proline because the +12 modification was tagged on lysine. Yet, we cannot conclude whether methyleneation happened exclusively on lysine or is a result of cleavage of a methylene bridge by collision induced dissociation during the MS analysis.

For dipeptide 7(Gln-Lys, Figure S5) the peak at m/z 113.0 that is ascribed to the immonium ion of lys+12 can be detected, but as the Lys and Gln have isobaric masses, it is not possible to conclude whether the peak at 140.9 is due to lys+12 or Glu+12. However, because of the m/z 113.0 we can conclude that at least lysine is methylated.

From the reaction of Trp with formaldehyde, Metz et al. reported the formation of a single methyleneation product (imine formation on the nitrogen atom of the pyrrole ring without bridge formation) in a peptide with the sequence of Ac-VEL**W**VLL-OH [28]. They observed that the typical immonium ion of tryptophan (159 Da) was lost after the reaction with formaldehyde and a new fragment appeared (171 Da), indicating that the tryptophan residue was modified. They did not detect any other new mass related to cross-linked peptide. However, when the mentioned peptide was treated with glycine and formaldehyde, coupling of glycine to tryptophan via a methylene bridge occurred. In our dipeptide 12 (Trp-Lys), also the fragment at m/z 159.0 ascribed to the immonium ion of native tryptophan vanished after treating with APS/TEMED, while a new fragment at m/z 171.0 ascribed to the methylated immonium ion of tryptophan was observed (Figure S9). These MS data alone are not strong enough to draw a conclusion for single methyleneation. However, our NMR data from the modified Trp-Lys (Figure 7B) are indicative of methylene bridge formation, not with the lysine but with the amine terminus of the peptide through a ring closure with the CH in the pyrrole ring.

For the dipeptide 9 (His-Lys), the fragment of histidine +12 was detected (Figure S7B). No other fragments related to methyleneation of lysine or cross-linking between lysine and histidine were found. As the NMR data for tryptophan showed the bridge formation between the N terminus and the pyrrole ring, it may be anticipated that bridge formation between with the N terminus and the imidazole ring in histidine could have been occurred as well, to form a thermodynamically stable 6-membered ring (Figure 13).

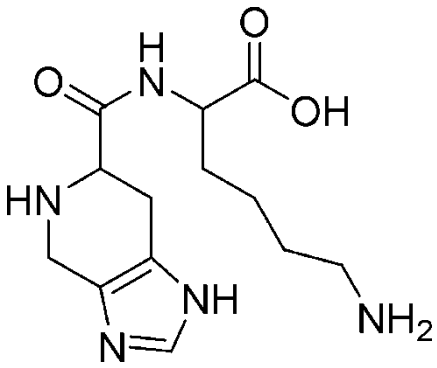

Figure 13. Proposed structure of methyleneation for His-Lys dipeptide.

The tandem mass spectra of native dipeptide 5 (Cys-Lys) and modified Cys-Lys are shown in figure S3. All ions related to lysine at m/z 84, 129 and 147 (y_1) can be detected in both the native and modified peptide which may indicate that methyleneation occurred preferentially at the cysteine residue, but these MS data alone are not strong enough to draw a definite conclusion for single methyleneation. Methyleneation was not observed in Met-Lys and can be explained due to the absence of free SH group in methionine.

In the Arg-Lys dipeptide, a peak with a 24 Da mass increase, although with very low intensity, was detected. Interestingly, dimethylene bridge formation has been reported by Metz et al. to occur between Lys and Arg upon reaction with formaldehyde, showing the great similarity between the quite different reaction conditions of TEMED and formaldehyde induced methyleneations. Besides, a mass increase of 24 Da was also observed in the Gln-Lys fragment of the decapeptide. However, this was not the case for the dipeptide Gln-Lys, which only showed the +12 Da modification. Unfortunately, subsequent fragmentation of the +24 Da modified decapeptide did not lead to the generation of a key fragment ion, which may be partly due to the isobaric mass of Lys and Gln which differ just 0.04 Da in mass [34].

4. Conclusion

A practical method for identifying methylated peptides was provided and we hypothesized the mechanism by which the methyleneation occurs in the presence of TEMED radicals. Mass spectrometric experiments have proven useful in detecting and identifying the modification sites and determining their relative reactivity. The studies on the simple peptide models presented here provided valuable insight into TEMED radical reactions.

It is shown that the TEMED radical is a carbon source to produce the methylated products. Taken together, the data from this work suggests that in most cases primary amines (lysine or N-terminus) are the initial reaction sites with the TEMED radical. In the case of lysine, the reaction with TEMED radical is driven to completion by a proton shift possibly catalyzed by a proper neighboring amino acid residue, and at least for Arg-Lys followed by methylene bridge formation. In the case of N-terminal Trp, NMR analysis indicated that methyleneation was followed by methylene bridge formation between the amino terminus of the

peptide and the pyrrole ring of Trp. Although identification of all possible intramolecular cross-links or single methyleneation products of TEMED-treated peptides still remains a tremendous job, the data from this study can be useful to predict the modifications that could happen on peptides and proteins in the presence of TEMED radicals.

As the stability of peptide and protein payloads or templates is crucial in in-situ polymerization for the preparation of drug delivery systems or molecularly imprinted polymers, avoiding the use of TEMED and replacing this catalyst with sodium bisulfite is recommended.

5. Experimental Procedures

5.1. Chemicals

Thymopentin of 99.0% purity was purchased from PROSPEC (Ness Ziona, Israel). Ammonium peroxodisulfate (APS) was obtained from FLUKA (Zwijndrecht, the Netherlands). Potassium peroxodisulfate (KPS) was purchased from Merck (Darmstadt, Germany). Sodium bisulfite ($NaHSO_3$) was from Acros Organics (Geel, Belgium). *N,N,N,N*-Tetramethylethylenediamine (TEMED), formic acid, trifloroacetic acid (TFA) and Hepes ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were purchased from Sigma-Aldrich Co (Zwijndrecht, the Netherlands). HPLC and MS grade acetonitrile were purchased from Biosolve (Valkenswaard, the Netherlands). A decapeptide (Gln-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly-Lys), which is a fragment of human immunoglobulin G [35] was from LifeTein (New Jersey, USA). 15 dipeptides with C-terminal lysine and different N-terminal amino acids were synthesized by Chinapeptides Co., Ltd.

5.2. Thymopentin imprinting

Thymopentin imprinted hydrogels were prepared by dissolving TP5 (4 µmol) in 600 µl of HEPES buffer (20 mM, pH 7.4) containing 400 µmol total monomers (methacrylamide as functional monomer and methylenebisacrylamide as a cross-linker) which polymerized by addition of 4 µmol TEMED and 4 µmol APS. Subsequently the vials were seal and incubated at 50 ˚C for 16 h. Alternatively the monomers were polymerized after addition of 4 µmol $NaHSO_3$ and 4 µmol KPS followed by incubation at room temperature for 16 h. Extracting of the template performed by sodium chloride solution[23,36].

5.3. Incubation of peptides with APS/TEMED

The different peptides (4 µmol) were incubated for 16 h with 4 µmol TEMED and 4 µmol APS in an eppendorf vial containing 600 µl of HEPES buffer (20 mM, pH 7.4) at 50˚C. Besides, thymopentine was also incubated at room temperature at otherwise similar conditions. Solutions of each peptide (4 µmol) in 600 µl in the same buffer were used as controls. Next, the samples were diluted with MQ water to reach a concentration of 0.33 µmol peptide/ml for TP5 and decapeptide, and 1.33 µmol peptide/ml for the different dipeptides prior to analysis.

5.4. Peptide analysis

Thymopentin solutions were analyzed by ultra-performance liquid chromatography (Waters ACQUITY UPLC®) using a column (length, 50 mm; internal diameter, 2.1 mm) packed with 1.7 µm ACQUITY BEH 300 C18 material. A gradient method was performed at 30 °C. The mobile phase A was water-acetonitrile-trifloroacetic acid (95:5:0.1, v/v/v) and phase B was acetonitrile-trifloroacetic acid (100:0.1, v/v). Eluent A linearly changed from 100% to 30% in 5 min with a flow rate of 0.25 ml/min. UV detection was done at 275 nm and the sample injection volume was 5 µl.

LC–MS experiments were performed using a Shimadzu 10A HPLC system (Kyoto, Japan) coupled to an Agilent Technologies 6300 Series LC/MSD ion-trap mass spectrometer (Santa Clara, CA, USA). A HPLC column (150 x 4.6 mm) packed with 3.5 µm XBridge™ BEH130 C18 material was used at ambient temperature. For analysis of the TP5 samples, a gradient method was used with the mobile phase A water-acetonitrile-formic acid (95:5:0.1, v/v/v) and mobile phase B acetonitrile-formic acid (100:0.1, v/v). The eluent A after 5 min linearly changed from 100% to 50% in 25 min with a flow rate of 0.5 ml/min. For the decapeptide, an isocratic method was used with a mobile phase of water-acetonitrile-formic acid (70:30:0.1, v/v/v). The sample injection volume was 5 µl. For the different dipeptides, the mobile phase was changed to water-methanol-trifloroacetic acid. The gradient method was the same as mentioned above but the sample injection volume was 10 µl. Electrospray ionization (ESI) was performed in the positive ion mode using an Agilent Technologies ion source and interface. The MS settings were: a capillary voltage of 2 kV, a nebulizer pressure of 60 psi, a dry gas flow of 11 L/min, a dry gas temperature, 350˚C and a scan range of m/z 50–1500. MS^2 and

MS^3 experiments were performed using an isolation width of 2 Da and a fragmentation amplitude of 1.0 V. Accurate mass spectra of thymopentin and modified thymopentin were obtained by direct infusion of a incubated sample by APS/TEMED into a Bruker (Bremen, Germany) ESI- time-of-flight mass spectrometer.

The Trp-Lys from the dipeptide library, treated with APS/TEMED, was separated by using multiple injections on the HPLC using water-acetonitrile-trifloroacetic acid (95:5:0.1, v/v/v) which after 5 min linearly changed to acetonitrile-trifloroacetic acid (100: 0.1, v/v) in 25 min with a flow rate of 1 ml/min and the fractions that contained methylated Trp-Lys were collected and after freeze-drying of the pooled fractions analyzed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra were recorded on a Bruker DRX-500 spectrometer operating at 500 MHz, using DMSO-d_6 as the solvent; the DMSO peak at 2.52 ppm was used as the reference line. The NMR peak assignments are based on ChemDraw calculation software.

Appendix: Supporting Information

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Appendix

Supporting Information

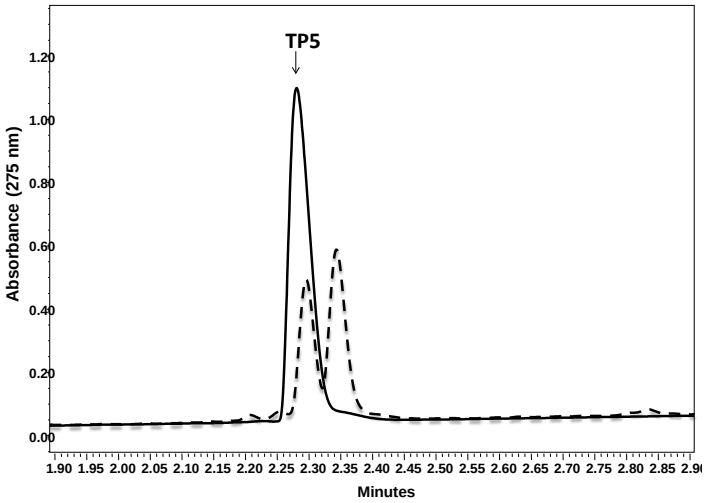

Figure S1. RP-UPLC chromatogram of native TP5 showing a single peak at 2.28 min (solid line), and extracted template after imprinting of TP5 in a polyacrylamide gel prepared by APS/TEMED at 50˚C showing an additional degradation product at 2.36 min (dashed line).

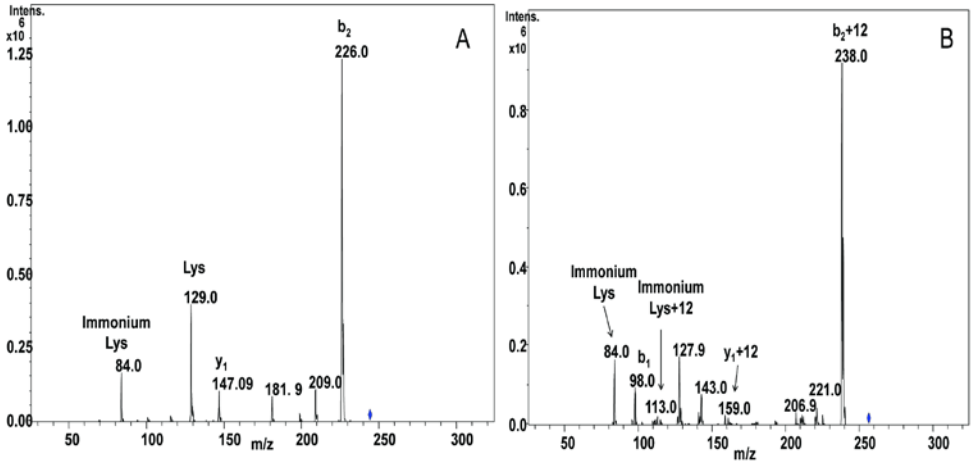

Figure S2. Tandem mass spectrum of dipeptide No.3 (Pro-Lys); (A) native, precursor ion at m/z 244.0,(B) modified, precursor ion at m/z 256.0.

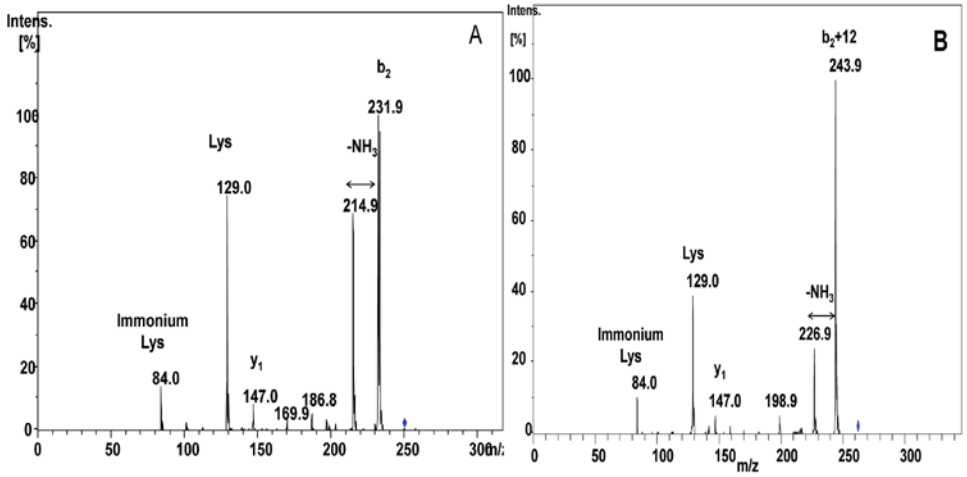

Figure S3. Tandem mass spectrum of dipeptide no.5 (Cys-Lys): (A) native, precursor ion at m/z 249.9, (B) modified, precursor ion, m/z 261.9.

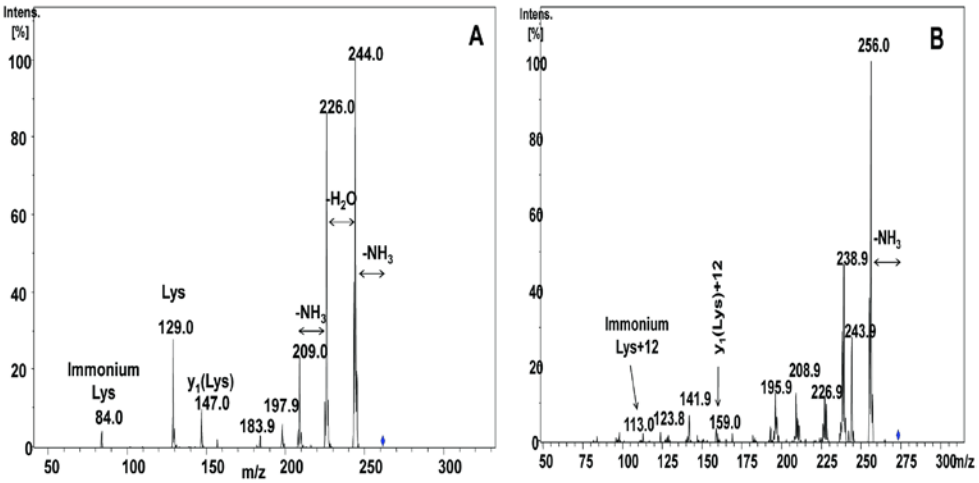

Figure S4. Tandem mass spectrum of dipeptide no.6 (Asn-Lys): (A) native, precursor ion at m/z 261.0, (B) modified, precursor ion at m/z 273.0

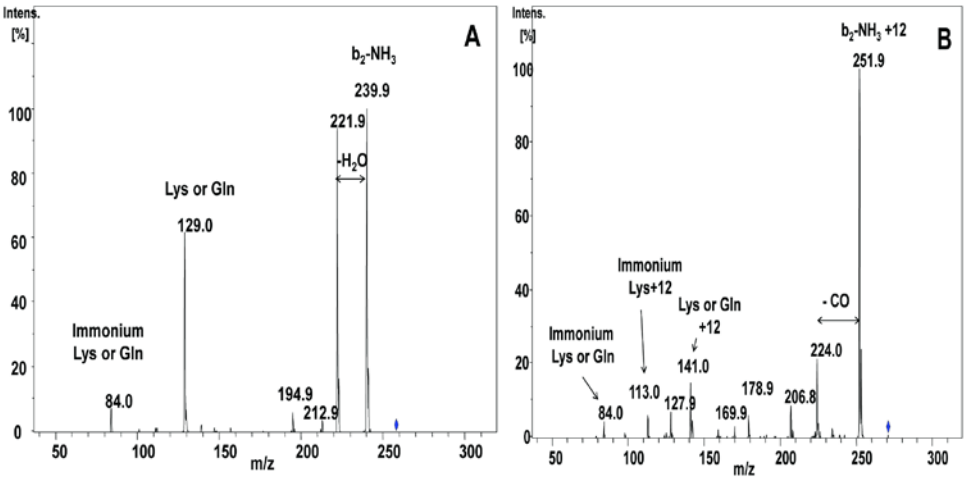

Figure S5. Tandem mass spectrum of dipeptide no.7 (Gln-Lys) -NH_3: (A) native, precursor ion at m/z 258.0, (B) modified, precursor ion at m/z 270.0

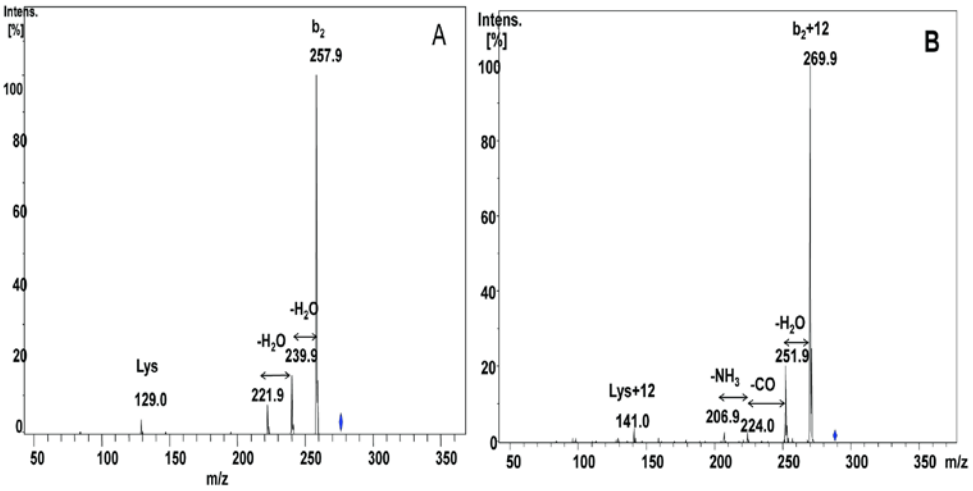

Figure S6. Tandem mass spectrum of dipeptide no.8 (Glu-Lys): (A) native, precursor ion at m/z 276.1, (B) modified, precursor ion at m/z 288.1.

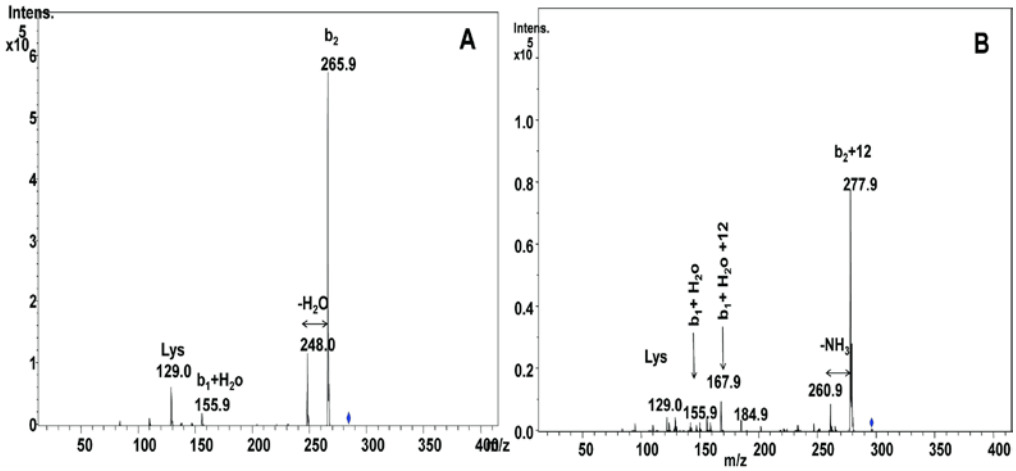

Figure S7. Tandem mass spectrum of dipeptide no.9 (His-Lys): (A) native, precursor ion at m/z 283.0, (B) modified, precursor ion at m/z 296.0.

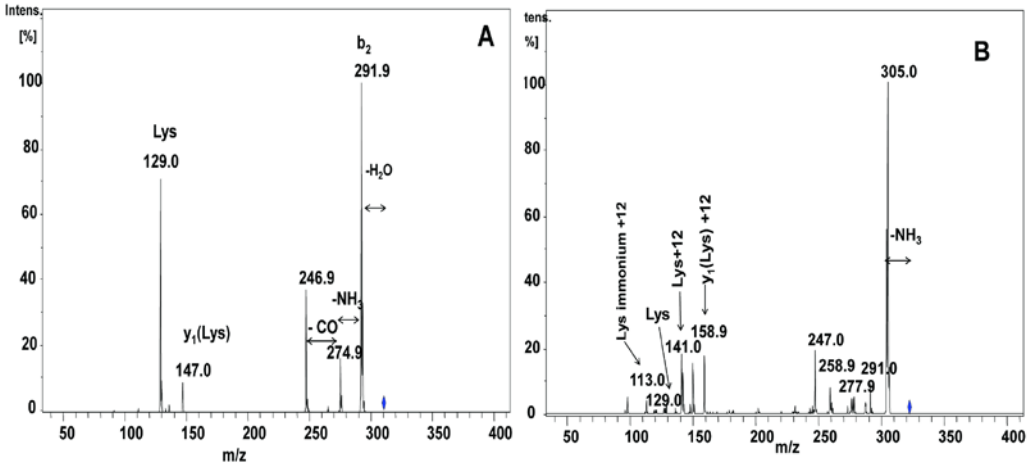

Figure S8. Tandem mass spectrum of dipeptide no.11 (Tyr-Lys): (A) native, precursor ion at m/z 310.0, (B) modified, precursor ion at m/z 322.0.

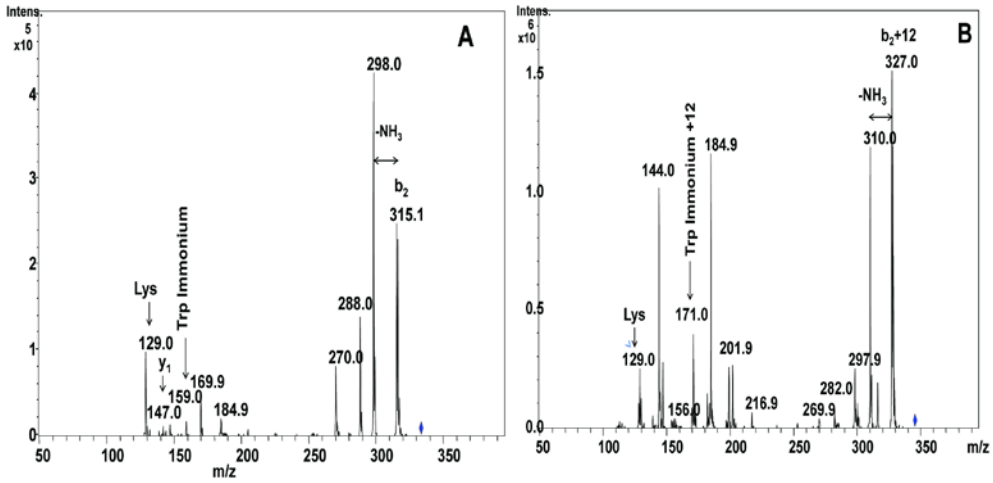

Figure S9. Tandem mass spectrum of dipeptide no.12 (Trp-Lys): (A) native, precursor ion at m/z 333.0, (B) modified, precursor ion at m/z 345.0.

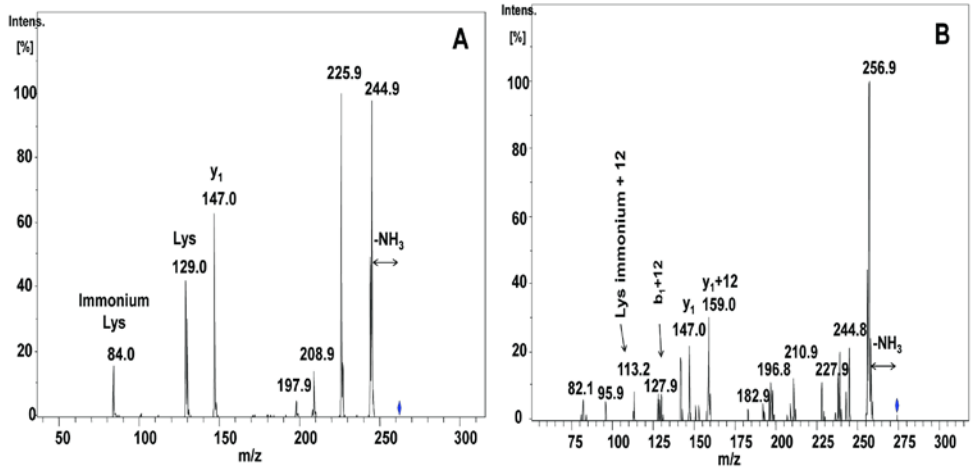

Figure S10. Tandem mass spectrum of dipeptide no.14 (Asp-Lys): (A) native, precursor ion at m/z 262.0, (B) modified, precursor ion at m/z 274.0.

Table S1: Accurate mass measurements of thymopentin and its modified product

Name	Molecular formula	Observed *m/z*	Calculated *m/z*	Error (mDa)
Thymopentin	$[C_{30}H_{49}N_9O_9+H]^+$	680.3726	680.3726	0.0
Modified thymopentin	$[C_{31}H_{49}N_9O_9+H]^+$	692.3734	692.3726	-0.8

Chapter 3

Identification and assessment of octreotide acylation in polyester microspheres by LC–MS/MS

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Abstract

Polyesters with hydrophilic domains, i.e. poly(D,L-lactic-*co*-glycolic-*co*-hydroxymethyl glycolic acid) (PLGHMGA) and a multiblock copolymer of poly(ε-caprolactone)-PEG-poly(ε-caprolactone) and poly(L-lactide) ((PC-PEG-PC)-(PL)) are expected to cause less acylation of encapsulated peptides than fully hydrophobic matrices. Our purpose is to assess the extent and sites of acylation of octreotide loaded in microspheres using tandem mass spectrometry analysis. Octreotide loaded microspheres were prepared by a double emulsion solvent evaporation technique. Release profiles of octreotide from hydrophilic microspheres were compared with that of PLGA microspheres. To scrutinize the structural information and localize the actual modification site(s) of octreotide, liquid chromatography ion-trap mass spectrometry (LC-ITMS) was performed on the acylated adducts. Hydrophilic microspheres showed less acylated adducts in comparison with PLGA microspheres. LC-MS/MS showed that besides the N-terminus and primary amine of lysine, the primary hydroxyl of the end group of octreotide was also subjected to acylation. Nucleophilic attack of the peptide can also occur to the carbamate bond presented in (PC-PEG-PC)-(PL) since 1,4-butanediisocyanate was used as the chain extender. Hydrophilic polyesters are promising systems for controlled release of peptide because substantially less acylation occurs in microspheres based on these polymers. LC-ITMS provided detailed structural information of octreotide modifications via mass analysis of ion fragments.

1. Introduction

In recent years, there has been an increasing interest in formulations for therapeutic peptides and proteins based on aliphatic polyesters to achieve targeted and/or sustained release of these therapeutics [1-3]. Particularly microspheres of poly(D,L-lactic-*co*-glycolic acid) (PLGA) have been extensively used for prolonged release of bioactive peptides and proteins [4,4-6]. However, one of the major obstacles in formulation of therapeutic peptides/proteins with PLGA is the modification of the actives as a result of acylation with lactic and glycolic units. Nucleophilic groups, particularly the N-terminus and primary amine groups of lysine residues, attack the electrophilic carbonyls of the ester groups of the PLGA backbone, which results in covalent addition of glycolyl or lactyl groups on the released peptide [7,8]. It was found that the low pH that is generated inside degrading PLGA microspheres catalyzes these acylation reactions [9]. Importantly, acylation can result in unwanted change of activity, immunogenicity and toxicity due to the structural changes of peptides/proteins [10,11] and should therefore be avoided .

Octreotide is a synthetic octapeptide analogue of somatostatine which is clinically used for the treatment of acromegaly as well as certain endocrine tumors [12]. Octreotide has poor pharmacokinetics (half-life of 100 min in humans) and therefore a sustained formulation based on PLGA microspheres has been developed which is presently used in the clinic [13]. Octreotide has a free N-terminus and a lysine amine group and has therefore been studied in depth regarding acylation reactions that occur in matrices of PLGA and related aliphatic polyesters [14-20]. For instance, Murty et al.[21] studied the acylation of octreotide acetate, formulated in microspheres of PLGA of varying molecular weight and comonomer composition. They found that due to the steric hindrance of the α-methyl groups of lactic acid units as compared to glycolic acid units, PLGA polymers with higher lactic acid content were less amenable to formation of acylation adducts as compared to PLGA with higher glycolic acid content. They also showed that microspheres prepared from PLGA 50:50 (9 kDa) released 54 % of loaded octreotide within 50 days and, importantly, 66 % of the released peptide was acylated. Sophocleous et al. studied the nature of peptide interaction with PLGA to get insight how peptide or PLGA properties effect sorption and acylation. They suggested that peptide sorption to PLGA is the first

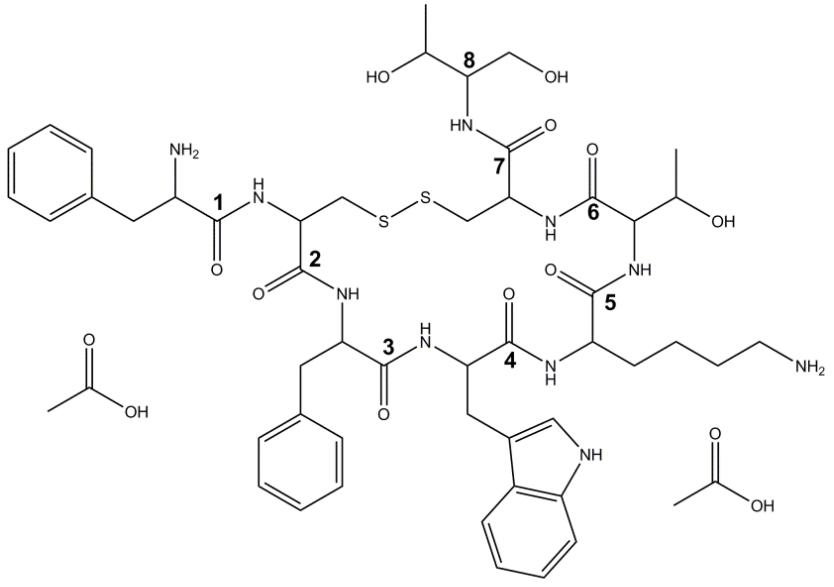
step to peptide acylation. They prevented the peptide sorption to PLGA by adding divalent cationic salt which indeed resulted to attenuation of acylation [22]. Ghassemi et al. compared the acylation of octreotide that occurs in poly(lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA) microspheres [23,24], with the commercial octreotide microsphere formulation (Sandostatin LAR®, which is based on PLGA-glucose star polymer). They found that less acylated octreotide adducts were formed in PLHMGA microspheres than in Sandostatin LAR® most likely due to the lack of glycolic acid units and lower extent of acidification during degradation [9,18]. In the studies mentioned above, it was shown that the extent of acylation depends on the type of polymer used. Further, no detailed information has been reported at which site acylation preferentially occurs (N-terminus or lys). Therefore, in the present study we further explored acylation and prevention of acylation using more hydrophilic matrices than PLGA and also assessed the sites of acylation using tandem mass spectrometry analysis. To this end, octreotide was encapsulated in microspheres based on PLGHMGA (poly(lactic-co-glycolic-co-hydroxymethyl glycolic acid)), i.e. copolymers containing hydrophilic HMGA units but also containing glycolic acid units [25], as well as those based on a multiblock copolymer of poly(ε-caprolactone)- PEG-poly (ε-caprolactone) with poly(L-lactide)). LC–MS/MS analysis was applied to identify the extent and sites of acylation by using ion-trap mass spectrometry (ITMS) employing electrospray ionization (ESI).

2. Materials and methods

2.1. Chemicals

Octreotide acetate (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol, MW= 1,018.8 Da; Figure 1) was obtained from Feldan-bio (Quebec, Canada). Polyvinyl alcohol (PVA; MW 30,000-70,000; 88% hydrolyzed) was from Sigma-Aldrich, Inc., USA. Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) were obtained from Merck. Sodium azide (NaN_3, Bio Ultra, ≥ 99.5%) was purchased from Sigma (Germany). HPLC and MS grade acetonitrile (ACN), peptide grade dichloromethane (DCM) and tetrahydrofurane (THF) were purchased from Biosolve (The Netherlands). Formic acid was purchased from Sigma-Aldrich Co (Zwijndrecht, the Netherlands). Dithiothreitol was from Sigma-Aldrich Co (Canada). PLGA (acid terminated 5004A with D,L-lactide/glycolide molar ratio 50:50, IV = 0.4 dl/g) was obtained from Purac, The Netherlands. Poly(lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA)

(82/18 lactic/hydroxymethyl glycolic acid ratio (L/HMG) and M_w of 22.5 kg/mol) and poly(lactic-*co*-glycolic-*co*-hydroxymethyl glycolic acid) (PLGHMGA) (64/18/18 lactic/glycolic/hydroxymethyl glycolic acid (L/G/HMG) ratio, M_w of 44 kg/mol) were synthesized as described by Leemhuis et al. [23,24]. The Polymers are further referred to as PLGA, PLHMGA and PLGHMGA, respectively. The company Innocore (The Netherlands) kindly provided 20CP10C20-LL40 (IV= 0,67 dl/g), which is a multiblock copolymer that was prepared by chain extension of a ABA-triblock copolymer of poly(ε-caprolactone) and 1000 Da PEG with a poly(L-lactide) block with a molecular weight of 4000, using 1,4-butanediisocyanate as a chain extender [26,27]. This copolymer is further referred to as (PC-PEG-PC)-(PL). The polymer structures are shown in Figure 2.



H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol

Figure. 1 Structure of octreotide acetate

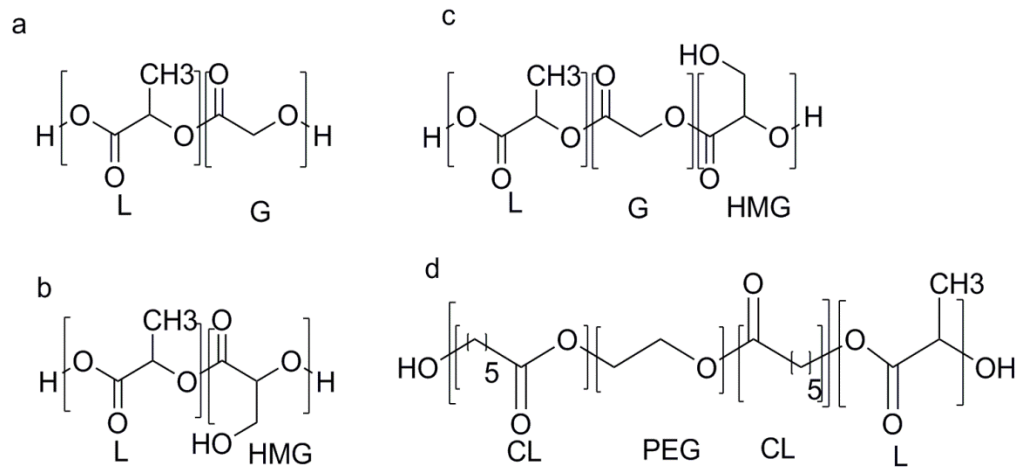


Figure. 2 Structural formulas of the polymers used in this study: a) Poly(D,L-lactic-*co*-glycolic acid) (PLGA 50/50), b) Poly(D,L-lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA 82/18), c) poly(D,L-lactic-*co*-glycolic-*co*-hydroxymethyl glycolic acid) (PLGHMGA 64/18/18), d) poly((ε-caprolactone-*b*-PEG-*b*-ε-caprolactone)-*b*-L-lactide)) (PC-PEG-PC)-(PL).

2.2. Microspheres preparation

Octreotide loaded microspheres were prepared by a double emulsion (W/O/W) solvent evaporation technique [18]. Briefly, 50 µl of an octreotide solution in milliQ water (200 mg/ml) was emulsified in 500 µl of a dichloromethane solution of polymer (220 mg, 25% w/w) by using an IKA homogenizer (IKA Labortechnik Staufen, Germany) for 30 s at the highest speed (30,000 rpm) to get the primary emulsion. Next, 500 µl of a PVA solution (1% w/w in 30 mM sodium phosphate buffer, pH 7.4) was added, and the mixture was vortexed for 30 s at 30,000 rpm. The w/o/w emulsion was subsequently transferred into an external aqueous solution (5 ml) containing PVA 0.5% (w/w) in 30 mM phosphate buffer pH 7.4 while stirring. Continuous stirring at room temperature for 2 h resulted in extraction/evaporation of DCM. Finally, hardened microspheres were collected by centrifugation (Laboratory centrifuge, 4 K 15 Germany) at 3,000 g for 3 min, subsequently washed 3 times with 50 ml RO water and freeze dried at −50 °C and at 0.5 mbar in a Chris Alpha 1−2 freeze-dryer (Osterode am Harz, Germany) for 16 h. The dried microspheres were stored at −25°C.

2.3. Characterization of microspheres

The Microspheres' size and size distribution were analyzed by a laser blocking technology (Accusizer 780, Optical particle sizer, Santa Barbara, California, USA) after dispersing the freeze dried microspheres in water. The morphology of the freeze dried microspheres was analyzed by scanning electron microscopy using a Phenom™ SEM (FEI Company, the Netherlands). The samples were mounted onto a 12 mm diameter aluminum specimen stub (Agar Scientific Ltd., England) using double-sided adhesive tape and were coated with 6 nm platinum prior to analysis. The octreotide loading was determined by dissolving about 10 mg of microspheres in 2 ml of THF with gentle shaking. Thereafter, 2 ml of solution of 0.2% w/v glacial acetic acid, 0.2% w/v sodium acetate and 0.7% w/v sodium chloride in water was added to precipitate the polymer. Next, the mixture was incubated at room temperature for 20 min, and the precipitated polymer was spun down by centrifugation at 5,000 g for 2 min. The octreotide content in the supernatant was measured by ultra performance liquid chromatography (Waters ACQUITY UPLC®) using an ACQUITY BEH 300 C18 column (1.7 µm, 2.1 mm × 50 mm). A gradient elution method was used with a mobile phase A (95% H_2O, 5% ACN + 0.1% TFA) and a mobile phase B (100% ACN + 0.1% TFA). The eluent linearly changed from 100% A to 70% B in 5 min with a flow rate of 0.25 ml/min. Octreotide standards (5–100 µg/ml, 7 µl injection volume) were used for calibration, and detection was done both at UV 210 nm and using fluorescence with setting excitation at 280 nm and emission a 330 nm. Loading efficiency (LE) of the peptide in the microspheres is reported as the encapsulated peptide divided by the total amount of peptide used for encapsulation. Loading capacity (LC) is defined as the encapsulated amount of octreotide divided by dry weight of the microspheres.

2.4. *In vitro* release studies

Octreotide release from different microspheres was studied in PBS (0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05% (w/w) NaN_3, pH 7.4). About 30 mg of microspheres (accurately weighted) were suspended into 1.5 ml of PBS buffer in eppendorf tubes and incubated at 37°C under mild agitation using a circular mixer (ASSISTANT RM 5). At the different time points, the dispersion was centrifuged (3,000 g, 3 min), and 1 ml of the supernatant was replaced with 1 ml

of fresh buffer. The microspheres were resuspended by gentle shaking, and the dispersion was incubated at 37°C. The released samples were kept in -20ºC until measurement by UPLC and mass analysis .

2.5. LC–MS/MS analysis

LC–MS experiments were performed using a Shimadzu 10A HPLC system (Kyoto, Japan) coupled to an Agilent Technologies 6300 Series LC/MSD ion-trap mass spectrometer (Santa Clara, CA, USA). A HPLC column (150 x 4.6 mm) packed with 3.5 µm XBridge^{TM} BEH130 C18 material was used at ambient temperature.

A gradient method was used with a mobile phase A (95% H_2O, 5% ACN + 0.1% formic acid) and a mobile phase B (100% ACN + 0.1% formic acid). The eluent linearly changed from 100% A to 100% B in 20 min with a flow rate of 0.5 ml/min. The injection volume was 10 µl. Electrospray ionization (ESI) was performed in the positive ion mode using an Agilent Technologies ion source and interface. The MS settings were: capillary voltage,2 kV; nebulizer pressure, 60 psi; dry gas flow, 11 L/min; dry gas temperature, 350˚C; scan range, *m/z* 50–1500. MS/MS experiments were performed using an isolation width of 2 Da and a fragmentation amplitude of 1.0 V.

Since the disulfide bond between the two cysteine residues in octreotide hinders peptide fragmentation in the mass spectrometer, 500 µl of the released octreotide samples were incubated with 50 µl of dithiothreitol (DDT) 10 mg/ml in water at 37˚ C for 4 hours before MS–MS analysis [28].

3. Results and discussion

3.1. Preparation and characterization of octreotide-loaded microspheres

Octreotide-loaded microspheres of the different aliphatic polymers of different composition and hydrophilicity (structures shown in Figure 2) were prepared using a common double emulsion/solvent evaporation method. The characteristics of the microspheres are summarized in Table I. The microspheres have the average size ranging from 20-53 µm and the loading capacity varies between 2.1 and 2.8 %. SEM pictures are shown in Figure. 3, and demonstrate that particles are spherical and have some pores (formulation B and D) or are non-porous (formulation A and C).

Table I. Characteristics of Octreotide-Loaded Microspheres (n=3)

Polymer	Volume weight diameter (μm)	LE[a] (%)	LC[b] (%)
PLGA	20.5 ± 3.0	54.1± 5.1	2.5 ± 0.2
PLGHMGA	23.7 ± 2.5	58.1± 6.1	2.7 ± 0.3
PLHMGA	24.8 ± 3.3	62.9± 7.5	2.8 ± 0.3
(PC-PEG-PC)-(PL)	53.6 ± 3.5	45.9± 4.2	2.1 ± 0.2

[a]*LE* = Loading efficiency [b]*LC* = Loading capacity

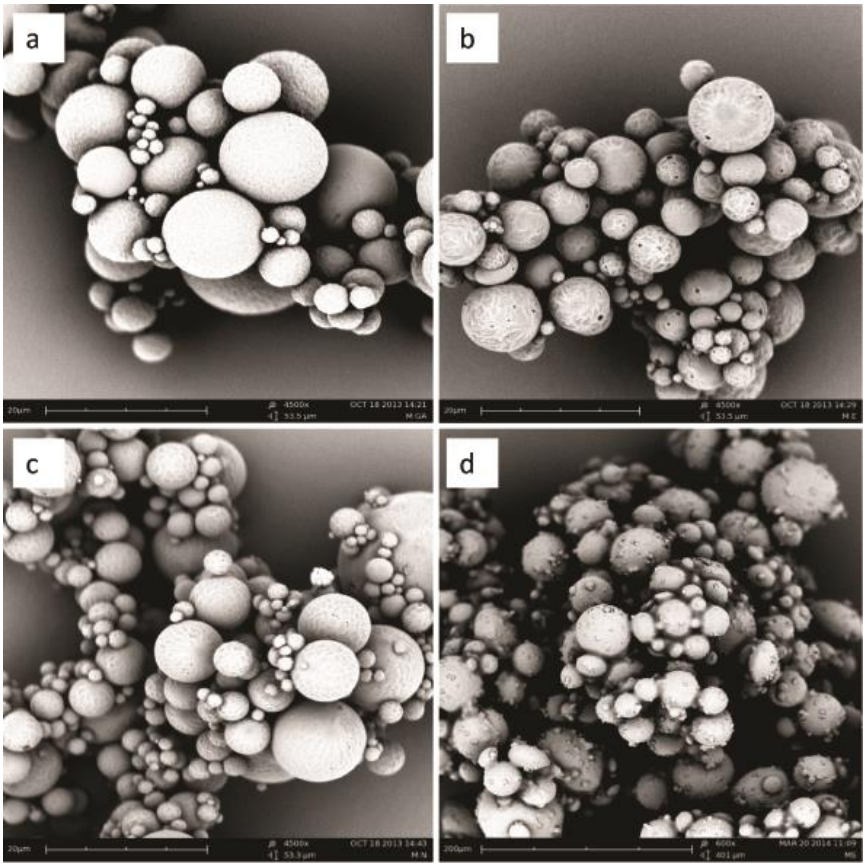


Figure. 3 SEM pictures of octreotide loaded microspheres based on (a) PLGA, (b) PLHMGA, (c) PLGHMGA, (d) (PC-PEG-PC)-(PL).

3.2. *In vitro* release of octreotide-loaded microspheres

UPLC-UV chromatograms of octreotide released after 45 days from PLGA, PLGHMGA and PLHMGA, respectively, and from (PC-PEG-PC)-(PL) after 28 days, are shown in Figure 4. The main peak eluting after approx. 2.5 minutes corresponds to native octreotide, while the extra peaks with longer retention times are originating from acylated octreotide adducts (see LC–MS analysis). It is remarkable that the extent of acylated adduct is significantly more pronounced for octreotide released from PLGA microspheres than that released from the other microspheres. It should be noted that only native octreotide was detected when the microspheres were dissolved immediately after preparation, demonstrating that the manufacturing process did not cause the acylation of the peptide. In line herewith, it has been observed that acylation of peptides occurs during the *in vitro* release from PLGA microspheres when the polymer starts to degrade [14]. Peptide acylation is catalyzed by the low pH that is generated inside degrading PLGA microspheres due to the accumulation of acid degradation products, i.e. glycolic and lactic acid and soluble oligomers thereof [9,14,29,30].

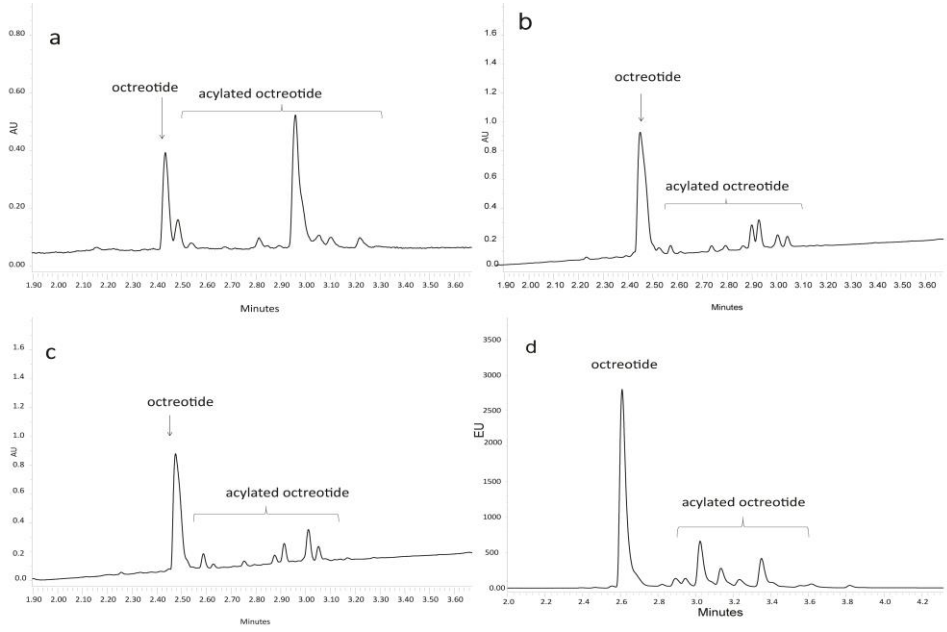


Figure. 4 UPLC of octreotide released after 45 days in PBS pH 7.4 at 37 ˚C from (a) PLGA, (b) PLGHMGA, (c) PLHMGA and (d) (PC-PEG-PC)-(PL) (the latter after 28 days).

Figure 5 shows the cumulative release of octreotide from the different microspheres in PBS pH 7.4 at 37˚C. These graphs show that acylation of octreotide is significantly higher when released from PLGA microspheres than of the others. Peptide release from PLGA microspheres started after 15-20 days without burst release and continued for the next 70 days until a plateau at 80% of the loaded amount was reached. UPLC analysis showed that only 31 % of the released peptide was in its native form, while 69% was acylated (assuming the same UV-absorbance response for native and acetylated product). PLGHMGA microspheres showed after a burst release of 10% consisting of only native peptide, a phase of low release of about 25 days (~10% of the loading was released). Faster release of octreotide (both native and acylated) started at day 25 reaching 90% of the loading at day 60. UPLC analysis showed that 72% of the released octreotide was in its native form while only 28% was acylated. For PLHMGA microspheres, in line with the result of PLGHMGA, initially only native octreotide was released, and both native and acylated peptide started to be released after day 25. Finally, around 78% of the released peptide was native octreotide, whereas 22% was acylated adducts. For (PC-PEG-PC)-(PL) microspheres, the release was faster than from the other formulations and it seems that the release is governed by diffusion rather than polymer degradation [26]. This is understandable because PEG increases the hydrophilicity of the polymer matrix which in turn results in water absorption allowing diffusion of peptide through the (channels or pores of) hydrated particles. The (PC-PEG-PC)-(PL) microspheres particles released the peptide in a continuous manner for 30 days and >90% of the loading was released at that time point. HPLC analysis showed that 75% of the released octreotide was in its native form while 25% was acylated. Figure 4d also shows that both native and acylated peptides were released from the start of the experiment. Although the presence of polymer degradation products can catalyze peptide acylation, the role of water should be considered as well. Liang et al. showed that a bell shape relation exists between the water content and the extent of acylation of exenatide (a polypeptide drug with a molecular weight of 4200 Da, which is clinically used as an adjunct for glycemic control in type 2 diabetes) when drug-loaded PLGA microspheres were incubated at different relative humidity. They showed that acylation kinetics depends on the water content of the microspheres. At low water content, water acts as plasticizer resulting in more acylation of the unfolded peptide (unfolding occurs due to the hydrophobic polymer matrix). According to the authors, at

higher water contents, bulk water present in the matrix will cause conformation recovery of the peptide resulting in a state in which it is less susceptible for acylation reaction [31]. The presence of PEG in (PC-PEG-PC)-(PL) microspheres will result in rapid hydration of the particles during the initial stage of the incubation in buffer and facilitate acylation of octreotide. This explains that already at early time points of release acylated octreotide adducts were detected (Figure 5d). However at later time points the ratio between native and acylated octreotide did not change suggesting that acylation occurs only during the initial stages likely because in later stages the water content of the microspheres became so high that acylation is prevented. Further the high water content facilitates release of the polymer degradation products.

The observation that acylation adduct formation of octreotide in PLGA (50/50) was substantially higher than in the other polymers can be explained by its high glycolic acid content and by the low water content that favors interactions between the peptide and the polymer matrix. Octreotide released form either PLGHMGA or PLHMGA microspheres was substantially less acylated than the peptide released from PLGA. Most likely, the hydroxyl pendent groups of PLHMGA and PLGHMGA increase the water absorption of the particles and facilitates the release of formed acid degradation products [18]. Further, octreotide released from PLGHMGA microspheres (containing glycolic acid as monomer) was slightly more acylated than the peptide released from PLHMGA microspheres lacking glycolic acid monomers (28 and 22 % respectively). Indeed, nucleophile attack is less hindered with glycolic ester units as compared to hydroxymethyl glycolic ester and lactic ester units [21].

The particle size studied in this paper is slightly smaller than most depots. Although some papers (e.g. Dunne et al. [32]) show that the polymer degradation rate (and release profile) depends on the particle size, it is difficult to predict the rate of acylation with particle size. One effect could be that peptides have a shorter pathway to diffuse through the pores in small particles, and the diffusion of degraded polymers such as monomer and oligomer is also easier, from which it might be anticipated that the peptide is in less contact with polymers and less acylation occurs. On the other hand, if the accumulation of acidic oligomer inside the bigger particles cause autocatalytic degradation of polymer and faster release of the peptide may cause easier release and less acylation.

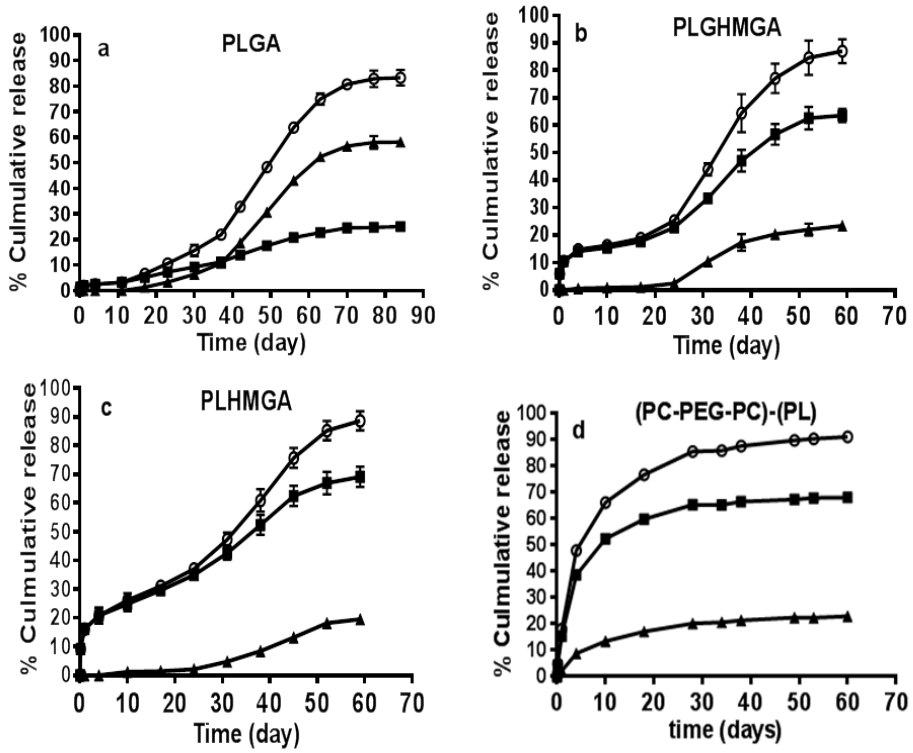


Fig. 5 *in vitro* release of octreotide from (a) PLGA, (b) PLGHMGA, (c) PLHMGA and (d) (PC-PEG-PC)-(PL) microspheres in PBS pH 7.4: native octreotide (squares), acylated octreotide (triangles) and total octreotide (sum of native and acylated adducts, circles)

3.3. LC–MS & LC–MS/MS analysis

LC–MS analysis confirmed that the extra peaks in the chromatograms (Figure 4) indeed represent acylated adducts. The assignments of observed masses of products released from PLGA, PLHMGA and PLGHMGA microspheres are summarized in Table II.

Table II. Acylated octreotide adducts observed by LC-ESI-MS in release samples of PLGA, PLHMGA and PLGHMGA microspheres.

Observed $[M+H]^{1+}$ m/z	Δm	Assigned structure of peptides		
		PLGA	PLHMGA	PLGHMGA
1021*	0	Octreotide	Octreotide	Octreotide
1079	+58	Octreotide-GA	N.D.	Octreotide-GA
1093	+72	Octreotide-LA	Octreotide-LA	Octreotide-LA
1109	+88	N.D.	Octreotide-HMGA	Octreotide-HMGA
1137	+116	Octreotide-GA-GA	N.D	Octreotide-GA-GA
1151	+130	Octreotide-GA-LA	N.D	Octreotide-GA-LA
1167	+146	N.D	N.D	Octreotide-GA-HMGA

*The m/z of octreotide is 1019, the increase with 2 Da is due to the reduction of disulfide bond by DDT.

N.D. not detected. GA, LA and HMGA are glycolyl, lactyl and hydroxymethyl glycolyl adducts**,** respectively.

In order to exactly determine the extent and site of acylation LC–MS–MS was performed. With Bruker Compass DataAnalysis software, the m/z values of the analyte of interest were extracted from the entire data set for each chromatographic run. For example, the extracted ion chromatogram (EIC) at m/z 1079 which is ascribed to reduced octreotide+GA (1021+58) showed 3 main peaks with retention times of 12.1, 12.8 and 13.3 min (Figure 6).

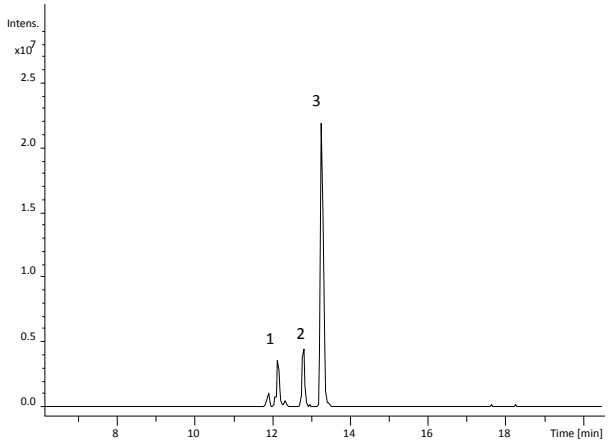


Figure.6 Extracted-ion chromatogram at m/z 1079 (= reduced octreotide, 1021+58) obtained by LC–MS of octreotide released from PLGHMGA after 45 days.

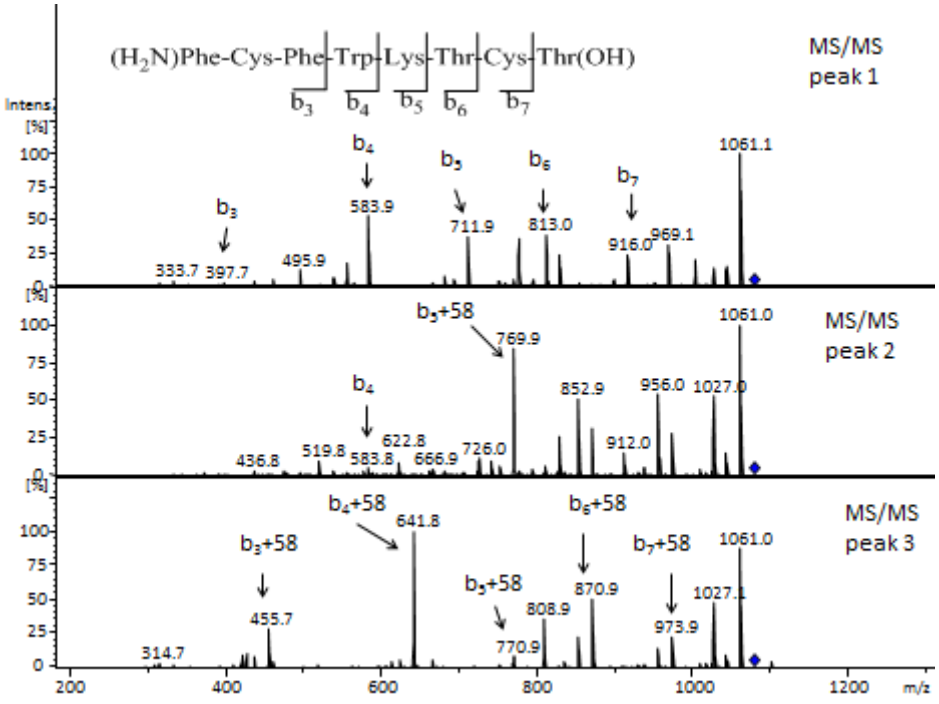


Figure. 7 MS/MS spectra of observed peaks in Figure 6 at m/z 1079.

Figure 7 shows the LC–MS/MS analysis of the peaks observed in the EIC at m/z 1079. MS/MS revealed complete amino acid sequence information by a comprehensive series of b-ions which carry the N-terminus. The MS/MS of peak 1 (in Figure 6) shows that all b ions up to b_7 can be attributed to the sequence of the native peptide, indicating that acylation had occurred on the last amino acid (position 8, Figure 1). The MS/MS results however cannot distinguish whether the primary or the secondary hydroxyl group of this position 8 group has been acylated. However, no acylation was observed on the secondary hydroxyl of threonine at position 6 and therefore it is concluded that the primary hydroxyl of position 8 is susceptible for acylation. In the second peak of the chromatogram in Figure 6, the b_4 ion was found unaffected while b_5 incremented by 58 Da, indicating that lysine (position 5) has been acylated. In the last peak (number 3), all b-ions where shifted by +58 Da, demonstrating that the amine of the N-terminus was acylated. The ion at m/z 1109 of octreotide released from PLGHMGA microspheres corresponds with addition of one HMGA unit to the native peptide (Table II). The extracted-ion chromatogram of this ion also showed 3 peaks pointing to the same sites of modification as observed for the octreotide-GA adduct (Figure S1 & S2). Figure S3 shows four peaks for the extracted ion chromatogram at m/z 1093, , which corresponds with addition of one lactic acid unit to octreotide. The MS/MS spectrum (Figure S4) of the first peak indicates the addition of the LA unit on the primary hydroxyl of the terminal amino acid, whereas the second peak is ascribed to addition on Lys, while peaks 3 & 4 have the exact same fragmentation pattern both indicating the addition of LA on the N-terminus. We presume that this is the result of the formation of diastereoisomers because the polymer contained both D- and L-lactic acid units. The same analysis was performed for all the observed ions that are mentioned in Table II for the peptide released from the other two microspheres (PLGA and PLHMGA);the distributions of acylation adducts are summarized in Figure 8. One can conclude that the N-terminus of octreotide is the most susceptible site for acylation, with the acylation by glycolic acid (if present in the copolymer) being the most abundant.

Lucke et al. applied enzymatic cleavage of salmon calcitonin (sCT) released from PLGA microspheres in order to identify the amino acids that are potential sites for acylation. They treated sCT with Endoproteinase Glu-C which selectively cleaves the peptide bond on the carboxyl site of glutamate. It was concluded that besides

the N-terminal amine group, lysine, tyrosine and serine could be acylated, although without specifying exactly which amino acid were susceptible for derivatization [14]. For octreotide, up to now only lysine and N-terminal phenylalanine were reported in the literature as the sites of acylation. Murty et al. reported that at least 11 additional peaks after the native octreotide peak were detected in the chromatogram of the product that was released from PLGA 50/50 microspheres. They identified 9 out of 11 observed acylated compounds and mentioned adduct formation at either the N-terminus or lysine residue with a higher degree of modification of the N-terminus. They also suggested the presence of diglycolyl, trigycolyl or lactyl-glycolyl adducts on the single lysine to explain the large number of different species formed [21]. However, no convincing evidence was presented to substantiate these claims. We have no evidence of dimer or trimer addition on lysine. De Jong et al. showed that at acidic pH the hydrolysis of lactic acid oligomers proceeds via fairly rapid chain-end scission [33]. Therefore, possible dimeric or trimeric aducts on the peptide will easily be hydrolyzed to form single adducts. Indeed, we now clearly show by LC-ITMS that three different species for each type of single acylated product can be identified (i.e. acylation on the N-terminus, Lys and Thr). Including the different species that can theoretically be formed with the two glycolyl and with the one glycolyl plus one lactyl unit observed at m/z = 1137 and 1151, respectively (see Table II), this would give rise to 15 different possible combinations.

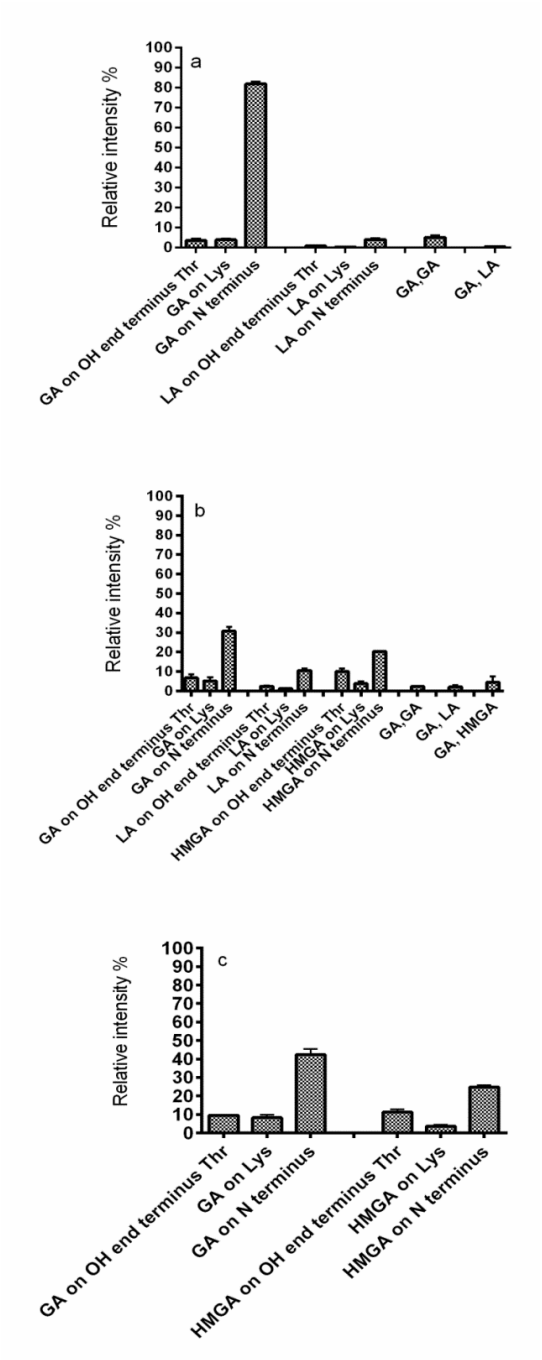


Figure. 8 Relative intensity of released acylated adducts of octreotide after 45 days from microspheres of a) PLGA b) PLGHMGA c) PLHMGA.

Table III**.** Acylated octreotide adducts observed by LC-ESI-MS in octreotide released from (PC-PEG-PC)-(PL) microspheres

Observed $[M+H]^{1+}$ m/z	Δm	Assigned structure	% Relative intensity of released acylated adducts
1091	+72	Octreotide-LA	63.5±2.5
1231	+212	Octreotide-butanediisocyanate-LA	36.5±2.5

For octreotide released from the (PC-PEG-PC)-(PL), two main peaks were observed in LC–MS with m/z values that were 72 and 212 Da higher than observed for native octreotide (Table III). The peak with m/z 1091 is ascribed to LA addition to octreotide. Since 1,4-butanediisocyanate has been used as the chain extender connecting the PC-PEG-PC with poly(lactic acid) to form the poly((ε-caprolactone-b-PEG-b-ε-caprolactone)-b-L-lactide)) multiblock copolymer, we propose that nucleophiles of the peptide can react with the carbonyl group of the carbamate bond (Figure 9). Consequently, after full hydrolysis of the remaining ester groups, a butanediisocyanate bound by a carbamate bond to one lactic acid unit remains coupled to the peptide. Indeed, the molecular weight of this group is 212 Da and explains the presence of the m/z 1231 ion. It should be noted that for quantitative measurement of the acylated products of the peptide released from (PC-PEG-PC)-(PL) microspheres (Figure 4d & 5d), fluorescence was used for detection in order to avoid the overestimation of acylated adducts at UV 210 because of the absorbance by the carbamate bond at this wavelength [34].

Table IV shows the monomer feed ratio for the synthesis (PC-PEG-PC)-(PL), as provided by the manufacturer. Interestingly, although the feed contained only 1.1 mole % of 1,4-butanediisocyanate (as compared with 75.9 % of L-lactide), the relative intensities of the peaks in the LC–MS chromatogram were 63.5:36.5 for 1,4-butanediisocyanate-LA and LA, respectively. Likely, the carbonyl group of the carbamate group is more accessible for attack by the nucleophile of the peptide than the secondary ester groups of the PLLA blocks. Also the higher hydrophilicity of the carbamate group than the ester group might contribute to its higher reactivity . Importantly, no addition of ε-caprolactone of released octreotide was

observed pointing to a low reactivity of the caprolactone ester groups. This is likely due to the hydrophobicity of the caprolactone blocks in combination with a low local solubility of the peptide in caprolactone rich domains.

Table IV. Monomer feed ratio for the synthesis of (PC-PEG-PC)-(PL)

	wt.%	Mole %
1,4-butanediol	1.9	1.5
1,4-butanediisocyanate	2.2	1.1
ε-caprolactone	9.6	6
PEG1000	9.6	15.5
L-lactide	76.8	75.9

Interestingly, the extracted ion chromatogram of the octreotide adduct with one LA unit released from (PC-PEG-PC)-(PL) (see Figure S5), shows three peaks as opposed to the four peaks that we observed for the LA adducts of the other polymers (*vide supra*). This can be explained by the fact that for the synthesis of (PC-PEG-PC)-(PL) just L-lactide had been used, giving only a single diastereomeric adduct.

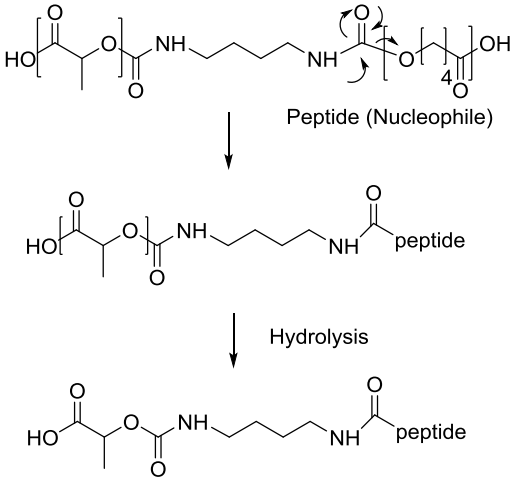


Figure. 9 Proposed mechanism of reaction between peptide and carbamate group in (PC-PEG-PC)-(PL) multiblock copolymer.

4. Conclusion

Our data demonstrate that acylation of the model peptide octreotide occurring in polyester microspheres depends on the hydrophilicity of the polymer and accessibility of the carbonyl ester groups for nucleophile attack by the peptide. The N-terminus of octreotide is most sensitive for acylation, and besides the primary amine of lysine in octreotide also the primary OH of the end group of octreotide was subjected to acylation. In (PC-PEG-PC)-(PL) polymer besides formation of lactic acid adducts, also formation of butanediisocyanate-LA was observed. Understanding the possible sites of acylation can be used to rationally develop methods and/or excipients that inhibit or preferably prevent this unwanted reaction. Several strategies for minimizing and preventing the peptide acylation in PLGA formulations have been proposed and studied in the past, such as the effects of pH-modifying excipients [35], water-soluble divalent cationic salts [17] and PEGylation of the peptide for inhibiting acylation [19]. We are also working on inhibition of acylation by protecting the nucleophilic sites of the peptide, which will be the subject of a next publication.

Appendix: Supporting Information

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Appendix

Supporting Information

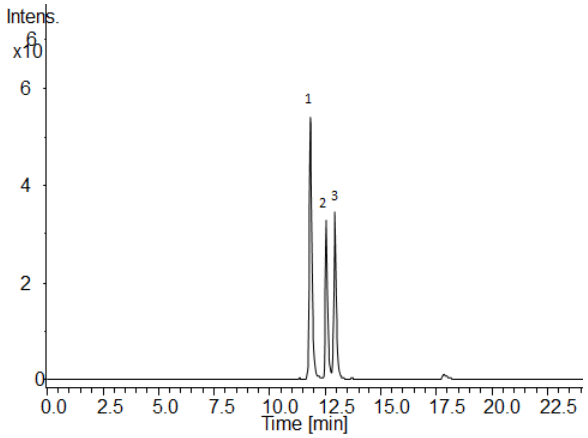

Figure. S1 Extracted-ion chromatogram at m/z 1109 (reduced octreotide +HMGA, 1021+88) obtained by LC-MS from octreotide released PLGHMGA after 45 days.

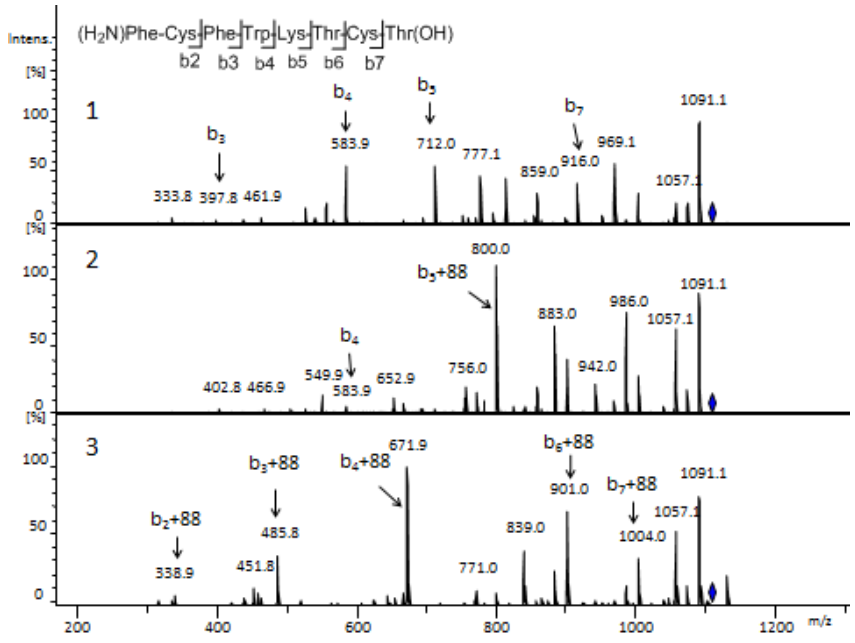

Figure. S2 MS/MS spectra at m/z 1109 of observed peak in figure S1

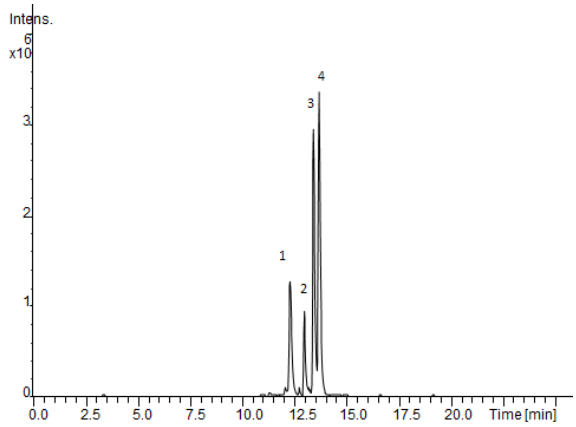

Figure. S3 Extracted-ion chromatogram at m/z 1093 (reduced octreotide+LA, 1021+72) obtained by LC-MS from octreotide released PLGHMGA after 45 days.

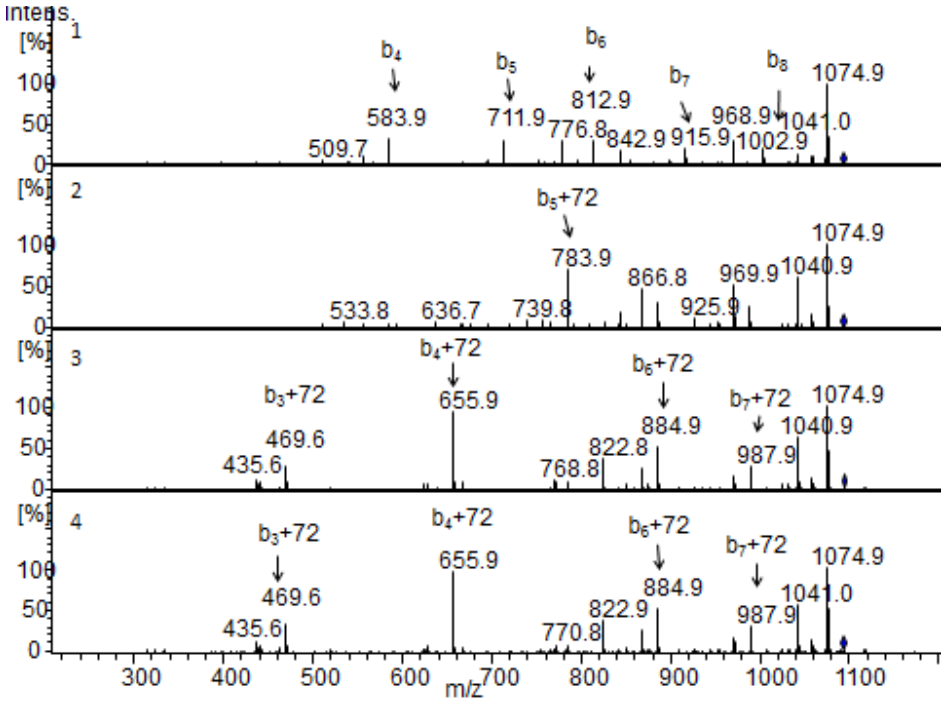

Figure. S4 MS/MS spectra at m/z 1093 of observed peak in figure S3

Chapter 4

Acylation of arginine in goserelin-loaded PLGA microspheres

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Submitted for publication

Abstract

Acylation of peptides is a well-known but unwanted phenomenon in polyester matrices such as poly(D,L-lactic-*co*-glycolic acid) (PLGA) microspheres used as controlled release formulations. Acylation normally occurs on lysine residues and the N-terminus of the peptide. The purpose of the present work is to assess other possible acylation sites on peptides. Goserelin was used as a model peptide that lacks lysine and a free N-terminus, but contains other nucleophilic residues, i.e. serine, tyrosine, histidine and arginine, which potentially can be acylated. Goserelin loaded PLGA microspheres were prepared by a double emulsion solvent evaporation technique. Liquid chromatography ion-trap mass spectrometry (LC-ITMS) was used for determining and monitoring acylation of released goserelin. It is demonstrated that arginine is subjected to acylation with glycolic acid and lactic acid units of PLGA, which was followed by loss of NH_3 from the guanidine group to obtain 2-oxazolin-4-one and 5-methyl-2-oxazolin-4-one residues with masses that are 41 and 55 Da higher, respectively, than the native goserelin. There was no evidence for acylation of serine, tyrosine and histidine in goserelin. Our results demonstrate that beside lysine also acylation of arginine can occur in peptides and proteins that are loaded and released from PLGA matrixes.

1. Introduction

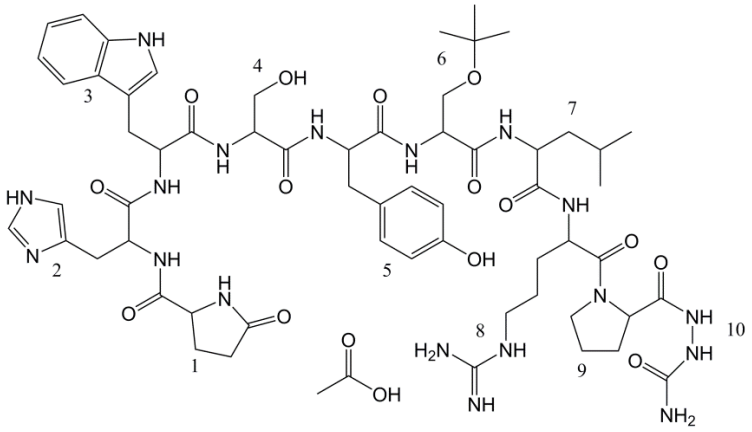
Recent developments in formulation of biopharmaceuticals, e.g. therapeutic peptides and proteins, have increased the need for investigation of the chemical stability of the bioactive molecule during formulation and administration [1-6]. Several commercially available therapeutic peptides, including leuprolide acetate (Lupron Depot®), octreotide acetate (Sandostatin LAR®), and goserelin acetate (Zoladex® implant), are formulated in poly(D,L-lactic-*co*-glycolic acid) (PLGA) polymer [7]. PLGA is a biocompatible and biodegradable polymer and used in FDA-approved drug delivery systems. Although peptides are often assumed to be stable during the manufacturing process [8], interaction between the loaded peptide and polymer may cause unwanted modification of peptides, such as deamidation, acylation and proteolysis [2,9-11]. Peptide acylation is unwanted because it may potentially result in structural changes, loss of activity and even immunogenicity [12].

Acylation involves the nucleophilic attack of peptide residues such as the N-terminus or lysine side chains to the electrophilic carbonyl groups of the lactate or glycolate esters of PLGA. This aminolysis reaction results in the formation of a peptide-PLGA adduct. After hydrolysis of the polyester chain, the released peptide is eventually modified with glycolic or lactic acid units through a hydrolytically stable amide bond. The extent and kinetics of this reaction depends on polymer characteristics and the nucleophilicity of the drug [13-16]. For example, Murty et al. studied the influence of polymer parameters such as lactide/glycolide ratio and found that acylation decreases with increasing lactide composition [15]. Also Ghassemi et al. studied the effect of polymers compositions and showed that formulation of octreotide in microspheres of a hydrophilic aliphatic polyester with pendant hydroxyl groups (i.e. poly(D,L -lactic-*co*-hydroxymethyl glycolic acid, pLHMGA) resulted substantially less acylated adducts. Most likely, increasing water absorption of the particles facilitates the release of formed degradation products, which caused less acidification inside the microspheres during biodegradation [13,16].

Most studies investigating acylation have been carried out on peptides formulated in PLGA microspheres [8,15,17]. It has been shown that the N-terminus and lysine side residues of octreotide are prone to acylation with the N-terminus being most susceptible for acylation [15]. We recently showed that besides the N-terminus and primary amine of lysine in octreotide also the primary OH of the end group of

octreotide was subjected to acylation [18]. Na et al. assessed the acylation reactions of salmon calcitonin (sCT), human parathyroid hormone 1–34 (hPTH1–34) and leuprolide, in PLGA microspheres. They reported that for sCT and hPTH1–34 the primary amine groups of these peptides were acylated inside degrading microspheres, whereas for leuprolide having no primary amines (it contains tyrosine, serine and arginine) no peptide adduct derivatives were detected. Ghalanbor et al. suggested that the free cysteine residue in BSA (Cys34) might undergo acylation via thioester bond formation in PLGA microspheres [19,20].

Goserelin acetate is a potent synthetic decapeptide analogue of luteinizing hormone-releasing hormone (LHRH), also known as a gonadotropin releasing hormone (GnRH) agonist analogue and is clinically used for the treatment of hormone-sensitive prostate cancer [21]. Its chemical structure is pyro-Glu-His-Trp-Ser-Tyr-D-Ser(Bu^t)-Leu-Arg-Pro-Azgly-NH_2 acetate (Fig. 1). Like leuprolide, this peptide neither contains lysine residues nor a primary amine group at the N-terminus, because the N-terminus is a pyroglutamyl residue. However, it contains serine, tyrosine, histidine and arginine as nucleophilic residues. Yet no research has been focused on the possibility of acylation of goserelin formulated in a PLGA matrix. We have recently shown that LC-MS/MS using an ion-trap mass analyser is a powerful tool to investigate in detail the sites of acylation of octreotide [18]. In the present study, we examined the possible acylation of goserelin acetate formulated in PLGA microspheres by this technique.



Pyr-His-Trp-Ser-Tyr-D-Ser(tBu)-Lue-Arg-Pro-Azagly-NH_2

Figure 1. Structural formula of goserelin acetate

2. Materials and methods

2.1. Chemicals

PLGA (5004A: acid terminated with D,L-lactide/glycolide molar ratio 50:50, IV = 0.4 dl/g) was purchased from Purac, The Netherlands. Goserelin acetate was obtained from BCN (Barcelona, Spain). Polyvinyl alcohol (PVA; molecular weight 30,000-70,000; 88% hydrolyzed) was from Sigma-Aldrich, Inc., USA. Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) were obtained from Merck. Sodium azide (NaN_3, Bio Ultra, ≥ 99.5%) was purchased from Sigma (Germany). HPLC and MS grade acetonitrile (ACN), peptide grade dichloromethane (DCM) and tetrahydrofuran (THF) were purchased from Biosolve (The Netherlands). Formic acid was purchased from Sigma-Aldrich Co (Zwijndrecht, the Netherlands).

2.2. Microspheres preparation

Goserelin loaded microspheres were prepared by a double emulsion (W/O/W) solvent evaporation technique [22]. Briefly, 50 µl of goserelin solution in milliQ water (200 mg/ml) was emulsified with 500 µl of dichloromethane solution of PLGA (220 mg, 25% w/w) by using an IKA homogenizer (IKA Labortechnik Staufen, Germany) for 30 s at the highest speed (30,000 rpm) to get the primary emulsion. Next, 500 µl of a PVA solution (1% w/w in 30 mM phosphate buffer, pH 7.4) was added slowly, and the mixture was vortexed for 30 s at 30,000 rpm. The emulsion was subsequently transferred into an external aqueous solution (5 ml) containing PVA 0.5% (w/w) in 30 mM phosphate buffer pH 7.4 while stirring. Continuous stirring at room temperature for 2 h resulted in extraction/evaporation of DCM. Finally, hardened microspheres were collected by centrifugation (Laboratory centrifuge, 4 K 15 Germany) at 3,000 g for 3 min, subsequently washed 3 times with 50 ml RO water and freeze dried at −50 °C and at 0.5 mbar in a Chris Alpha 1−2 freeze-dryer (Osterode am Harz, Germany) overnight. The dried microspheres were stored at −25°C.

2.3. Microspheres characterization

A laser blocking technology (Accusizer 780, Optical particle sizer, Santa Barbara, California, USA) was used to measure the size of the PLGA microparticles. The morphology of the microspheres after freeze-drying was analyzed by scanning electron microscopy using a Phenom™ SEM (FEI Company, the Netherlands). The samples were mounted onto a 12 mm diameter aluminum specimen stub (Agar

Scientific Ltd., England) using double-sided adhesive tape and were sputter-coated with platinum.

The goserelin loading in the microspheres was determined by dissolving about 10 mg of microspheres in a mixture of 1 ml of THF and 1 ml of ACN with gentle shaking. Next, 2 ml of an aqueous solution (0.2% w/v glacial acetic acid, 0.2% w/v sodium acetate and 0.7% w/v sodium chloride in water) was added to precipitate the polymer. The mixture was kept at room temperature for 30 min, and the precipitated polymer was spun down by centrifugation at 5,000 g for 3 min. The peptide content in the supernatant was measured by high performance liquid chromatography (Waters HPLC) using a Sunfire C18 column (5 µm, 4.6 mm × 150 mm). A gradient method was used with mobile phase A (95% H_2O, 5% ACN + 0.1% TFA) and mobile phase B (100% ACN + 0.1% TFA). The eluent linearly changed from 100% A to 100% B in 10 min with a flow rate of 1 ml/min. Goserelin standards (5–100 µg/ml, 20 µl injection volume) were used for calibration, and detection was done at 220 nm.

The loading efficiency (LE) of the peptide in microspheres is reported as the encapsulated peptide divided by the total amount of peptide used for encapsulation. The loading capacity (LC) is defined as encapsulated amount of goserelin divided by dry weight of the microspheres.

HPLC-MS experiments were performed by coupling the HPLC instrument to an Agilent Technologies 6300 Series LC/MSD ion-trap mass spectrometer (Santa Clara, CA, USA). employing electrospray ionization (ESI) in positive ion mode. The eluent was the same as mentioned above except that TFA was replaced by formic acid. The MS settings were: capillary voltage, 2 kV; nebulizer pressure, 60 psi; dry gas flow, 11 L/min; dry gas temperature, 350˚C; a scan range, *m/z* 50–1500. MS^2 and MS^3 experiments were performed using an isolation width of 2 Da and a fragmentation amplitude of 1.0 V.

2.4. In vitro release studies

The goserelin release from PLGA microspheres was studied in PBS (0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05% (w/w) NaN_3, pH 7.4). About 30 mg of microspheres (accurately weighed) were suspended into 1.5 ml of PBS buffer in Eppendorf tubes and incubated at 37°C under mild agitation using a circular mixer (ASSISTANT RM 5). At the different time points, the dispersion was centrifuged (3,000 g, 3 min), and 1 ml of the supernatant was replaced with 1 ml

of fresh buffer. The microspheres were resuspended by gentle shaking, and further incubated at 37°C. The released samples were kept in -20°C until measurement by HPLC and mass analysis.

3. Results and discussion

3.1. Preparation and characterization of goserelin-loaded microspheres

PLGA microspheres loaded with goserelin were successfully prepared using a double emulsion/solvent evaporation technique as described in section 2.2. The microspheres had a volume weighted mean diameter of 21.6 ± 2.8 µm. The loading efficiency and loading capacity of goserelin were 88.0 ± 3.9 % and 3.9 ± 0.2 %, respectively (n=3). SEM analysis (Fig. 2) showed that the goserelin-loaded microspheres were spherical with a smooth and non-porous surface.

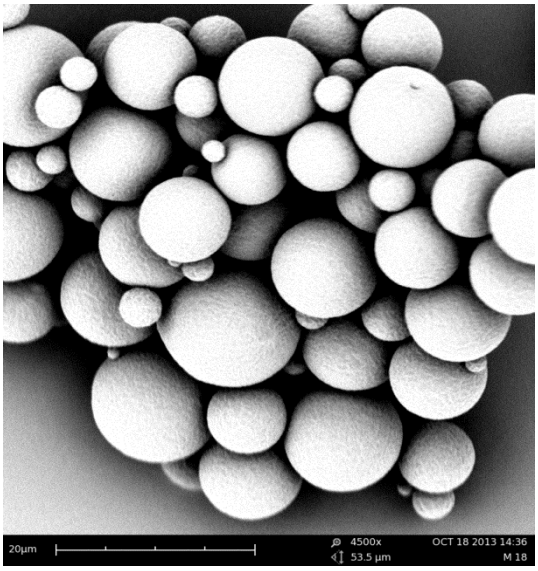


Figure 2. SEM analysis of goserelin loaded PLGA microspheres.

3.2. In vitro release of goserelin loaded microspheres

Figure 3 shows the cumulative *in vitro* release of goserelin from PLGA microspheres in PBS pH 7.4 at 37˚C . No burst release was observed, and after a lag phase of approximately two weeks, 70 % of drug was released in 70 days. The release after the lag time is due to the diffusion of peptide through the water-filled pores that have been formed during degradation, which is the main

reported mechanism for release of macromolecular drugs such as proteins and peptides from PLGA based systems [23]. The release profile of goserelin is comparable with that of octreotide from PLGA [13].

Figure 4A shows the single peptide peak in the HPLC chromatogram of native goserelin. In the chromatogram of goserelin released from PLGA microspheres two additional small peaks with longer retention time were detected (Fig. 4B). Table 1 shows the identification of the observed peaks by LC-MS.

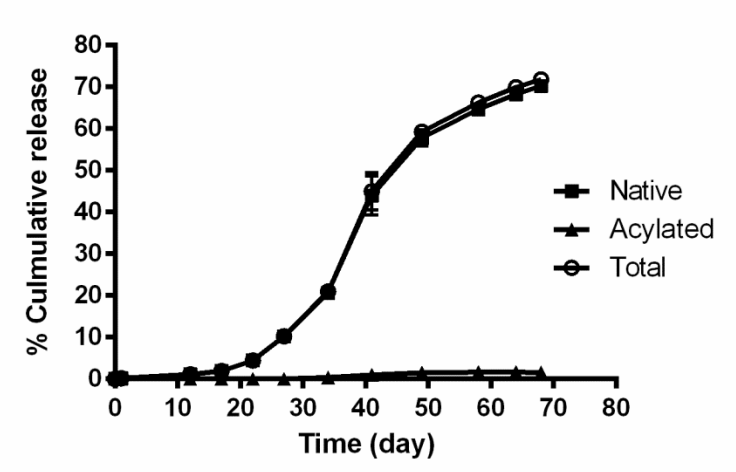


Figure 3. *In vitro* release (n=3) of goserelin from PLGA in PBS pH 7.4 at 37 ºC: native goserelin (squares), acylated goserelin (triangles) and total goserelin (sum of native and acylated adducts, circles).

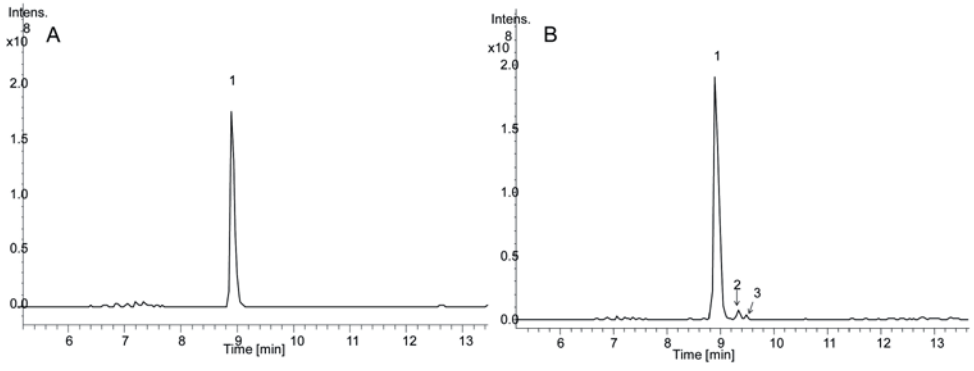


Figure 4. HPLC chromatogram of (A) goserelin in PBS buffer, and (B) after 42 days released from PLGA microspheres.

Table 1. Identification of observed peaks in Figure 4

Peak number	Observed $[M+H]^+$ m/z	Observed $[M+2H]^{2+}$ m/z	Δm	Assigned structure
1	1269.3	635.2	0	Goserelin (native)
2	1310.3	655.7	+41	Goserelin+GA(-17)
3	1324.3	662.7	+55	Goserelin+LA(-17)

MS analysis showed $[M+H]^+$ of the first main peak at m/z 1269.3, which corresponds with the mass of native goserelin (MW=1268.3 Da). Peaks 2 and 3 showed $[M+H]^+$ at m/z 1310.3 and 1324.3, which corresponds with compounds with 41 Da and 55 Da higher mass than the parent compound, respectively (Table 1). In order to obtain detailed information about the site and nature of the observed peptide modification, MS^2 detection was performed on precursor ions with m/z 635.2 (goserelin), 655.7 and 662.7 (modified goserelin). Figure 5 shows the resulting tandem mass spectra. Although the MS^2 analysis of goserelin did not lead to complete fragmentation of the peptide, a close inspection of MS^2 spectra shows two informative ions, i.e. b_7 and b_8 ions. The signal at m/z 941.4 is related to the b_7 ion which is the N-terminus side of the peptide after cleavage of the peptide bond between leucine and arginine. This signal can be observed in all three spectra indicating that all amino acids up to arginine remained intact. The signal at m/z 1097.4 that is seen for native goserelin is the b_8 ion. However, this signal is absent in the spectra of peaks 2 and 3 (Fig. 5A & 5B), and replaced by new signals with incremented mass (+41 and +55 Da). This indicates that the modification occurred at the arginine residue of goserelin. Moreover, also the signal at m/z 328.9 related to y_3 (the C-terminal side of the peptide after cleavage of the peptide bond between leucine and arginine) was shifted to 41 and 55 Da higher mass in the spectra of peaks 2 and 3.

The most intense peak with $[M+2H]^{2+}$at m/z 607.2 in native goserelin (Fig. 5A) is ascribed to the mass of the peptide that lost the t(Bu) (-56 Da) on the serine residue. This signal is observed in spectra of peaks 2 and 3 at m/z 627.8 and 634.8, indicating again modifications with 41 and 55 Da mass increments, respectively. To elucidate the chemical nature of these most intense peaks, they were fragmented in a third MS cycle. Figure 6 shows the HPLC/MS^3 spectra of precursor

$[M+2H]^{2+}$ ions at m/z 607.2 and 627.8 for native and +41 modified goserelin, respectively. The resulting b and y ions of new fragments are in accordance with a loss of 56 Da due to cleavage of t(Bu) from the serine residue. Most importantly, one can nicely see from the new b and y ions that only the y_3 and b_8 ions have the mass increase of +41 Da, which again confirms that modification occurs on the arginine residue. There is no evidence for modification of other amino acids, such as serine, tyrosine and histidine, in goserelin.

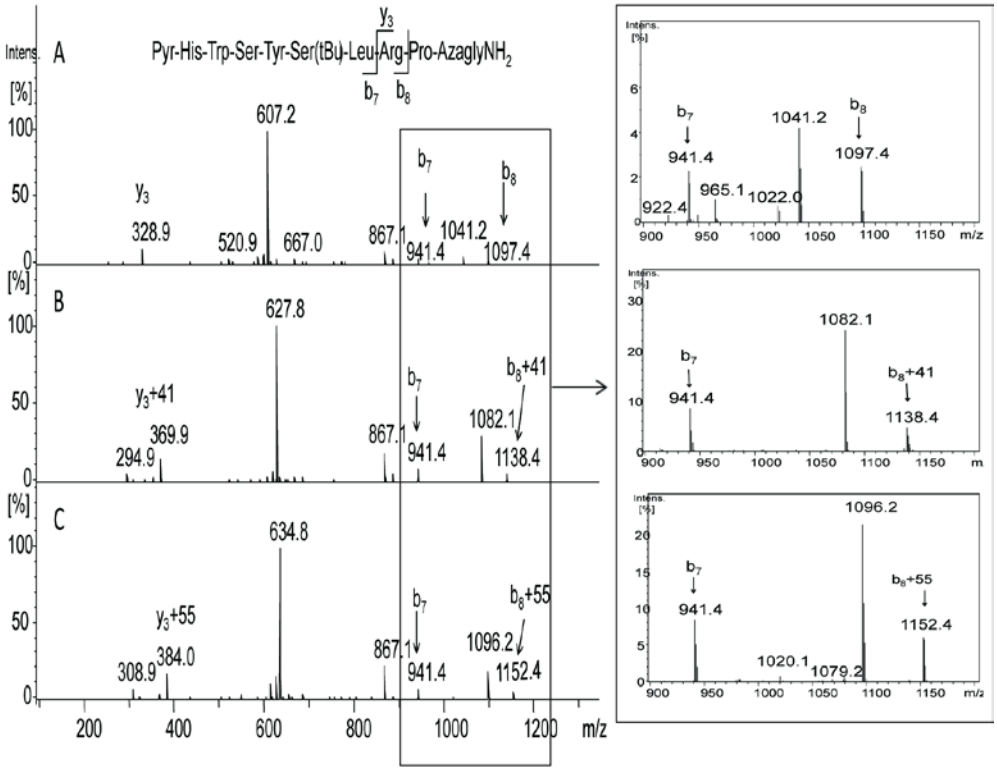


Figure 5. MS/MS spectra of (A) native goserelin (precursor ion, $[M+2H]^{2+}$= 635.2), (B) modified goserelin with precursor ion $[M+2H]^{2+}$= 655.7, (C) modified goserelin with precursor ion $[M+2H]^{2+}$= 662.7.

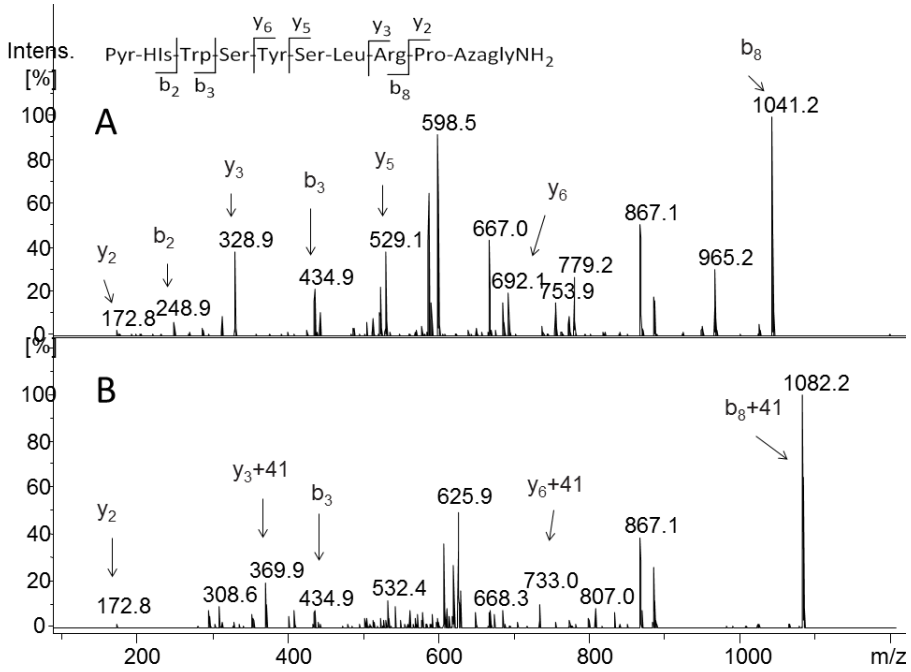


Figure 6. MS^3 spectra of (A) goserelin (precursor ions 635.2 → 607.2), (B) modified goserelin (precursor ions 655.7 → 627.7).

The total mass of the two released goserelin compounds that are modified at the arginine residue differ by 14 Da, which corresponds with the mass difference of glycolyl (58 Da) and lactyl (72 Da) units of PLGA. This implies that goserelin is modified with either a glycolyl or a lactyl unit. However, the total mass increment by this modification is not 58 and 72 Da, respectively, but 17 Da less.

Fig. 7 shows the possible mechanism of the reaction that results in arginine acylation. We propose that the NH of the guanidine group of arginine acts as a nucleophile to attack an electrophilic carbonyl ester in PLGA and substitutes the carbonyl ester causing the formation of an amide bond with the polymer (Fig. 7). Then, as a consequence of the hydrolysis of the PLGA chains, soluble fragments may initially be released that contain short oligomeric remains of the polyester. However, they can hardly or not be detected because these intermediate products will rapidly hydrolyze further to eventually generate the stable single-acylated forms (see Fig. 7) [10]. Finally, the terminal hydroxyl of the glycolyl or lactyl unit adds to the double bond of NH and the stable cyclic form is obtained by subsequent elimination of NH3 (-17 Da), i.e. resulting in 2-oxazolin-4-one (oxazolinone) and 5-methyl-2-oxazolin-4-one (methyl oxazolinone).

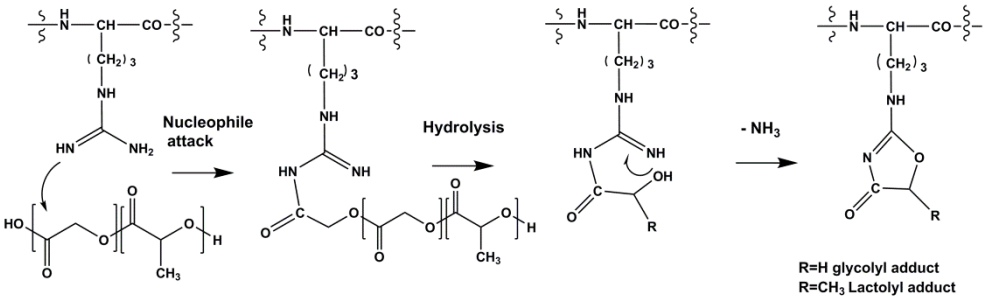


Figure 7. Proposed mechanism of reaction between an arginine residue of a peptide and PLGA.

To the best of our knowledge, this is the first report of acylation of a peptide's arginine in a polyester matrix. Na et al. studied the possibility of acylation of leuprolide formulated in PLGA (50:50, acid terminated) microspheres using capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Leuprolide is also an analogue of gonadotropin-releasing hormone and has a similar chemical structure as goserelin, including arginine (i.e. pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt). However, Na et al. did not detect the formation of acylation products of leuprolide released from degrading microspheres over 28 days [17]. This could be because of the fact that we used an extended release time of up to 70 days. We observed that the acylated peptide only appeared after about 35 days, eventually resulting in 2.2% of the released peptide being acylated.

Although in PLGA the LA/GA molar ratio is 1/1, HPLC analysis showed that the extent of goserelin modification with glycolyl adducts is higher than that with lactyl adducts (approx. 3/1 based on the area's under the curve in the HPLC chromatogram of Fig. 4B). This shows that in PLGA the glycolic acid units are more reactive than the lactic acid units, which is in line with previous studies and can be explained by the steric hindrance of the methyl group in the lactic acid units of PLGA [15,18].

Although the total amount of acylated goserelin (2.2%) is much less than for instance with the 69% acylation that was reported before for octreotide that contains more reactive amine groups of the N-terminal end and lysine [18], our new results show that arginine as a possible site of acylation should certainly be taken into account. The extent of acylation may be influenced not only by the reactivity of the residue, but also by the accessibility and its position in the amino

acid sequence, and might therefore be more pronounced in other peptides or proteins. Arginine is a positively charged amino acid at a neutral pH, whereas acylated arginine is uncharged. This may increase the hydrophobicity of the peptide or protein, leading to changes in structure and function and even possibly to aggregation [24]. Moreover, the oxazolinone ring that is formed in the peptide structure may act as a hapten due to its structural similarity to some reported haptens such as 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one [25] which might induce antibody formation [26,27].

4. Conclusions

Detailed knowledge of peptide modifications in PLGA matrixes is of major importance for safe delivery of peptide and protein drugs because the modification may change the structural and biological activity and/or cause adverse effects. This study shows that acylation of arginine residues can happen, through the formation of a stable covalent cyclic ring, and therefore should always be taken into account as a possible site of modification of proteins/peptides loaded in PLGA matrices.

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Chapter 5

Inhibition of octreotide acylation inside PLGA microspheres by derivatization of the amines of the peptide with a self immolative protecting group

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Submitted for publication

Abstract

Acylation of biopharmaceuticals such as peptides has been identified as a major obstacle for the successful development of PLGA controlled release formulations. The purpose of this study was to develop a method to inhibit peptide acylation in poly(D,L-lactide-co-glycolide) (PLGA) formulations by reversibly and temporarily blocking the amine groups of a model peptide (octreotide) with a novel self immolative protecting group (SIP), *O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate*. The octreotide with two self-immolative protecting groups (Oct*diSIP*) on the N-terminus and lysine side chain was synthesized by reaction of the peptide with *O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate*, purified by preparative RP-HPLC and charachterized by mass spectrometry. Degradation studies of Oct*diSIP* in aqueous solutions of different pH showed that protected octreotide was stable at low pH (pH 5) whereas the protecting group was eliminated at physiological pH, especially in the presence of an esterase, to generate native octreotide. Oct*diSIP* encapsulated in PLGA microspheres, prepared using a double emulsion solvent evaporation method, showed substantial inhibition of acylation as compared to the unprotected octreotide: 52.5% of unprotected octreotide was acylated after 50 days incubation of microspheres in PBS pH 7.4 at 37 °C, whereas Oct*diSIP* showed only 5.0 % acylation in the same timeframe. In conclusion, self immolative protection provides a viable approach for inhibition of acylation of peptides in PLGA delivery systems.

1. Introduction

Biodegradable and biocompatible poly(D,L-lactide-co-glycolide) (PLGA) has been widely used for the design of sustained release formulations for therapeutic peptides and proteins [1-3]. However, the stability of peptides and proteins within PLGA matrices has been identified as a major challenge for the successful development of PLGA controlled release systems [4-6]. PLGA can interact with nucleophiles, such as the N-terminus and primary amine groups of lysine residues of peptides/proteins, resulting in an acylation reaction between the peptide/protein and the polymer. This reaction involves the nucleophilic attack of an amine of the peptide on a lactate or glycolate ester of the polymer resulting in aminolysis, followed by hydrolysis of the conjugated polymer chain. Finally, this results in covalent connection of al glycolyl or lactyl group through an amide bond on the peptide [7,8]. Peptide acylation may potentially result in loss of activity, a change of receptor affinity, or even immunogenicity [9] and should therefore be avoided.

Several methods to minimize peptide acylation within PLGA microparticles have been proposed. Lucke et al. investigated whether a blockcopolymer of poly(ethylene glycol) (PEG) and poly(D,L -lactic acid) (PLA) would reduce peptide acylation since PEG could in principle reduce the accumulation of acid degradation products in the degrading matrices and peptide adsorption onto the PLGA surface. However, this approach did not show a favorable effect concerning peptide acylation inside degrading PLGA microspheres [10]. Houchin et al. studied the effects of pH-modifying excipients, such as a proton sponge compound (1,8-bis-(dimethylamino)naphthalene), magnesium hydroxide, ammonium acetate, and magnesium acetate, on the chemical stability and acylation of a model peptide (VYPNGA) in PLGA films. They showed that addition of these excipients to PLGA formulations does not prevent acylation reactions, and even an increased acylation was observed likely due to the increased nucleophilicity of the peptide amine [11]. Sophocleous et al. suggested that peptide sorption to PLGA is the first step to peptide acylation. They prevented peptide sorption to PLGA by adding a divalent cationic salt to a peptide/PLGA mixture, which indeed resulted in attenuation of acylation [12,13]. In another study from this group, the prevention of acylation of octreotide in the presence of divalent cationic salt inside PLGA microspheres prepared by a double emulsion solvent evaporation method was

investigated. Addition of divalent cationic salts to both the inner and the outer water phase of the double emulsion indeed decreased octreotide acylation: after 28 days, 76.3 % of octreotide was in its native form inside the microspheres without any salt, as compared to 90.9 % that was in its native form using $MnCl_2$ as excipient [14]. In a more recent study [15] they evaluated the effect of addition of divalent cation salts as well as carboxymethyl chitosan (CMCS) on inhibition of acylation of octreotide, salmon calcitonin (sCT), and human parathyroid hormone (hPTH). Divalent cation salts decreased the extent of acylation of octreotide and hPTH but not of sCT. Addition of CMCS alone was ineffective, whereas a combination of inorganic cations with CMCS improved the stability of octreotide and sCT but it had no effect on hPTH stability [15]. From this study it may be concluded that the effects of excipients on inhibition of acylation varied depending on the properties of the individual peptides. Therefore, selective addition of such stabilizers to PLGA formulations needs to be considered on an individual peptide on a case-by-case basis [15]. Feng et al. evaluated the inhibitory effect of dications on the acylation of an acidic peptide (exenatide) outside or inside PLGA microspheres. They showed that Ca^{2+} did not prevent acylation, Mn^{2+} resulted in some inhibition of acylation whereas Zn^{2+} possessed the greatest inhibitory effect [16].

Chemical modification (e.g. PEGylation) of peptide therapeutics is an attractive option to improve their physical stability and resistance to proteases, to reduce immunogenicity, and to increase half-life [17-19]. In some cases, PEGylation may also prevent acylation, however it may cause changes in the affinity of the peptide/protein for the target receptor [20,21]. Na et al. presented a PEGylation strategy to prevent acylation of octreotide. However, PEGylation of the lysine residue of octreotide resulted in significant loss of its biological activity [22,23]. It should be remarked that in this study PEGylated octreotide was incubated with PLGA polymer dispersed in phosphate buffer pH 7.4 instead of encapsulating the PEGylated derivatives in the microparticles.

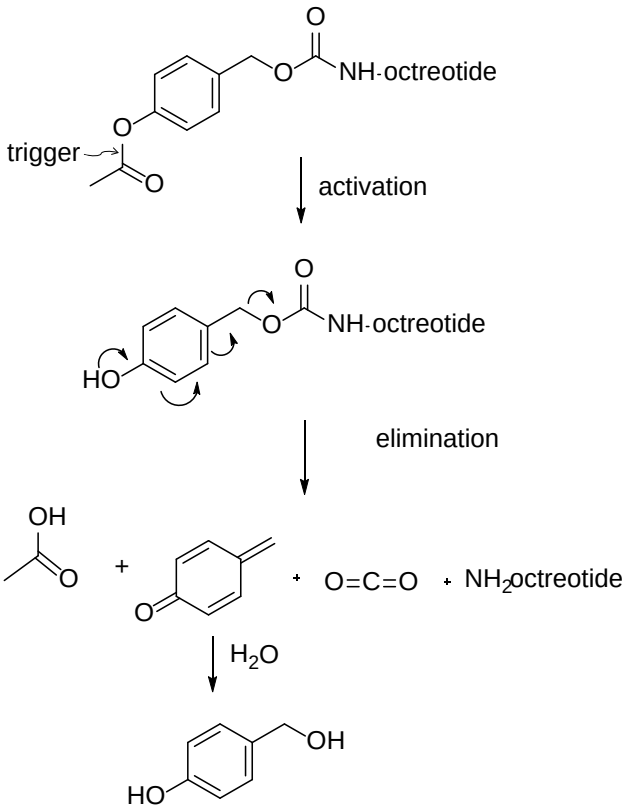
Reversibly or temporarily blocking the amine groups could be an interesting approach to prevent acylation. Ahn et al. investigated reversible blocking of the amine groups of octreotide with maleic anhydride (MA) to prevent its acylation in PLGA particles. The hydrolysis of maleiated amines is acid catalyzed [24]. The hydrolytic degradation of PLGA results in the formation of acid degradation

products that accumulate in the polymer matrix, which results in pH decrease [25,26], and the MA conjugate was because of its acid-sensitivity supposed to be converted into intact peptide that is subsequently released into the surrounding medium. Indeed, a substantial inhibition of the formation of acylated octreotide was observed. However, the maleiated octreotide was released faster than that it was converted into intact octreotide inside the PLGA film [27]. Since de-blocking after release is rather slow at physiological conditions, the possible toxicity of maleiated octreotide should be evaluated. Vaishya et al. reported minimizing octreotide acylation by masking the reactive nucleophilic amine of octreotide with a reversible hydrophobic ion-pairing complex which is stable at acidic pH inside degrading the PLGA particles and can dissociate at physiological pH to yield native octreotide. Dextran sulfate A-octreotide and dextran sulfate B-octreotide complexes that were encapsulated in PLGA microparticles showed <7 % acylation during *in vitro* release in a gel matrix based on a ABCBA pentablock polymer of polylactic acid (A), poly(ε-caprolactone (B) and PEG (C). However, they did not show the extent of acylation of unprotected octreotide as a reference under the same conditions in the gel matrix [28].

Despite to all efforts, none of the approaches described above could fully and safely prevent acylation of peptides in PLGA matrices. Therefore, a new approach for modifying the peptide by an enzyme cleavable/self immolative protecting group is investigated in this paper. Self immolative prodrugs have been widely used to generate an active drug form an inactive precursor/prodrug in the body [29]. Self immolative elimination is the spontaneous and irreversible fragmentation of a compound into its building blocks through a cascade of electronic elimination processes. This phenomenon is most commonly observed for electron-rich aromatic species. The electron donating substituent is required to lower the energy barrier of de-aromatisation [30].

This study aims for the synthesis of a new self immolative group and its attachment to the amine sites the therapeutic peptide octreotide. The structure of the investigated group is shown in scheme 1. The design of this protecting group is such that it is stable at acidic pH (inside degrading PLGA microspheres) and is de-protected either by enzymatic hydrolysis by esterases or due to the higher pH outside the microspheres. The first trigger step, which is the hydrolysis of the acetate group, is rate-limiting in the generation of the parent octreotide, as

the remaining part of the self immolative group is rapidly eliminated and split into non-toxic compounds like carbon dioxide, acetic acid and 4-hydroxy-benzyl alcohol (scheme 1), which are safe and non-toxic compounds [31]. Octreotide was used as a model peptide to protect its most susceptible sites of acylation (the N-terminus and lysine residue [32]) using this self immolative protecting group.



Scheme 1. Self immolative conversion of Oct*diSIP* into native octreotide

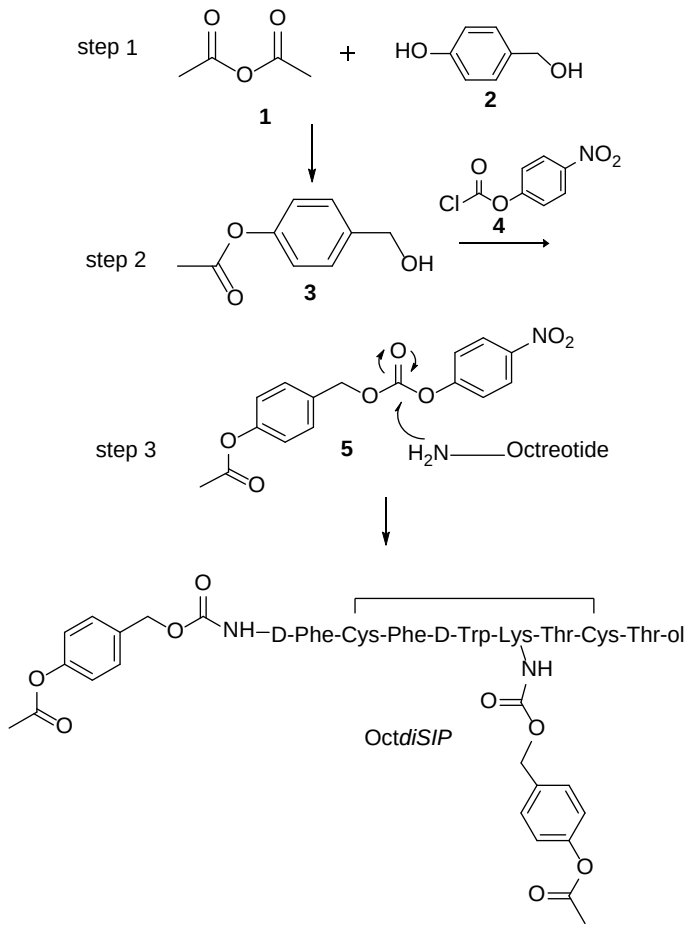
2. Materials and methods

2.1. Chemicals

All materials were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise and used without further purification. Octreotide acetate (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol, molecular weight of 1019.2 Da) was obtained from Biorbyt (USA). 4-Hydroxybenzylalcohol was obtained from Aldrich (Switzerland), 4-nitrophenyl chloroformate was purchased from Sigma (China) and triethylamine from Sigma-Aldrich (Belgium). Polyvinyl alcohol (PVA; MW 30,000-70,000; 88% hydrolyzed) and esterase from porcine liver was obtained from Sigma-Aldrich (USA). Disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) were obtained from Merck. Sodium azide (NaN_3, Bio Ultra, ≥ 99.5%) was purchased from Sigma (Germany). HPLC and MS grade acetonitrile (ACN), peptide grade dichloromethane (DCM), dimethylformamide (DMF), tetrahydrofuran (THF) and ethyl acetate were purchased from Biosolve (The Netherlands). PLGA (acid terminated 5004A with D,L-lactide/glycolide molar ratio of 50:50, IV = 0.4 dl/g) was obtained from Purac.

2.2. Synthesis of self immolative protecting group

Scheme 2 shows the pathway of the synthesis of the protecting group. The chemical structures of intermediate and final compounds were confirmed by ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) analysis measured on an Agilent 400-MR NMR spectrometer (Agilent Technologies, Santa Clara, USA). Copies of all NMR spectra, UPLC chromatograms and MS spectra can be found in the supporting information section.



Scheme 2. Synthesis of the self immolative protecting group and its conjugation to octreotide.

2.2.1. *4*-Acetoxybenzyl alcohol (3)

4-Hydroxybenzyl alcohol **2** (0.62 g, 5 mmol) and triethylamine (0.4 mL, 3 mmol, 0.6 eq.) were dissolved in THF (13 mL) which was dried over 3 Å molecular sieve, and purged with dry nitrogen for 15 min. The solution was cooled on ice and acetic acid anhydride **1** (0.57 mL, 6 mmol, 1.2 eq.) followed by triethylamine (0.4 mL, 3 mmol, 0.6 eq.) were added drop wise during 3 min. The mixture was stirred at 0 °C under nitrogen atmosphere till the spot of 4-hydroxybenzyl alcohol could not be detected on TLC (~30 min). The solvent was evaporated and the residue was redissolved in ethyl acetate (50 mL). The organic layer was washed with brine

three times and dried over $MgSO_4$, filtered and evaporated to dryness. The residue was purified by silica gel chromatography, using ethyl acetate/n-hexane (9:1 v/v) as eluent. Alcohol **3** was a faint yellow oil and was obtained in 52% yield. ^{1}H NMR (CD_3CN): δ = 2.28 (s, 3H, OC(O)-CH_3), 4.61 (s, 2H, CH_2O), 7.09 (d, 2H, -C_6H_4), 7.41 (d, 2H, -C_6H_4) (Figure S1, supporting information); ^{13}C NMR (CD_3CN): δ = 169.77, 149.94, 139.69, 127.81, 121.64, 63.24, 20.33. (Figure S2).

2.2.2. O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate (5)

4-Nitrophenyl chloroformate **4** (1.1 g, 5.5 mmol, 1.1 eq.) dissolved in 3 mL dry accetonitril was added drop wise during 20 min to a stirred solution of 4-acetoxybenzyl alcohol **3** (0.83 g, 5 mmol) and triethylamine (0.8 mL, 6 mmol, 1.2 eq.) in anhydrous acetonitrile (10 mL). The obtained reaction mixture was stirred at room temperature under nitrogen atmosphere for 1 h, during which triethylamine hydrochloride precipitated from the reaction mixture. Subsequently, ethyl acetate (50 mL) was added and the obtained solution was washed with cold water (3×15 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to yield the crude product as a white solid, which was recrystallized from ethyl acetate/petroleum ether in 60% yield [33]. Melting point was determined at 114.7 ^{o}C using differential scanning calorimetry (DSC) Q2000 apparatus (TA Instruments, New Castle, DE, USA), (Figure S5). ^{1}H NMR (CD_3CN): δ = 2.27 (s, 3H, OC(O)-CH_3), 5.30 (s, 2H, -CH_2OC(O)O), 7.16 (d, 2H, -C_6H_4OAc), 7.46 (d, 2H, -C_6H_4OAc), 7.51 (d, 2H, -$C_6H_4NO_2$), 8.30 (d, 2H, -$C_6H_4NO_2$) (Figure S3).; ^{13}C NMR (CD_3CN): δ = 169.49, 155.66, 152.46, 151.25, 145.65, 132.54, 129.84, 125.30, 122.26, 70.00, 20.25 (Figure S4).

2.2.3. Conjugation of the protecting group (compound 5) to octreotide

A solution of compound **5** (16 mg, 0.05 mmol in 0.5 mL DMF) was added drop wise to a mixture of octreotide (20 mg, 0.02 mmol) and triethylamine (0.015 mL, 0.11 mmol) in DMF (0.5 mL) during 10 min. The obtained reaction mixture was stirred at room temperature for 24 h. During this period of stirring, the reaction mixture turned yellow due to formation of 4-nitrophenol. Preparative HPLC (Akta purifier) was used for purification of the desired product. Separation was carried out on a C-18 column (19×150mm, 5µm, Sunfire). A linear gradient elution was run from 30% to 60% (v/v) acetonitrile in water, each containing 0.1% (v/v) TFA, for 27 min. The flow rate was 10 mL/min, and UV detection at λ 280 nm was used

for monitoring the separation. The collected fractions were identified by direct infusion into an ion-trap mass spectrometer (Agilent 1100 Series LC/MSD SL) equipped with an electrospray ionization source (Agilent Technologies) using a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). The fraction of interest, i.e. octreotide with two self-immolative protecting (SIP) groups (Oct*diSIP*), was collected, freeze-dried and molecular weight was measured by mass spectrometry (Figure S6B). The yield was 21 %. The purity of the obtained Oct*diSIP* was calculated by UPLC analysis from the areas under the curve of the UPLC chromatogram (Figure S6A).

2.3. Hydrolytic and enzymatic deprotection of Oct*diSIP*

The hydrolytic deprotection of Oct*diSIP* was studied in aqueous solutions of pH 5.0, 7.4 and 9.0 using acetate buffer for pH 5.0 and phosphate buffer for pH 7.4 and 9.0. The buffer concentration was 100 mM and the ionic strength was adjusted to 0.3 with sodium chloride. A solution of Oct*diSIP* (75 µg/mL) was prepared in buffer:acetonitrile 2:1 (v/v), divided in different Eppendorf tubes, and incubated at 37 °C with mild shaking. At selected time intervals, samples were taken and stored in a freezer at –20 °C in order to prevent further hydrolysis prior to analysis with UPLC. The degradation rate constant k_{obs} was determined from the slope of the plot of the natural logarithm of the residual Oct*diSIP* concentration versus time.

To evaluate the instability of the protecting group in the presence of an esterase, Oct*diSIP* was incubated at pH 7.4 with porcine liver esterase. In detail, 4.2 µL from a stock solution of 1 mg/mL of porcine esterase in PBS 7.4 was added to a 1 mL of a solution of 15 µg/mL of Oct*diSIP* in PBS pH 7.4 that also contained 3% acetonitrile to solubilize the peptide. After one minute of incubation at 37 °C the solution was analyzed by UPLC measurement. Prior to investigation of the esterase-catalyzed conversion of Oct*diSIP*, the enzymatic activity of porcine liver esterase in PBS buffer and in the presence of 3% acetonitrile was determined by measuring the enzyme catalyzed hydrolysis of p-nitrophenyl acetate (pNPA) to p-nitrophenol (pNP), which has an absorption maximum about 405 nm, using a Shimadzu UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Enzyme assays were carried out in 100 mM PBS pH=7.4 at 37 °C. A pNP dilution series was used for calibration. One unit of the enzyme is defined as the amount of enzyme that releases 1 µmol p-nitrophenol per min in pH 7.4 at 37 °C [34].

2.4. Preparation and characterization of PLGA microspheres loaded with Oct*diSIP* or octreotide

PLGA microspheres loaded with octreotide or Oct*diSIP* were prepared by a double emulsion (W/O/W) solvent evaporation technique [32]. Briefly, 10 mg of an octreotide solution in 50 µL milliQ water was emulsified in 500 µL of dichloromethane (DCM) solution of PLGA (220 mg, 25% w/w) by using an IKA homogenizer (IKA Labortechnik Staufen, Germany) for 30 s at the highest speed (30,000 rpm) to get the primary emulsion. In the case of Oct*diSIP*, which has a poor solubility in water due to the hydrophobic protecting groups, 10 mg of Oct*diSIP* was dissolved in 50 µL of 50:50 (v/v) water/DMF prior to emulsification in the organic solution. Next, 500 µL of a PVA solution (1% w/w in 30 mM phosphate buffer, pH 7.4) was added slowly, and the mixture was vortexed for 30 s at 30,000 rpm. The emulsion was transferred into an external aqueous solution (5 mL) containing PVA 0.5% (w/w) in 30 mM phosphate buffer pH 7.4 while stirring. Continuous stirring at room temperature for 2 h resulted in the extraction and evaporation of DCM. Finally, hardened microspheres were collected by centrifugation (Laboratory centrifuge, 4 K 15 Germany) at 3,000 g for 3 min, subsequently washed 3 times with 100 mL RO water and freeze dried at −50 °C and at 0.5 mbar in a Chris Alpha 1−2 freeze-dryer (Osterode am Harz, Germany) for 16 h. The dried microspheres were stored at −25 °C.

The size of the microspheres and the size distribution were analyzed by a laser blocking technology (Accusizer 780, Optical particle sizer, Santa Barbara, California, USA) after dispersing the freeze dried microspheres in water.

The octreotide or Oct*diSIP* loading was determined by dissolving about 10 mg of microspheres (accurately weighted) in 2 mL of acetonitrile with gentle shaking. Subsequently, 2 mL of solution of 0.2% w/v glacial acetic acid, 0.2% w/v sodium acetate and 0.7% w/v sodium chloride in water was added to precipitate the polymer. Next, the mixture was incubated at room temperature for 20 min, and the precipitated polymer was spun down by centrifugation at 5,000 g for 2 min. The octreotide or Oct*diSIP* content in the supernatant was measured by ultra-performance liquid chromatography (UPLC, *vide infra*). Loading efficiency (LE) of the peptide in the microspheres is reported as the encapsulated peptide divided by the total amount of peptide used for encapsulation. Loading capacity (LC) is

defined as the encapsulated amount of peptide divided by dry weight of the microspheres.

2.5. Peptide stability inside PLGA microspheres

For stability study of the peptides inside the microspheres, freeze-dried microspheres (4-10 mg) were suspended in 1.5 mL PBS (0.033 M NaH_2PO_4, 0.066 M Na_2HPO_4, 0.056 M NaCl and 0.05% (w/w) NaN_3, pH 7.4) in Eppendorf tubes. The samples were incubated at 37 °C while gently shaken. At different time points, three tubes were taken, and the microspheres were collected after centrifugation at 3000 × g for 5 min and washed with 1 mL of SDS 1 % to remove the released peptide that was associated with the particles, then washed again with 1 mL PBS buffer pH 7.4. Subsequently, the microspheres were dissolved in acetonitrile, and the peptides retained in the microspheres were analyzed similarly as for peptide loading as mentioned above.

2.6. UPLC analysis

UPLC analysis was carried out on a Waters ACQUITY UPLC® system using an ACQUITY BEH 300 C18 column (1.7 µm, 2.1 mm × 50 mm). A gradient elution method was used with a mobile phase A (95% H_2O, 5% acetonitrile + 0.1% TFA) and a mobile phase B (100% acetonitrile + 0.1% TFA). The eluent linearly changed from 95 % A to 100 % B in 7.5 min with a flow rate of 0.25 mL/min. Octreotide standards (5–100 µg/mL, 7 µl injection volume) were used for calibration, and detection was done using fluorescence with setting excitation at λ 280 nm and emission at λ 330 nm. Moreover, the UV detection at λ 210, 260 and 280 nm, respectively, was also recorded.

UPLC-MS experiments were performed by coupling the UPLC instrument to an Agilent Technologies 1100 Series LC/MSD SL ion-trap mass spectrometer employing electrospray ionization (ESI) in positive ion mode. The eluent was the same as mentioned above except that TFA was replaced by formic acid. The MS settings were: capillary voltage, 2 kV; nebulizer pressure, 60 psi; dry gas flow, 11 L/min; dry gas temperature, 350 °C; a scan range, *m/z* 50–2000.

3. Results and discussion

3.1. Synthesis and characterization of self-immolative protected octreotide

As shown in scheme 2, the first step in the synthesis of the protecting group was *O*-acetylation of the phenol group of 4-hydroxybenzyl alcohol to yield 4-acetoxybenzyl alcohol. The yield was 52 % and the identification was carried out by NMR analysis (Figure S1 and S2, supporting information). Subsequently, the self-immolative protecting group (SIP, *O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate*, compound **5**) was successfully synthesized by the reaction of the 4-acetoxybenzyl alcohol with 4-nitrophenyl chloroformate (step 2). SIP was obtained in a yield of 60 % and characterized by NMR (Figure S3 and S4). A single sharp melting point was observed by DSC at 114.7 °C (ΔH=87.1 J/g) (Figure S5). To the best of our knowledge, this compound has not been synthesized before. The carbonate intermediate in compound **5** is highly reactive toward amines because it provides a good leaving group (4-nitrophenol) to form a carbamate bond between an amine group of octreotide and carbonate of SIP. Thus, compound **5** was conjugated to octreotide by substitution of nitrophenyl carbonate by the two primary amines of the peptide, i.e. the N-terminus and the ε-amine of the lysine residue. A mixture of products was obtained, including di-protected peptide and some byproducts. The desired di-protected octreotide (Oct*diSIP*) was isolated by preparative HPLC in a yield of 21 % and with a purity of >95% according to the areas under the curve of its UPLC chromatogram (Figure. S6). The product was identified by mass spectrometry and showed the correct molecular weight of 1403 Da.

3.2. *In vitro* hydrolytic and enzymatic degradation of Oct*diSIP*

To study the rate of the conversion of the Oct*diSIP* into native octreotide, the kinetics of the hydrolytic degradation of Oct*diSIP* was determined in (2:1 v/v) water/acetonitrile mixture at different pH values at 37 °C. It should be mentioned that acetonitrile was added as cosolvent since Oct*diSIP* has a very low solubility in water. Figure 1 shows the UPLC chromatograms of the incubation mixtures at pH 7.4 at 0, 48 and 120 hours, respectively. The main degradation products that gradually appeared in time were identified by UPLC-MS as being the two expected mono-protected peptides, with the remaining SIP on either the N-terminus or the ε-amine of the lysine residue (Oct*monoSIP*, *m/z* 1212), and native octreotide (*m/z*

1020). However, minor peaks were observed that were identified by MS as octreotide with one acetyl group (acetylated octreotide, *m/z* 1062) and octreotide with one SIP and one acetyl group (two isomers of acetylated Oct*monoSIP, m/z* 1256: acetyl on N-terminus with SIP on lysine or SIP on N-terminus with acetyl on lysine) (Table 1). This can be explained by aminolysis of an acetate group of a remaining SIP unit by a free amine that is formed after deprotection (either by an inter or an intra molecular reaction). Scheme 3 depicts the full reaction scheme of degradation of Oct*diSIP* in neutral or basic media. The two isomeric peptides with one acetyl and one SIP group (acetylated Oct*monoSIP*) are obtained by reaction between an amine of Oct*monoSIP* and a SIP-acetate group from another peptide molecule (Scheme 3: k_5, k_6). After deprotection of the remaining SIP unit (k_7, k_8) these intermediate isomers will finally result in mono-acetylated octreotide. On the other hand, intramolecular reaction of the amine of Oct*monoSIP* with the remaining SIP on the same peptide will directly result in mono-acetylated octreotide (k_{11} and k_{12} in scheme 3, see scheme 4 for the proposed reaction mechanism). Also, reaction of native octreotide with the acetate group of protected octreotide (k_9, k_{10}), will finally result in the formation of acetylated octreotide as the main byproduct.

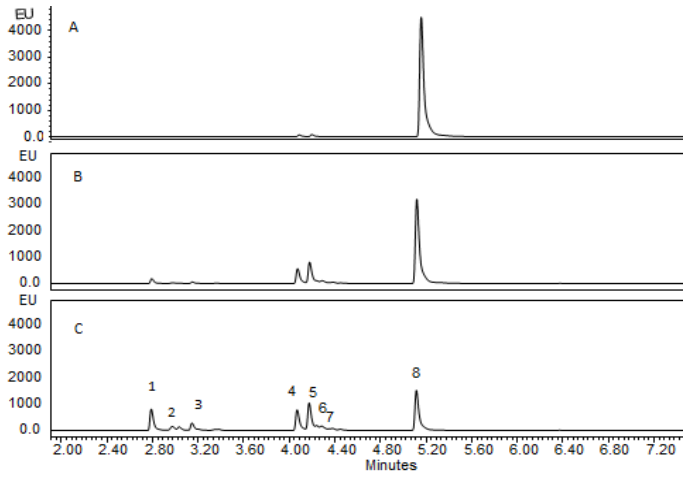
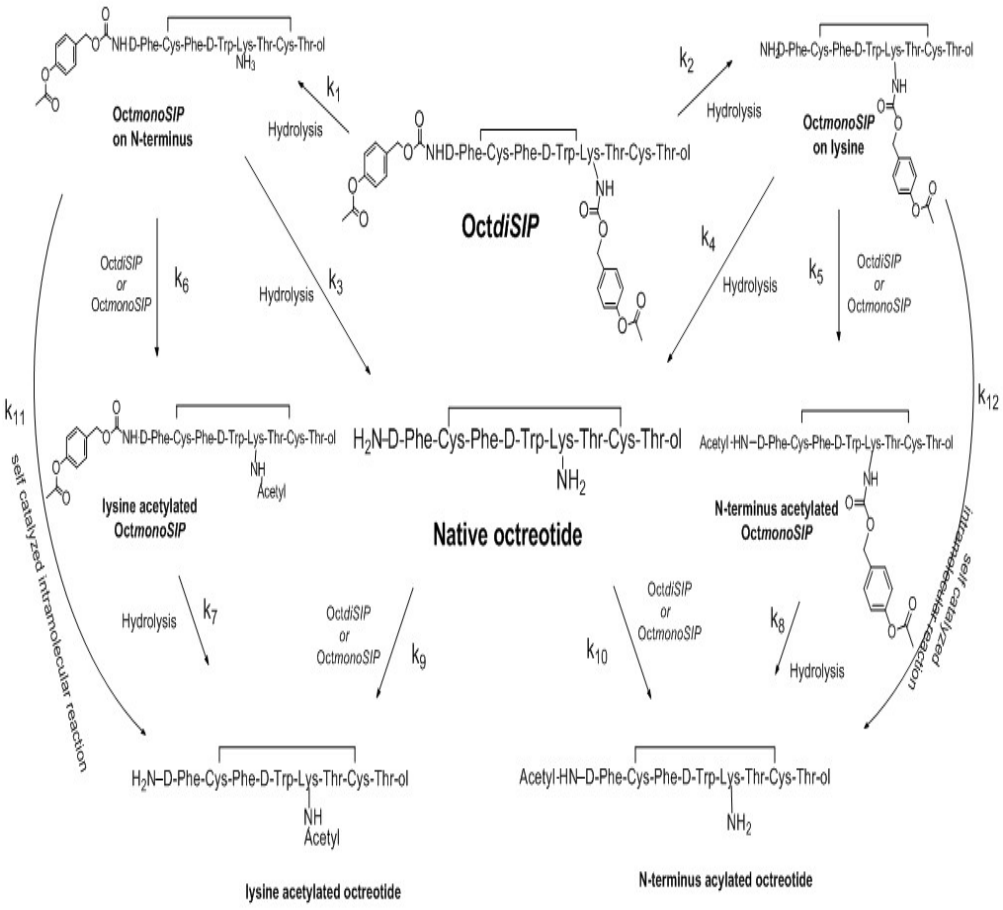


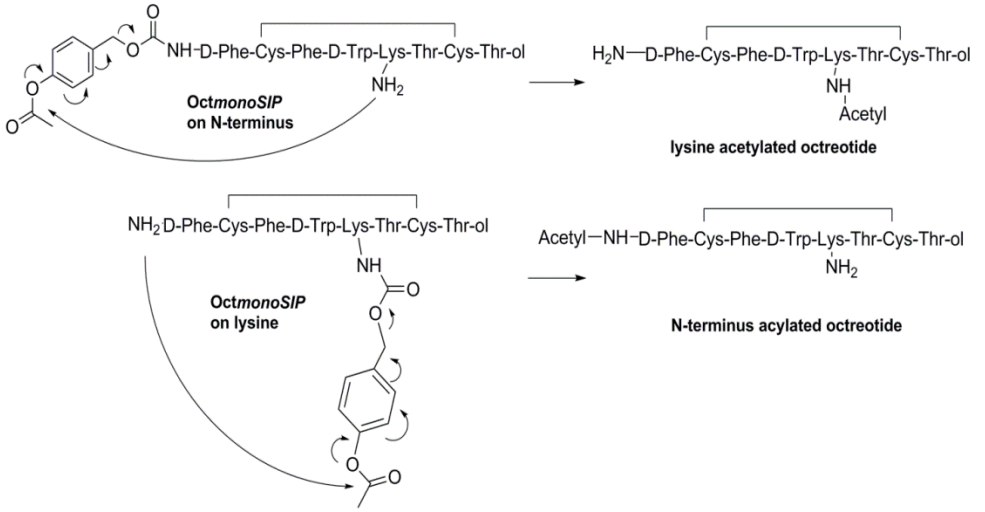
Figure 1. UPLC of 75 µg/mL Oct*diSIP* incubated in buffer:acetonitrile (2:1) (v/v) at pH 7.4 and 37 °C : A) t=0 B) t=48 h and C) t=120 h. Fluorescent detection was used with excitation at λ = 280 nm and emission at λ = 330 nm. The identification of the numbered peaks is shown in Table 1.

Table 1. Identification of the observed peaks in Figure 1 by UPLC-MS.

Peaks number	Observed $[M+H]^+$ *m/z*	Δm (Da)	Assigned structure
1	1020	0	octreotide (native)
2, 3	1062	+42	acetylated octreotide
4, 5	1212	+192	Oct*monoSIP*
6, 7	1254	+234 (192+42)	acetylated Oct*monoSIP*
8	1404	+384 (192+192)	Oct*diSIP*



Scheme 3. Scheme of proposed degradation pathway for Oct*diSIP* in neutral or basic media.



Scheme 4. Proposed mechanism of intramolecular aminolysis.

The effect of pH on the degradation kinetics and hydrolysis rate constants of Oct*diSIP* was analyzed by monitoring the degradation of Oct*diSIP* in aqueous solution at pH 5.0, 7.4 and 9.0 by UPLC (Figure 2). Oct*diSIP* is quite stable in pH 5 (Figure 2A) while the conversion into Oct*monoSIP*, native octreotide and the other byproducts occurs at elevated pH (7.4 and 9.0; Figure 2B and 2C, respectively). These figures also show that the amount of the mono-protected intermediate products first increases and then decreases in time because it is formed from hydrolysis of one of the protecting groups at either the N-terminus or lysine and are subsequently converted into native octreotide or one of the acylated products. Eventually, the final degradation products are native octreotide and acetylated octreotide, which are formed in almost equal amounts (Figure 2B and 2C). Table 2 shows the rate constants of the degradation of Oct*diSIP* (C=75 µg/mL in buffer:acetonitrile 2:1) at different pH at 37 °C. This table shows that the rate constant at pH 5.0 is much lower than at neutral and basic pH. The half-life of Oct*diSIP* in pH 5.0 at 37 °C s estimated to be 1050 hours in comparison with 63 hours at pH 7.4. Ester hydrolysis is catalyzed by H^+ or OH^- ions, and for that reason the trigger to release the SIP group by hydrolysis of the acetate unit is pH dependent, with the lowest hydrolysis rate at around pH 5.0. This is in line with general principle of ester hydrolysis with the minimum rate constant at ~ pH 4.5 [35,36].

It must be stressed that the values reported in table 2 are the rate constants for hydrolysis in water/acetonitrile (2:1) mixtures. The Influence of the dielectric constant (ϵ) of the medium on the k_{obs} of ester hydrolysis was studied by de Jong et al. [37]. They have shown higher k_{obs} values at higher dielectric constant of the medium ranging from ϵ = 47.7 to 68.3. The dielectric constant of water: acetonitrile (2:1) that we used in our studies was 64.8 according to the formula ϵ = ($\epsilon_{acetonitrile}$ × acetonitrile (%) + ϵ_{water} × H_2O (%))/100, with $\epsilon_{acetonitrile}$ = 37.5 and ϵ_{water} = 78.5. Assuming the same dependence of ϵ on the degradation rate constant as found by de Jong et al. [37] for oligo(lactic acid) hydrolysis, we anticipate that the rate constant of Oct*diSIP* is 6.3-fold higher in water than in water: acetonitrile 2:1. The reaction rate constants and half-life times in both media are summarized in Table 2. We thus estimated the half life time of Oct*diSIP* to be around 10 h at physiological pH in water.

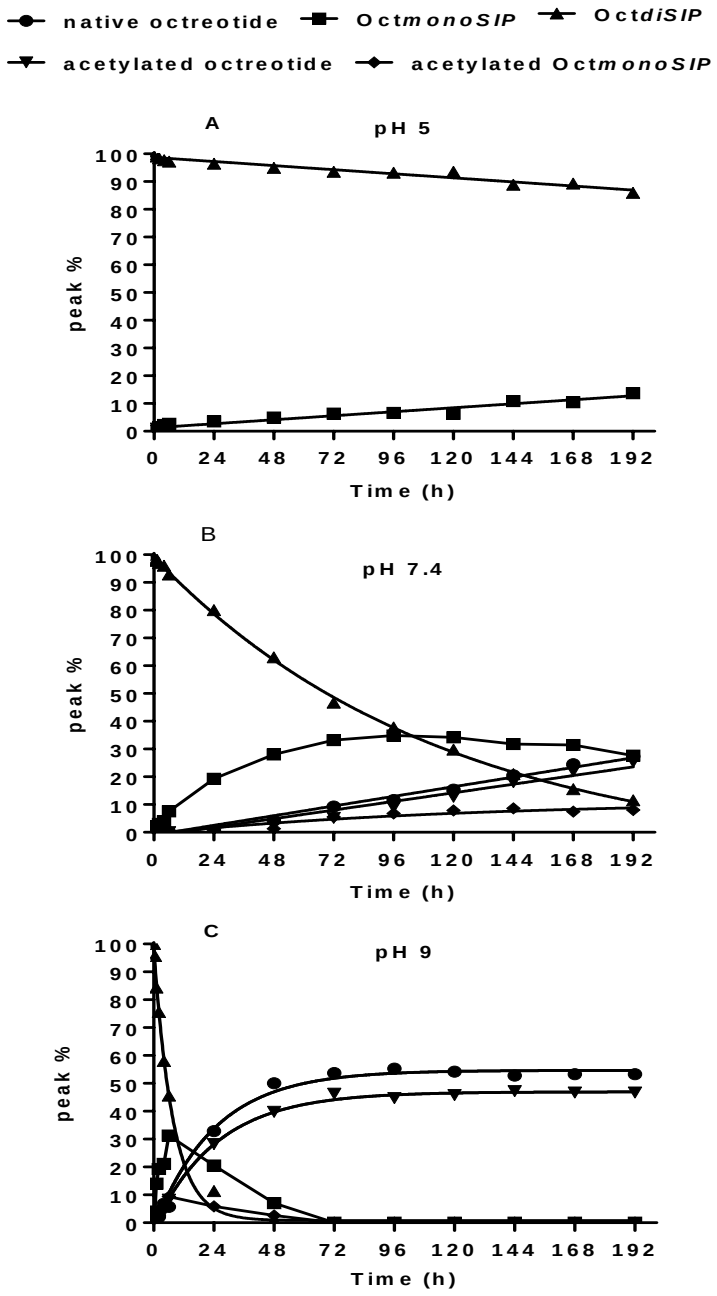


Figure 2. Degradation profile of Oct*diSIP* (75 µg/mL buffer:acetonitrile (2:1)) at (A) pH 5, (B) pH 7.4 and (C) pH 9.

Table 2. Reaction rate constants of degradation of Oct*diSIP (*c=75 µg/mL) at 37 °C at different pH.

pH	Buffer:acetonitrile (2:1) (measured)		Buffer (calculated)	
	k (h^{-1})	$t_{1/2}$ (h)	k (h^{-1})	$t_{1/2}$ (h)
5.0	0.66×10^{-3}	1050	4.16×10^{-3}	160
7.4	11.0×10^{-3}	63	69.3×10^{-3}	10
9.0	93×10^{-3}	7.4	590×10^{-3}	1.2

Importantly, the pH value inside degrading PLGA microspheres was reported to be approx. 4 [38-40]. For successful application of Oct*diSIP* to minimize acylation of peptide inside degrading PLGA microspheres, the stability of the protecting group at lower pH (Fig. 5A) is a clear advantage. In the ideal case, the protecting group must be eliminated completely after release to yield native octreotide. The half-life time of Oct*diSIP* in pH 7.4 is around 63 hours in buffer:acetonitrile 2:1 and estimated to be 10 h in buffer, although the formation of the acetylated byproducts was unexpected and is not desired. However, esterases present in the body can affect the conversion by catalyzing the hydrolysis of the acetate group of the SIP units [41], and thereby trigger the deprotection. Therefore, an enzymatic degradation study was done, in which we reduced the amount of cosolvent to 3 % acetonitrile to minimize enzyme inactivation (as compared to the hydrolytic degradation study where 33% acetonitrile was used). Under these conditions, the enzymatic activity was determined to be 5 units/mg, in comparison with 12.5 units/mg in PBS. The esterase (4.2 µg or 0.02 units) was added to 15 µg (0.01 µmol) of Oct*diSIP* in 1 mL PBS buffer pH 7.4 containing 3 % acetonitrile, an amount of enzyme in principle is suitable to hydrolyze the two protecting groups in octreotide (one unit of enzyme is able to hydrolyze one µmol ester bond per minute at 37 °C in PBS buffer pH 7.4 containing 3 % acetonitrile, so 0.02 unit which is also a physiologically relevant concentration [42] is able to hydrolyze 0.02 µmol ester bond, one mol of Oct*diSIP* contains two ester bonds). Figure 3 shows the relative peak area's in the UPLC chromatogram of Oct*diSIP* before and after addition of the esterase. The conversion was indeed complete after 1 min in pH

7.4 at 37 °C after addition of the enzyme and most importantly, without formation of any acetylated octreotide as opposed to the hydrolytic degradation study presented above. This is an important observation, which means that because of the carboxyl esterase in the body [42] the conversion is expected to be completely to native octreotide.

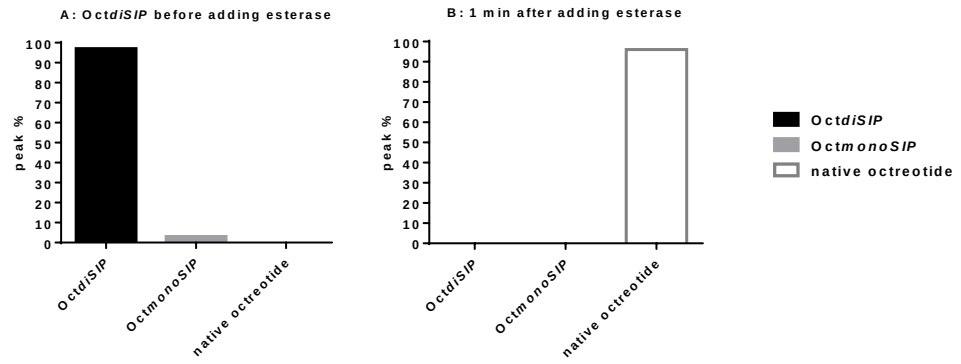


Figure 3. Relative peptide content A) before adding esterase and B) 1 min after adding esterase to a solution of Oct*diSIP* in PBS pH 7.4 containing 3% acetonitrile.

3.3. Preparation and characterization of microspheres

PLGA microspheres loaded with octreotide or Oct*diSIP* were prepared using a double emulsion/solvent evaporation technique. The average particle sizes of octreotide and Oct*diSIP* loaded microspheres were 23.5 ± 0.6 µm and 46.9 ± 11.2 µm, respectively. The Oct*diSIP* microspheres had a bigger particle size probably due to the addition of 50% DMF to the OctdiSIP solution to increase the solubility of the peptide. The loading efficiencies were 86.5 ± 2.4 % and 61.3 ± 6.7 % and loading capacities were 3.9 ± 0.1 % and 3.0 ± 0.3 % for octreotide and Oct*diSIP*, respectively (n=3).

3.4. Stability of Oct*diSIP* in the PLGA microspheres

To determine the stability of Oct*diSIP* inside the PLGA microspheres, the peptide and its degradation products were extracted from the microspheres at different time points of incubation in PBS buffer pH 7.4 at 37 °C. The extracted samples were analyzed by UPLC and MS and compared with peptides that were extracted from microspheres containing unprotected octreotide. Figures 4A and 4B show

the chromatograms of peptides still loaded in microspheres upon extraction from the PLGA microspheres after 50 days. Figure 4A shows a main peak eluting after approx. 2.6 min which corresponds to native octreotide, while extra peaks with longer retention times emerged that were attributed by MS analysis to acylated octreotide (glycolyl and lactyl adducts). Figure 4B shows the main peak eluting after approx. 5 min that corresponds to remaining Oct*diSIP*, while some conversion to Oct*monoSIP* (around 4 min) and native octreotide (2.6 min) had occurred and, as opposed to unprotected octreotide, only some small peaks related to acylation (i.e. glycolyl and lactyl adducts) with retention times of 3 min (majority glycolyl octreotide) and 4.3 min (glycolyl Oct*monoSIP*) were observed. Importantly, no acetylated compounds (like those that emerged in the hydrolysis study presented above) could be detected.

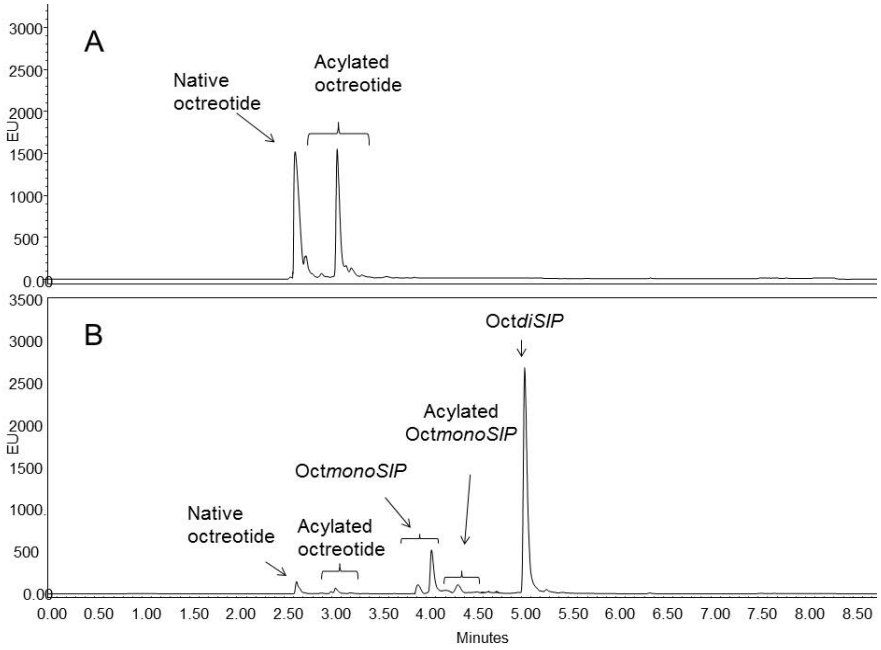


Figure 4. UPLC chromatograms of extracted peptides from PLGA microspheres after 50 days incubation in PBS buffer pH 7.4 at 37 °C: A) Octreotide and B) Oct*diSIP* loaded microspheres.

Figure 5 shows the relative peak areas of the peptides that were extracted from degrading PLGA microspheres loaded with Oct*diSIP*, at different time points. The

peptides that remained in the PLGA particles mostly consisted of the fully protected form (76.4 % after 50 days) and some monoprotected degradation products (13.5 % after 50 days). Native and acylated octreotide were only found back in minor amounts (at day 50: 4.9 % native octreotide, 1.5% acyl (mostly glycolyl) adducts of octreotide and 3.5 % are acyl (mostly glycolyl) adducts of Oct*monoSIP*). Importantly, the total amount of acylated products was significantly lower in the microspheres loaded with the protected peptide as compared to the unprotected peptide (5.0 % and 52.5%, respectively). Therefore, it can be anticipated accordingly that the released peptide from the Oct*diSIP* loaded microspheres will display a lower degree of acylation as well. The released protected octreotide will rapidly be converted *in vivo* by esterase to form native octreotide exclusively according to the previous experiments.

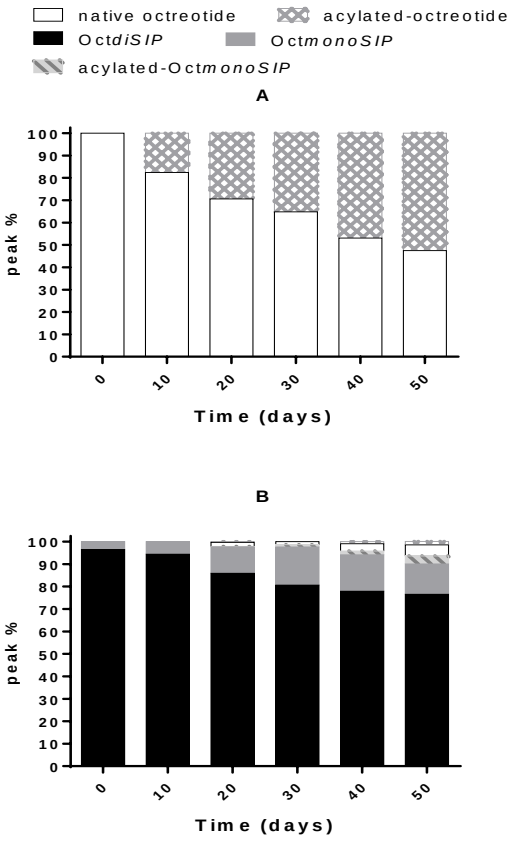


Figure 5. Change of relative peptide content in time, of PLGA microspheres loaded with A) octreotide and B) Oct*diSIP*, during incubation in PBS 7.4 at 37 °C. The contents are expressed as relative peak areas from UPLC analysis.

4. Conclusions

Octreotide was successfully protected by a novel self immolative group at the two sites that can undergo acylation side reactions in PLGA, and resistance of the protected peptide against acylation once loaded in PLGA microspheres was investigated. The kinetics of Oct*diSIP* deprotection was investigated in water/acetonitrile mixture of different pH and in the presence of an esterase. The protecting group was stable at the acidic pH inside degrading microspheres and was eliminated to yield octreotide at physiological pH. The protecting group was able to significantly inhibit the formation of lactyl and glycolyl adducts of octreotide in PLGA microspheres, as compared to unprotected octreotide in PLGA. The formation of unexpected acetylated byproducts was reduced by action of an esterase which triggered the elimination of the protecting groups. The novel self immolative protecting group presented here was successfully designed as a versatile and generally applicable approach to inhibit the interaction and subsequent acylation of amines of peptides and proteins in polyester-based controlled release systems. *In vivo* release, conversion and pharmacokinetics of Oct*diSIP* from PLGA microspheres should be studied further.

Appendix: Supporting Information

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Appendix

Supporting Information

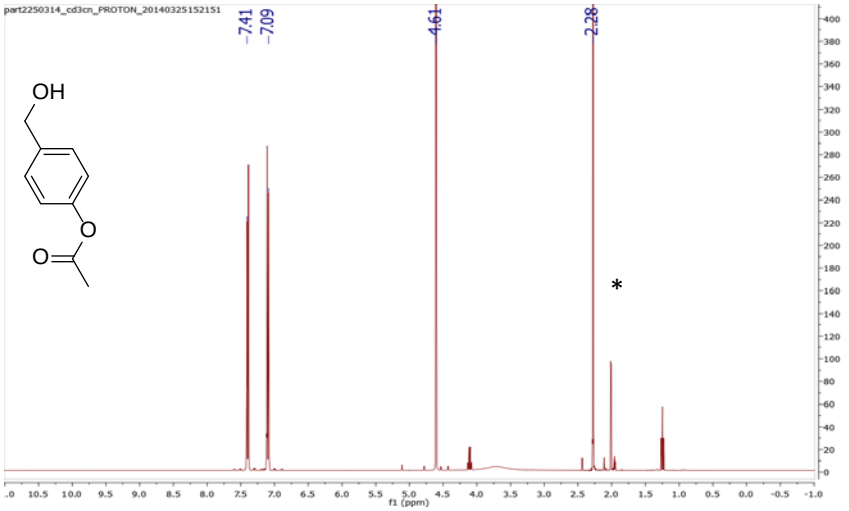

Figure S1. ^{1}H NMR (CD_3CN) spectrum of *4-acetoxybenzyl alcohol (**3**)*: δ = 2.28 (s, 3H, OC(O)-CH_3), 4.61 (s, 2H, CH_2O), 7.09 (d, 2H, -C_6H_4), 7.41 (d, 2H, -C_6H_4).* indicates the solvent (CD_3CN) peak.

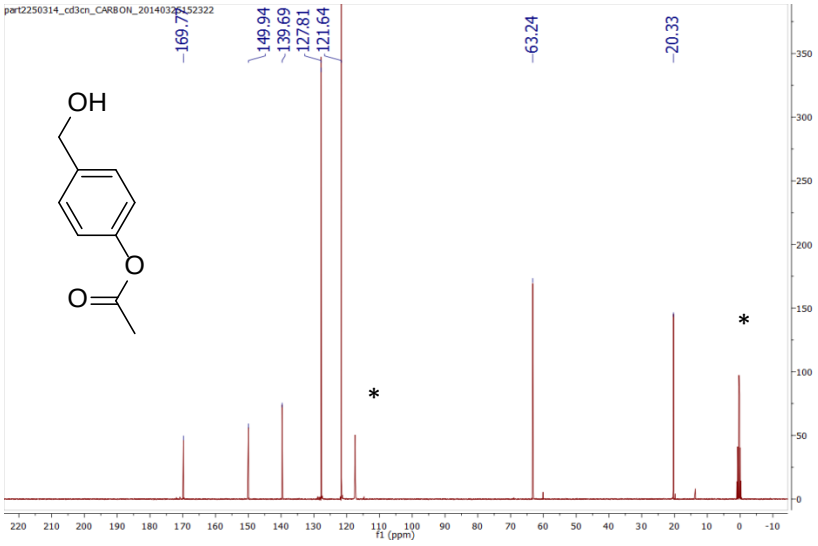

Figure S2. ^{13}C NMR (CD_3CN) of *4-acetoxybenzyl alcohol (**3**)*: δ = 169.77, 149.94, 139.69, 127.81, 121.64, 63.24, 20.33. * indicates the solvent (CD_3CN) peak.

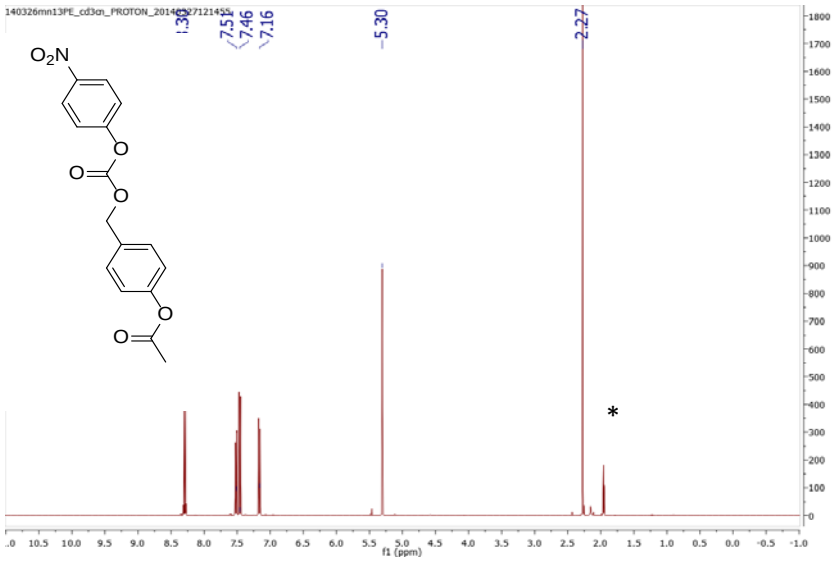

Figure S3. ^{1}H NMR (CD_3CN) *O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate* ***(5):*** δ = 2.27 (s, 3H, OC(O)-CH_3), 5.30 (s, 2H, -CH_2OC(O)O), 7.16 (d, 2H, -C_6H_4OAc), 7.46 (d, 2H, -C_6H_4OAc), 7.51 (d, 2H, -$C_6H_4NO_2$), 8.30 (d, 2H, -$C_6H_4NO_2$). * indicates the solvent (CD_3CN) peak.

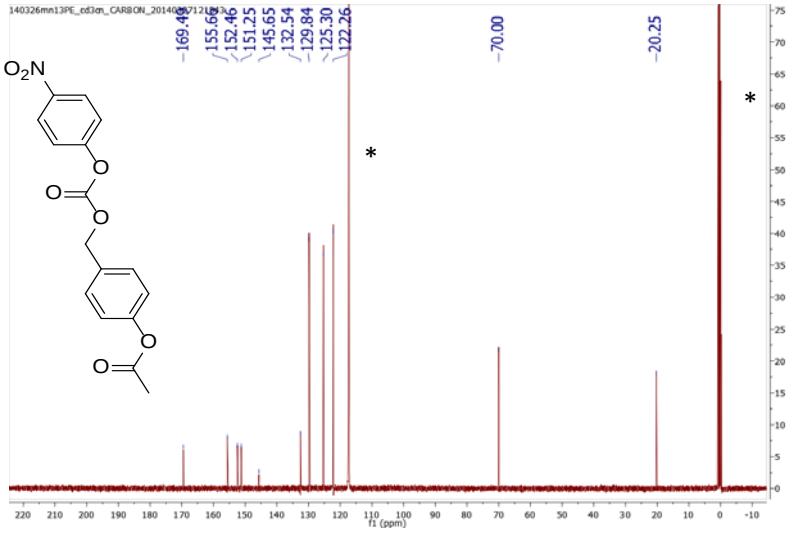

Figure S4. ^{13}C NMR (CD_3CN) *O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate* ***(5)***: δ= 169.49, 155.66, 152.46, 151.25, 145.65, 132.54, 129.84, 125.30, 122.26, 70.00, 20.25. * indicates the solvent (CD_3CN) peak.

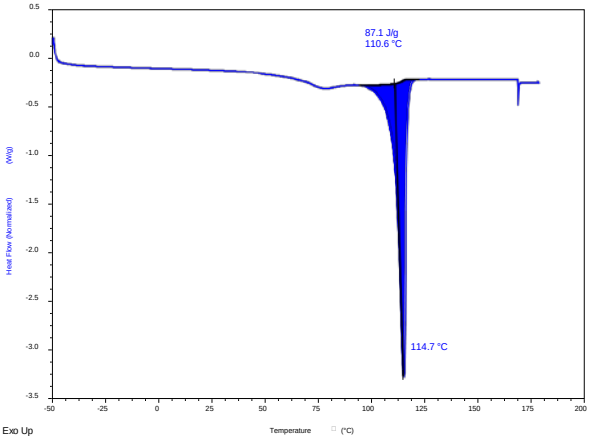

Figure S5. DSC thermogram of *O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate (**5**)*

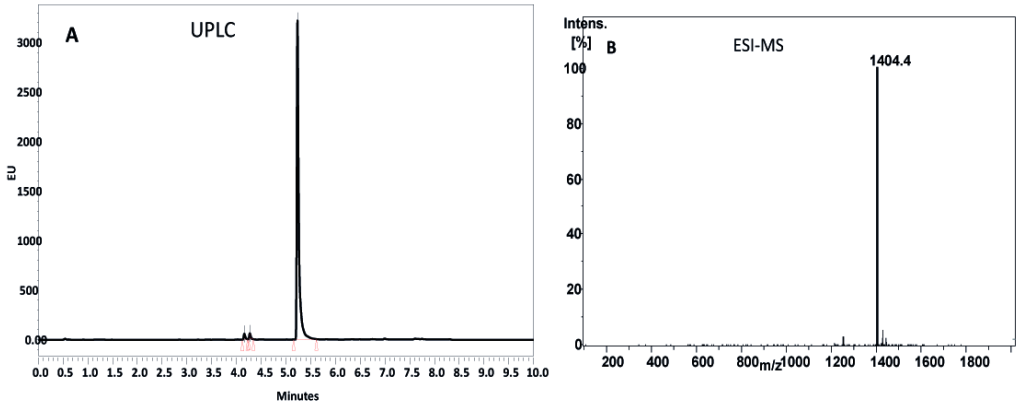

Figure S6. Octreotide di-protected with self immolative groups (Oct*diSIP*). A) UPLC chromatogram, B) Mass spectrum.

Chapter 6

Summary and perspectives

1. Summary

Peptide and protein drugs are presently an important class of pharmaceuticals due to their favorable properties, i.e. high and selective activity [1,2]. However, peptides and proteins are relatively sensitive for degradation and therefore there is need for investigation of the chemical stability of these biotherapeutics during formulation, storage and use [3]. Stability evaluation of a drug substance or drug product is the key to drug quality as it determines the efficacy and safety of any drug dosage form [4]. Moreover, in the area of molecular recognition there is an increasing interest in peptide and protein imprinted polymers (MIP), due to their ability to selectively recognize and bind their targets in competitive media [5,6]. The biggest issue with formulations intended for long-term controlled release or molecularly imprinted polymers is the stability of the peptides/proteins themselves [7].

This project started with an attempt to prepare thymopentin (an immunomodulatory peptide drug) [8] imprinted polymer for its application in solid-phase extraction and separation. A high-throughput and combinatorial technique was used to produce a library of thymopentin imprinted hydrogels based on N-isopropylacrylamide (NIPAm) and methacrylamide (MAm) with methylenebisacrylamide as a crosslinker. It was shown that the peptide was partly modified during the imprinting process as a result of the polymerization conditions. This triggered us to evaluate the chemical stability of pharmaceutical peptides in polymeric matrices during preparation and release and provide a rational solution to prevent the observed modifications. In this thesis, hydrogels and polyesters are used because these are frequently investigated systems for the design of long acting controlled release systems for biotherapeutics such as proteins and peptides.

Chapter 1 of this thesis provides a general introduction to therapeutic peptides/proteins, the most prevalent instabilities of peptides and their delivery challenges. A brief description about imprinted hydrogels and peptide delivery systems based on polyesters is also provided. Furthermore, approaches to prevent peptide acylation is rewired. Finally, the aims of this thesis are outlined.

Hydrogels are hydrophilic cross-linked polymer networks which have various biomedical applications, e.g., drug delivery, molecular imprinting and tissue engineering. One of the method to prepare crosslinked hydrogel networks is free

radical polymerization by using an initiator and catalyst. In **Chapter 2** we investigated the effect of an initiator (ammonium peroxodisulfates (APS)) and a catalyst (N,N,N,N-tetramethylethylenediamine (TEMED)) frequently used for hydrogel formation on the integrity of peptides that each contain one lysine residue. Stability of the therapeutic thymopentin (TP5), a decapeptide (epitope of IgG) and a library of dipeptides was assessed after exposure to APS/TEMED. LC-MS of APS/TEMED-exposed TP5 revealed a major reaction product with an increased mass (+12 Da) with respect to TP5. LC-MS^2 and LC-MS^3 were performed to obtain structural information on the modified peptide and localize the actual modification site. The obtained data demonstrate the formation of a methylene bridge between the lysine and arginine residue in the presence of TEMED, while replacing TEMED with a sodium bisulfite catalyst did not induce this modification in the peptide. Studies with the other peptides showed that the TEMED radical can induce methyleneation when lysine is next to arginine, proline, cysteine, aspargine, glutamine, histidine, tyrosine, tryptophan, and aspartic acid residues. In this chapter a practical method for identifying methylated peptides was provided, and it is shown that the TEMED radical is the carbon source to produce the methylated products. Finally, it is concluded that the stability of peptides and protein needs to be considered when using APS/TEMED in *in situ* polymerization systems. The use of an alternative catalyst such as sodium bisulfite may preserve the chemical integrity of peptides during *in situ* polymerization [9].

The biodegradable and biocompatible polyester of poly(D,L-lactic-co-glycolic acid) (PLGA) is widely used for the design of sustained release formulations of therapeutic peptides and proteins [10]. However, the stability of peptides and proteins within PLGA nano/microspheres and implants has been identified as a major challenge for the successful development of PLGA controlled release systems [3]. PLGA can interact and react with nucleophiles, such as the N-terminus and primary amine groups of lysine residues of peptides, resulting in an acylation reaction between the drug and the polymer [11]. In **Chapter 3** the extent of acylation of the model peptide octreotide in PLGA microspheres was compared with that in microspheres of polyesters with hydrophilic domains, i.e., poly(D,L-lactic-co-glycolic-co-hydroxymethyl glycolic acid) (PLGHMGA) and a multiblock copolymer of poly(ε-caprolactone)-PEG-poly(ε-caprolactone) and poly(L-lactide) ((PC-PEG-PC)-(PL)). Octreotide loaded microspheres were prepared by a double emulsion solvent evaporation technique. It was found that in microspheres of the more hydrophilic polyester less acylated adducts were

formed than in PLGA microspheres. With LC-MS/MS analysis the different sites of acylation on octreotide as well as their extent of derivatization were determined. We showed that besides the N-terminus and primary amine group of lysine, the primary hydroxyl of the end group of octreotide was also subjected to acylation. The N-terminus of octreotide was the most susceptible site of acylation. It was also found that a nucleophilic attack of the peptide can occur onto the carbamate bond present in (PC-PEG-PC)-(PLA) originating from 1,4-butanediisocyanate that was used as the chain extender, resulting in a butanediisocyanate-lactide adduct [12].

Based on the knowledge acquired in chapter 3 about the major sites of acylation in octreotide, in **Chapter 4** we assessed other possible acylation sites on peptides. Goserelin was used as a model peptide because it lacks lysine and a free N-terminus, but contains other nucleophilic residues, i.e. serine, tyrosine and arginine, which potentially can be acylated. Goserelin-loaded PLGA microspheres were prepared by a double emulsion solvent evaporation technique. LC-MS/MS was applied for monitoring the acylation and scrutinize the exact site of modification. It was shown that arginine is subjected to acylation with glycolic acid and lactic acid units of PLGA, which was followed by loss of NH_3 from the guanidine group to obtain 2-oxazolin-4-one and 5-methyl-2-oxazolin-4-one residues with masses that are, respectively, 41 and 55 Da higher than native goserelin. Acylation of serine and tyrosine in goserelin was not detected. The results of this chapter demonstrate that beside lysine also acylation of arginine can occur in peptides and proteins that are loaded in and subsequently released from PLGA matrixes.

Chapter 5 presents a new method to inhibit acylation in poly(D,L-lactide-co-glycolide) (PLGA) formulations by reversely blocking the amine groups of a model peptide (octreotide) with a novel self immolative protecting group. This self immolative protecting group, *O-4-nitrophenyl-O'-4-acetoxybenzyl carbonate,* was synthesized and characterized and attached to the amine groups of octreotide. Octreotide with two self-immolative protecting groups (Oct*diSIP*) on the N-terminus and lysine side chain was synthesized, subsequently isolated by preparative RP-HPLC and charachterized by MS. The kinetic deprotection profiles of Oct*diSIP* in solution were investigated at various pH conditions. It was shown that the protecting group was stable at acidic pH (that has been found inside degrading PLGA microspheres), but it was eliminated upon incubation at

physiological pH. It was shown that deprotection slowly occurred by chemical hydrolysis ($t_{½}$ is 63 hrs at 37 °C in buffer:acetonitrile (2:1, v/v) and estimated to be 10 hrs in buffer of pH 7.4 and the same temperature), but importantly within 1 min in the presence of a physiological relevant concentration of an esterase. Oct*diSIP* encapsulated PLGA microspheres were prepared using a double emulsion evaporation method. Protected octreotide showed substantial inhibition of acylation inside PLGA microspheres as compared to the unprotected octreotide. After 50 days incubation of microspheres in PBS pH 7.4 at 37 °C, 52.5% of unprotected octreotide was in an acylated form, whereas protected octreotide showed only 5.0 % acylation. From the of results of this chapter, it is concluded that the novel self immolative protecting group is a versatile and generally applicable approach to inhibit the interaction and subsequent acylation of amines of peptides and proteins in polyester-based controlled release systems.

2. Perspectives and discussion

The objective of this work was to increase the understanding of peptide integrity in hydrogels and polyester matrices. Detailed knowledge of unwanted modification pathways is prerequisite to develop strategies to prevent chemical modifications and to stabilize peptide and protein pharmaceuticals in polymer based pharmaceutical formulations.

Overall, in this thesis new unwanted modifications on pharmaceutical model peptides were identified and a novel strategy to prevent particularly acylation in polyester delivery systems was explored. There are still questions that arise as a result of this thesis and that need further investigations.

In **chapter 2** we showed that the TEMED radical induced methyleneation (Δm +12) that occurs on lysine residues of peptides depended on its neighboring amino acid. For both thymopentin and the dipeptide arginine-lysine, we have demonstrated by MS that a methylene bridge is formed between arginine and lysine residues after exposure to APS/TEMED. Moreover, in other dipeptides, methyleneation happened when lysine was next to proline, cysteine, aspargine, glutamine, histidine, tyrosine, tryptophan and aspartic acid residues. However, we could not differentiate a methylene bridge between two amino acid or single methyleneation of individual residue by MS. At the scale of the experiments, we only succeed to isolate enough material of modified tryptophan-lysine to perform NMR analysis. This technique showed that a methylene bridge was formed

between the N-terminus of the tryptophan and its pyrrole ring to form a thermodynamically stable six-membered ring. Therefore it is recommended to perform complementary NMR analysis of the other dipeptides that showed +12 modification when exposed to APS/TEMED. Moreover, it was demonstrated that upon exposure to the TEMED radical, bridge formation occurs between a lysine and arginine residue when they are direct neighbors in a peptide structure. An interesting question is whether this bridge formation can still occur when e.g. a glycine is placed between an arginine and lysine residue. Although identification of all possible intramolecular cross-links or single modification sites of APS/TEMED-treated peptides still remains a tremendous job, the data from this study can be useful to predict the modifications that could happen on peptides and proteins in the presence of TEMED radicals. Avoiding the use of TEMED and replacing this catalyst with sodium bisulfite is recommended to prevent methyleneation of peptides during *in situ* polymerization.

In **chapter 3** it is found that hydrophilic polyesters are promising systems for controlled release of peptides because substantially less acylation occurs in microspheres based on these polymers as compared with microspheres based on PLGA. However, *in vivo* studies are necessary to investigate the biocompatibility of these new polymers and their in vivo biodegrability and degradation kinetics. **Chapter 4** reports that acylation occurs on arginine in goserelin peptide that lacks lysine and a free N-terminus. The pK_a value of the α-amino group of a peptides is 7.8, whereas that of the ε-amino group and guanidine group are 10.1 and 12.5, respectively [13]. Therefore, the α-amino group of the N-terminus is likely more reactive than that of the lysine and arginine residue at acidic pH of degrading polymers. In line herewith, it has been found by us and others that octreotide acylation occurs preferentially on the N-terminus [11].

Future studies are suggested on a peptide that contains both a N-terminus and arginine residue to evaluate if arginine acylation can be detected in such a peptides or that acylation will be governed by N-terminus. In this thesis it is shown that the extent of acylation on arginine in goserelin is only 2.2 % (compare with the 69% acylation of octreotide **(chapter 3)**, but arginine as a possible site of acylation should certainly be taken into account. Due to its high pK_a value (12.5), arginine is a positively charged at a neutral pH, whereas acylated arginine is uncharged. This may increase the hydrophobicity of the peptide or protein, leading to changes in structure and function and even possibly to aggregation.

Finally, **chapter 5** reports on a novel self immolative protecting group that inhibits acylation by reversibly blocking the amine groups of octreotide. In an attempt to inhibit the acylation several excipients were added to the formulations. With the aim to prevent octreotide from reaction with PLGA, Pluronic F 68 (non-ionic surfactant), n-dodecyl-β-D-maltoside (non-ionic surfactant) and β (beta)-cyclodextrin (a cyclic oligosaccharide) were individually encapsulated with the peptide in PLGA microspheres. Further, in order increase water up take and facilitate the release of formed acid degradation products, sucrose was encapsulated with octreotide in PLGA microspheres. In another formulation polylysine was co-encapsulated in order to compete with the amine groups of octreotide for possible reaction with PLGA. However, none of the studied excipients resulted in less acylation of octreotide. The problem with co-encapsulating of excipients is their leakage during incubation of the particles in an aqueous medium and a subsequent change of the morphology of particles. As an example the particles loaded with sucrose as excipient showed a high burst release of the peptide likely due to the increase in porosity of the particles because of the leached excipient. Therefore, we came up with novel approach to covalently, but temporally, protect the amine groups of octreotide with a novel self immolative protecting group (SIP). The SIP is stable at slightly acidic pH but can be deprotected at physiological pH. It was further shown that the protecting group moiety can be rapidly cleaved by the action of an esterase. However, due to the decreased hydrophilicity of Oct*diSIP*, we faced difficulties to properly evaluate the *in vitro* drug release profile of his peptide, because it was difficult to maintain sink conditions. It is therefore recommended to add surfactants or proteins (e.g. albumin) in the release medium to solubilize the released Oct*diSIP* and in this way maintain sink conditions and to simultaneously investigate the conversion of the released peptide into unprotected octreotide. Moreover, *In vivo* release, conversion and pharmacokinetics of Oct*diSIP* from PLGA microspheres should be studied further.

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Appendices

Nederlandse samenvatting

List of publications

List of abbreviations

Acknowledgments

Curriculum Vitae

Samenvatting

Peptiden en eiwitten zijn tegenwoordig een belangrijke klasse geneesmiddelen vanwege hun hoge en selectieve therapeutische activiteit [1,2]. Peptiden en eiwitten zijn echter relatief gevoelig voor degradatie en er is daarom behoefte aan meer onderzoek naar hun chemische stabiliteit gedurende formulering, opslag en gebruik [3]. Evaluatie van de stabiliteit van een medicijn of geneesmiddel is van belang voor bewaking van de geneesmiddelkwaliteit aangezien het bepalend is voor de effectiviteit en veiligheid van het product [4]. Daarnaast is er op het gebied van moleculaire herkenning een toenemende interesse in het maken van polymeren met moleculaire afdrukken van peptiden en eiwitten, vanwege de mogelijkheid om daarmee selectief de afgedrukte moleculen te herkennen en te binden [5,6]. Het grootste probleem bij formuleringen die bedoeld zijn voor langdurig gereguleerde afgifte of bij polymeren met moleculaire afdrukken is de stabiliteit van de toegepaste peptiden/eiwitten [7].

Dit project ging van start met een poging om polymeren te synthetiseren met moleculaire afdrukken van thymopentine (een immunomodulatoir peptide geneesmiddel) [8] voor toepassing in vaste-fase extractie en scheiding. Een *high-throughput* en combinatoriële techniek werd toegepast om een bibliotheek te verkrijgen van thymopentine-afgedrukte hydrogelen op basis van *N*-isopropylacrylamide (NIPAm) en methacrylamide (Mam) met methyleenbisacrylamide als netwerkvormer. Er werd aangetoond dat het peptide deels gemodificeerd werd gedurende het afdrukproces ten gevolge van de polymerisatieomstandigheden. Dit bracht ons er toe om de chemische stabiliteit van farmaceutische peptiden in polymere matrixen tijdens bereiding en afgifte nader te gaan onderzoeken, om uiteindelijk rationele oplossingen te kunnen bedenken om de waargenomen modificaties te voorkomen. In dit proefschrift worden hydrogelen en polyesters onderzocht omdat dat materialen zijn die vaak gebruikt worden voor het ontwerpen van gereguleerde afgiftesystemen van biotherapeutica zoals eiwitten en peptiden met langdurige werking.

Hoofdstuk 1 van dit proefschrift geeft een algemene inleiding over therapeutische peptiden/eiwitten, de meest voorkomende vormen van instabiliteit van peptiden, en de uitdagingen die men aantreft met betrekking tot hun afgifte. Er wordt een korte beschrijving gegeven van het maken van moleculaire afdrukken in hydrogelen en van afgiftesystemen van peptiden op basis van polyesters. Verder

wordt een overzicht gegeven van benaderingen om acylering van peptiden te voorkomen. Tenslotte wordt het doel van dit proefschrift samengevat.

Hydrogelen zijn verknoopte hydrofiele polymeernetwerken met een variëteit aan biomedische toepassingen, o.a. in geneesmiddelafgifte, moleculaire afdrukken en weefselkweektechnologieën. Een van de methoden om verknoopte hydrogelnetwerken te maken is vrije-radicaalpolymerisatie, waarbij gebruik gemaakt wordt van een initiator en een katalysator. In **Hoofdstuk 2** onderzochten we het effect van een initiator (ammonium peroxodisulfaat (APS)) en een katalysator (*N,N,N,N*-tetrametylethyleendiamine (TEMED)), die veel gebruikt worden bij hydrogelvorming, op de integriteit van peptiden die allen een lysine eenheid bevatten. De stabiliteit van het therapeutische thymopentine (TP5), een decapeptide (een epitoop van IgG), en een bibliotheek van dipeptiden werd onderzocht na blootstelling aan APS/TEMED. LC-MS analyse van TP5 blootgesteld aan APS/TEMED liet voornamelijk een reactieproduct zien met een toegenomen moleculaire massa (+12 Da) ten opzichte van TP5. LC-MS^2 en LC-MS^3 werd uitgevoerd om structurele informatie te verkrijgen over het gemodificeerde eiwit en de plaats van modificatie te lokaliseren. De verkregen data tonen de vorming van een methyleenbrug aan tussen de lysine en arginine eenheden van het eiwit in de aanwezigheid van TEMED, terwijl het vervangen van TEMED door natriumbisulfiet als katalysator deze modificatie in het peptide niet tot stand bracht. Studies aan de andere peptiden lieten zien dat het TEMED-radicaal de methylenering kan veroorzaken wanneer lysine aanwezig is naast een van de residuen arginine, proline, cysteïne, aspargine, glutamine, histidine, tyrosine, tryptofaan of aspartinezuur. In dit hoofdstuk werd aldus een praktische methode ontwikkeld om gemethyleerde peptiden te identificeren, en er is aangetoond dat het TEMED-radicaal de koolstofbron is die de gemethyleerde producten genereert. Tenslotte is geconcludeerd dat de stabiliteit van peptiden en eiwitten in aanmerking moet worden genomen wanneer APS/TEMED wordt gebruikt bij *in situ* polymerisatiesystemen. Door het gebruik van een alternatieve katalysator zoals natriumbisulfiet kan de chemische integriteit van peptiden tijdens *in situ* polymerisatie worden behouden [9].

Het biologische afbreekbare en verdraagzame polyester poly(D,L-melkzuur-*co*-glycolzuur) (PLGA) wordt veel gebruikt voor het ontwerpen van formuleringen met geleidelijke afgifte van therapeutische peptiden en eiwitten [10]. De stabiliteit van peptiden en eiwitten in nano/microsferen en implantaten van PLGA

is bekend als een belangrijke uitdaging bij de succesvolle ontwikkeling van gereguleerde-afgiftesystemen op basis van PLGA [3]. PLGA kan interacteren en reageren met nucleofielen zoals de N-terminus en primaire aminogroepen van lysine eenheden in peptiden, hetgeen resulteert in een acyleringsreactie tussen het geneesmiddel en het polymeer [11]. In **Hoofdstuk 3** werd de mate van acylering van het modelpeptide octreotide in PLGA vergeleken met dat in microsferen van polyesters met hydrofiele domeinen, d.w.z. poly(D,L-melkzuur-*co*-glycolzuur-co-hydroxymethylglycolzuur) (PLGHMGA) en een multiblokcopolymeer van poly(ε-caprolacton)-PEG-poly(ε-caprolacton) en poly(L-melkzuur) ((PC-PEG-PC)-PL)). Octreotide-beladen microsferen werden bereid met een dubbele-emulsie-oplosmiddelverdamptechniek. Er werd gevonden dat er in microsferen van het hydrofielere polyester minder geacyleerde adducten werden gevormd dan in PLGA microsferen. Met behulp van LC-MS/MS analyse werden de verschillende acyleringsplaatsen op octreotide en de mate van derivatisering bepaald. We lieten zien dat naast de N-terminus en de primaire aminegroep van lysine ook de primaire hydroxyl van de eindgroep van octreotide werd geacyleerd. De N-terminus van octreotide was de positie die het meest gevoelig was voor acylering. Er werd ook gevonden dat een nucleofiele aanval van het peptide kan plaatsvinden op de in (PC-PEG-PC)-PL aanwezige carbamaatbinding, die zijn oorsprong heeft in 1,4-butaandiisocyanaat dat was gebruikt als ketenverlenger bij de synthese van het polymeer, resulterend in een adduct van het peptide met butaandiisocyanaat-lactide [12].

Gebaseerd op de kennis opgedaan in hoofdstuk 3 met betrekking tot de belangrijkste acyleringsplaatsen in octreotide, hebben we in **Hoofdstuk 4** ook andere mogelijke acyleringsplaatsen in peptiden bestudeerd. Gosereline werd daarbij gebruikt als modelpeptide omdat het geen lysine en geen vrije N-terminus bevat, maar wel andere nucleofiele eenheden die mogelijk kunnen worden geacyleerd, namelijk serine, tyrosine en arginine. Gosereline-beladen PLGA microsferen werden bereid met een dubbele-emulsie-oplosmiddelverdamptechniek. LC-MS/MS werd toegepast om de acylering te bestuderen en de exacte modificatieposities vast te stellen. Er werd aangetoond dat arginine werd geacyleerd met glycolzuur- en melkzuureenheden afkomstig van PLGA gevolgd door verlies van NH_3 van de guanidinegroep, waarbij 2-oxazolin-4-on en 5-methyl-2-oxazolin-4-on derivaten ontstonden met massa's die respectievelijk 41 en 55 Da groter waren dan het oorspronkelijke gosereline. Acylering van serine en tyrosine in gosereline kon niet worden vastgesteld. De

resultaten in dit hoofdstuk laten zien dat naast lysine ook acylering van arginine kan plaatsvinden in peptiden en eiwitten die beladen zijn in en vervolgens afgegeven worden uit PLGA matrices.

Hoofdstuk 5 presenteert een nieuwe methode om acylering in poly(D,L-melkzuur-*co*-glycolzuur) (PLGA)-formuleringen te remmen door de aminegroepen van een modelpeptide (octreotide) reversibel te blokkeren met een nieuwe, zichzelf vernietigende beschermgroep. Deze zichzelf vernietigende beschermgroep, *O*-4-nitrofenyl-*O*'-4-acetoxybenzylcarbonaat, werd gesynthetiseerd, gekarakteriseerd en gebonden aan de aminegroepen van octreotide. Octreotide met twee zichzelf-vernietigende beschermgroepen (Oct*diSIP*), op de N-terminus en lysinezijketen, werd gesynthetiseerd en vervolgens geïsoleerd met behulp van preparatieve RP-HPLC en gekarakteriseerd met MS. De kinetiek van ontscherming van Oct*diSIP* in oplossing werd onderzocht bij verschillende pH omstandigheden. Er werd aangetoond dat de beschermgroep stabiel was in zure pH (d.w.z. de omstandigheden aanwezig binnen in degraderende PLGA microsferen), maar dat het werd geëlimineerd tijdens incubatie bij fysiologische pH. Er werd aangetoond dat de ontscherming langzaam plaatsvond door chemische hydrolyse ($t_{½}$ is 63 uur bij 37 ^{o}C in buffer:acetonitril mengsel (2:1, v/v) en daarmee geschat op 10 uur in zuivere buffer pH 7.4 van dezelfde temperatuur), maar belangrijker nog dat dit proces binnen 1 minuut plaatsvond in de aanwezigheid van een fysiologisch relevante concentratie esterase. PLGA microsferen met ingesloten Oct*diSIP* werden bereid met een dubbele-emulsie-oplosmiddelverdamptechniek. Het beschermde octreotide liet substantiële remming van acylering in PLGA microsferen zien vergeleken met het onbeschermde octreotide. Na 50 dagen incubatie van microsferen in PBS pH 7.4 bij 37 ^{o}C was 52,5% van het onbeschermde octreotide in een geacyleerde vorm aanwezig, terwijl het beschermde octreotide slechts 5.0% acylering liet zien. Uit de resultaten van dit hoofdstuk wordt geconcludeerd dat de nieuwe, zichzelf vernietigende beschermgroep een veelzijdige en algemeen toepasbare benadering is om de interactie en daaropvolgende acylering van amines in peptide en eiwitten te remmen in gereguleerde afgiftesystemen op basis van polyesters.

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M. Shirangi, J.S. Torano, B. Sellergren, W.E. Hennink, G.W. Somsen, C.F. van Nostrum, Methyleneation ofpeptides by N,N,N,N-tetramethylethylenediamine (TEMED) under conditions used for free radical polymerization: a mechanistic study, Bioconjug. Chem. 26 (2015) 90-100.

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M. Shirangi, W.E. Hennink, G.W. Somsen, C.F. van Nostrum, Acylation of arginine in goserelin-loaded PLGA microspheres, submitted for publication.

M. Shirangi, M.Najafi, D.T.S Rijkers, R.J Kok W.E. Hennink, C.F. van Nostrum, Inhibition of octreotide acylation inside PLGA microspheres by derivatization of the amines of the peptide with a self immolative protecting group, submitted for publication.

Abbreviations and terms

Acyl	Glycolyl or lactyl
Acylated octreotide	Octreotide with a glycolyl or lactyl adduct
Acetylated octreotide	Octreotide with an acetyl adduct (Δm +42 Da)
APS	Ammonium peroxodisulfate
DDT	Dithiothreitol
ESI	Electrospray ionization
GA	Glycolic acid
HMGA	Hydroxymethyl glycolic acid
KPS	Potassium peroxodisulfate
LA	Lactic acid
LC	Loading capacity
LC-ITMS	Liquid chromatography ion-trap mass spectrometry
LE	Loading efficiency
Oct*diSIP*	Octreotide with two self-immolative protecting groups
Oct*monoSIP*	Octreotide with one self-immolative protecting group
PLGA	Poly(D,L-lactic-*co*-glycolic acid)
PLGHMGA	Poly(D,L-lactic-*co*-glycolic-*co*-hydroxymethyl glycolic acid)
PLHMGA	Poly(D,L-lactic-*co* -hydroxymethyl glycolic acid)
(PC-PEG-PC)-(PL)	Poly(ε-caprolactone)-PEG-poly(ε-caprolactone)- poly(L-lactide) multiblock copolymer
PVA	Polyvinyl alcohol
SEM	Scanning electron microscopy
TEMED	N,N,N,N-tetramethylethylenediamine
UPLC	Ultra performance liquid chromatography

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Mehrnoosh

Curriculum vitae:

Mehrnoosh Shirangi was born on 15th June 1975 in Gorgan, Iran. After finishing high school in 1993, she passed the National Academic Entrance Exam for Medical Sciences to study pharmacy at the Faculty of Pharmacy Tabriz University of Medical Sciences. Her Pharm D. research project entitled: "Evaluation of stability of forusemide in their products" was conducted under the supervision of Prof. M. Barzegar Jalali. Mehrnoosh successfully completed her Pharmacy Doctorate Degree (Pharm. D) in 1998. After 4 years working in hospital and private pharmacies in 2002 she started her work as a researcher in R & D department of TolidDaoru company. In 2004 she was appointed as a quality control manager in MehrDarou company. In 2010, she joined the department of pharmaceutics at Utrecht University, Utrecht, The Netherlands through an awarded scholarship founded by Iranian Ministry of Health. Her PhD research was supervised by Prof. Wim Hennink, Dr. Rene van Nostrum and Prof. Govert Somsen. The results of his work are presented in this thesis.