

Inhibition of Octreotide Acylation Inside PLGA Microspheres by Derivatization of the Amines of the Peptide with a Self-Immolative Protecting Group

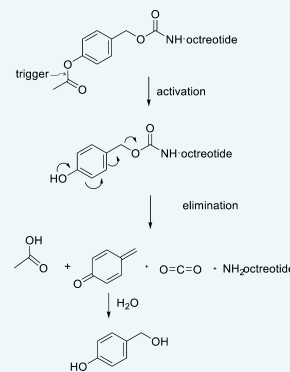
Mehrnoosh Shirangi,^{†,§} Marzieh Najafi,[†] Dirk T. S. Rijkers,[‡] Robbert Jan Kok,[†] Wim E. Hennink,[†] and Cornelus F. van Nostrum^{*,†}

[†]Department of Pharmaceutics and [‡]Medicinal Chemistry & Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3512 JE Utrecht, The Netherlands

[§]Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Science, Tehran 1417614411, Iran

S Supporting Information

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 self-immolative protecting group (SIP), *O*-4-nitrophenyl-*O'*-4-acetoxybenzyl carbonate. The octreotide with two self-immolative protecting groups (Oct*di*SIP) 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 characterized by mass spectrometry. Degradation studies of Oct*di*SIP in aqueous solutions of different pH values 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*di*SIP 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*di*SIP showed only 5.0% acylation in the same time frame. In conclusion, the incorporation of self-immolative protection groups provides a viable approach for inhibition of acylation of peptides in PLGA delivery systems.



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.^{1–4} 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.^{5–7} 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 a glycolyl or lactyl group through an amide bond on the peptide.^{8,9} Peptide acylation may potentially result in loss of activity, a change of receptor affinity, or even immunogenicity¹⁰ 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.¹¹ 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.¹² 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.^{13,14} In another study from this group, the prevention of acylation of octreotide in the presence of divalent cationic salt inside PLGA

Received: November 4, 2015

Revised: December 30, 2015

Published: January 4, 2016



microspheres prepared by a double emulsion solvent evaporation method was investigated. Addition of divalent cationic salts to both the inner and 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.¹⁵ In a more recent study¹⁶ 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.¹⁶ 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.¹⁶ 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 and Mn^{2+} resulted in some inhibition of acylation, whereas Zn^{2+} possessed the greatest inhibitory effect.¹⁷ Minimizing peptide acylation (with some degree of success) has been reported by modifications of polymer composition with alternating the polymer with more hydrophilic domains.^{18–20}

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.^{21–24} In some cases, PEGylation may also prevent acylation; however, it may cause changes in the affinity of the peptide/protein for the target receptor.^{25,26} 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.^{27,28} 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 microspheres.

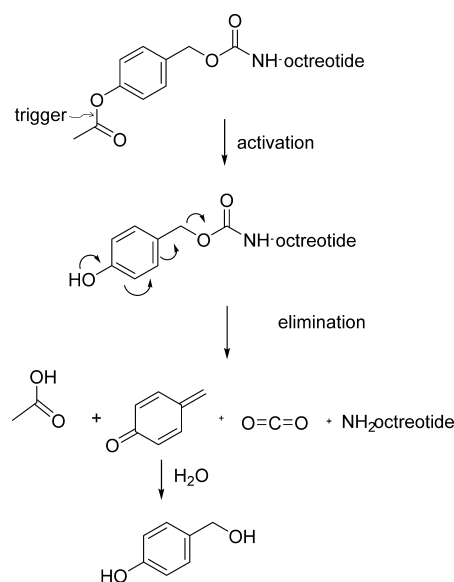
Reversibly or temporarily blocking the amine groups could be an interesting approach to prevent acylation. Ahn et al. investigated reversible blocking of the amine groups of octreotide with maleic anhydride (MA) to prevent its acylation in PLGA particles. The hydrolysis of maleoyl amides is acid catalyzed.²⁹ The hydrolytic degradation of PLGA results in the formation of acid degradation products that accumulate in the polymer matrix, which results in pH decrease,^{30,31} and the MA conjugate, because of its acid-sensitivity, was 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 maleoylated octreotide was released faster than it was converted into intact octreotide inside the PLGA film.³² Since deblocking after release is rather slow at physiological conditions, the possible toxicity of maleoylated 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.³³

Despite 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.³⁴ 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 dearomatization.³⁵

This study aims for the synthesis of a new self-immolative protecting group and its attachment to the amine functionalities of the therapeutic peptide octreotide. The structure of the investigated group is shown in Scheme 1 and is based on 4-

Scheme 1. Self-Immolative Conversion of OctdiSIP into Native Octreotide

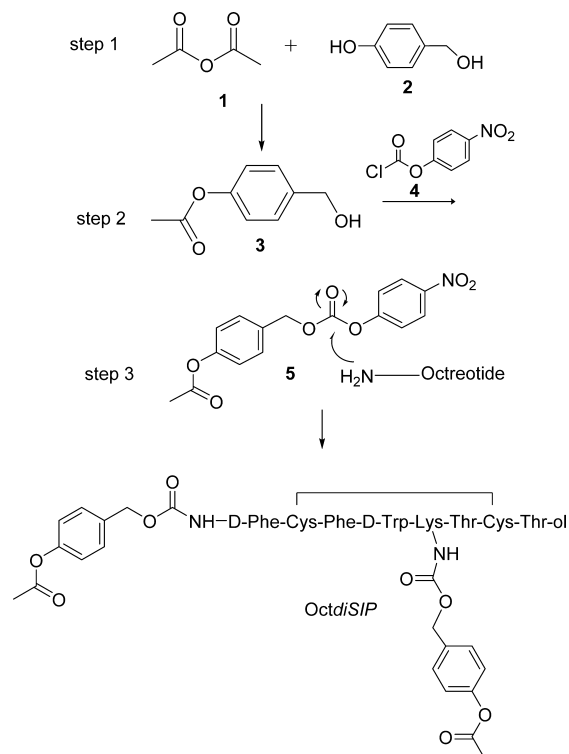


hydroxybenzyl alcohol which has been previously used for the development of self-immolative polymers.^{35–37} The design of this protecting group is such that it is stable at acidic pH (inside degrading PLGA microspheres) and decomposition is triggered by either enzymatic hydrolysis, e.g., esterases, or 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,³⁵ as the remaining part of the self-immolative group is rapidly eliminated and split into nontoxic compounds like carbon dioxide, acetic acid, and 4-hydroxybenzyl alcohol (Scheme 1).³⁸ Octreotide was used as a model peptide to protect its most susceptible sites of acylation (the N-terminus and lysine residue¹⁸) using this self-immolative protecting group.

RESULTS AND DISCUSSION

Synthesis and Characterization of Self-Immolative Protected Octreotide. As shown in Scheme 2, the first step

Scheme 2. Synthesis of the Self-Immolative Protecting Group and Its Conjugation to Octreotide



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 (Figures S3 and S4). A single, sharp melting point was observed by DSC at 114.7 °C ($\Delta H = 87.1$ J/g) (Figure S5). 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 diprotected peptide and some byproducts. The desired diprotected octreotide (OctdiSIP) 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.

In Vitro Hydrolytic and Enzymatic Degradation of OctdiSIP. To study the rate of the conversion of the OctdiSIP into native octreotide, the kinetics of the hydrolytic degradation of OctdiSIP was determined in (2:1 v/v) buffer/acetonitrile mixture at different pH values at 37 °C. It should be mentioned that acetonitrile was added as cosolvent since OctdiSIP 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 h, respectively. The main degradation products that gradually appeared in time were identified by UPLC-MS as being the two expected monoprotected peptides, with the remaining SIP on either the N-terminus or the ϵ -amine of the lysine residue (OctmonoSIP, 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

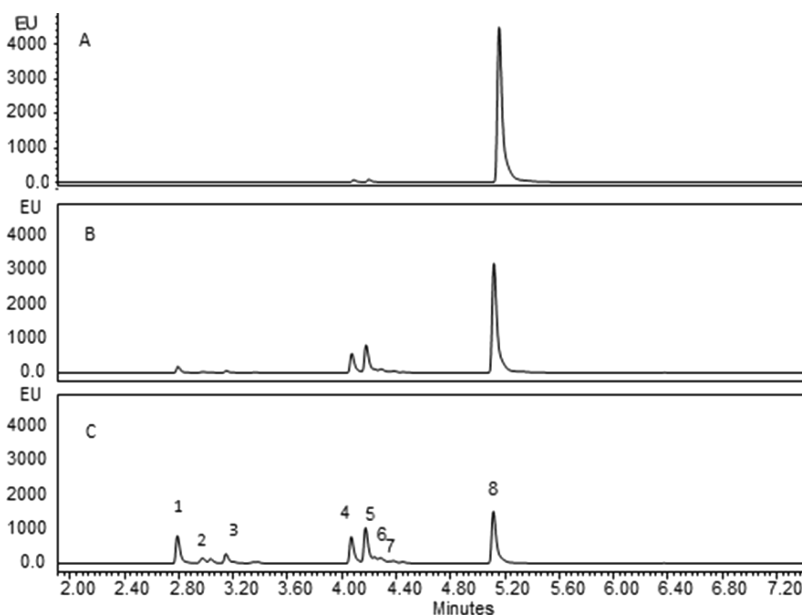


Figure 1. UPLC of 75 µg/mL OctdiSIP incubated in buffer/acetonitrile (2:1) (v/v) at pH 7.4 and 37 °C: (A) $t = 0$; (B) $t = 48$ h; and (C) $t = 120$ h. Fluorescent detection was used with excitation at $\lambda = 280$ nm and emission at $\lambda = 330$ nm. The identification of the numbered peaks is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

(acetylated octreotide, m/z 1062) and octreotide with one SIP and one acetyl group (two isomers of acetylated OctmonoSIP, m/z 1256: acetyl on N-terminus with SIP on lysine or SIP on N-terminus with acetyl on lysine) (Table 1). The formation of

Table 1. Identification of the Observed Peaks in Figure 1 by UPLC-MS

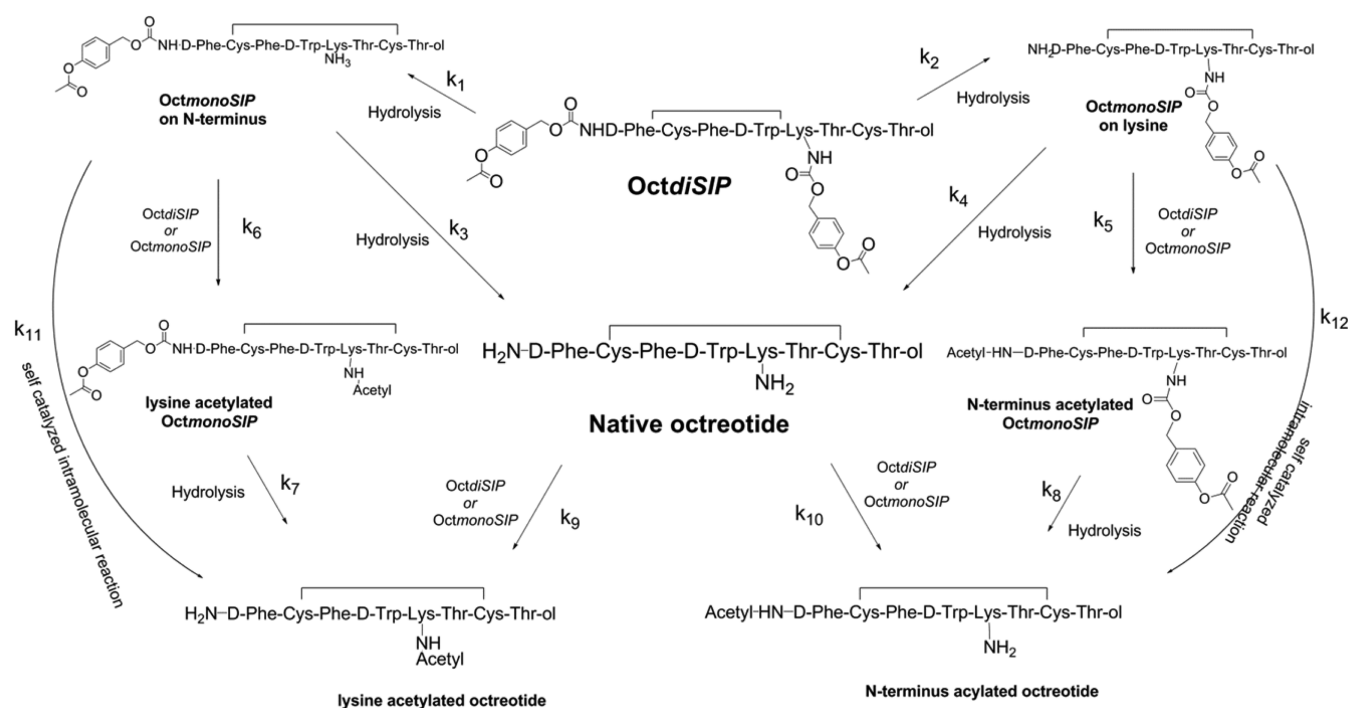
number of peaks	observed $[M + H]^+$ m/z	Δm (Da)	assigned structure
1	1020	0	octreotide (native)
2, 3	1062	+42	acetylated octreotide
4, 5	1212	+192	OctmonoSIP
6, 7	1254	+234 (192 + 42)	acetylated OctmonoSIP
8	1404	+384 (192 + 192)	OctdiSIP

acetylated products 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 OctdiSIP in neutral (or basic) media. The two isomeric peptides with one acetyl and one SIP group (acetylated OctmonoSIP) are obtained by reaction between an amine of OctmonoSIP 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 monoacetylated octreotide. On the other hand, intramolecular reaction of the amine of OctmonoSIP with the remaining SIP on the same peptide will directly result in monoacetylated 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.

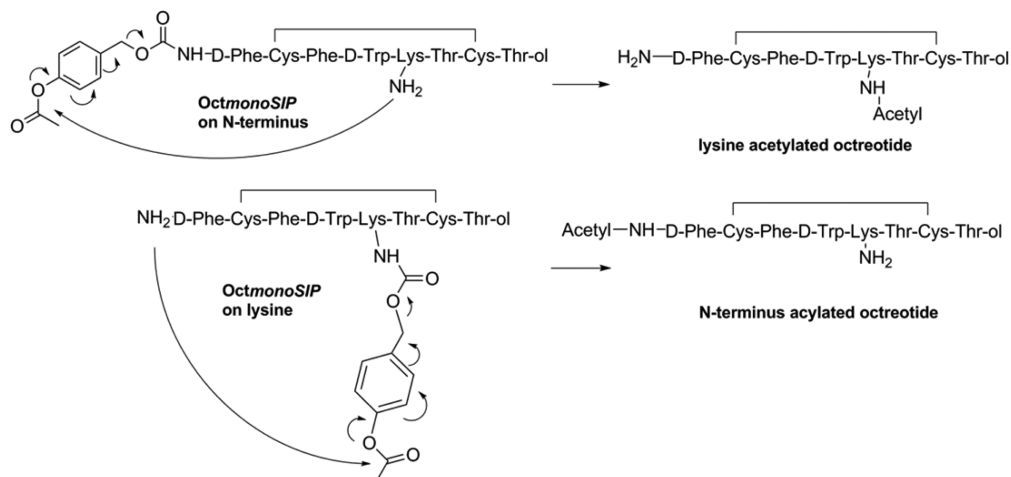
The effect of pH on the degradation kinetics and hydrolysis rate constants of OctdiSIP was analyzed by monitoring the degradation of OctdiSIP in buffer/acetonitrile at pH 5.0, 7.4, and 9.0 by UPLC (Figure 2); the UPLC chromatograms of the incubation mixtures at pH 5 and 9 at 0, 48, and 120 h are shown in Figure S7 and S8 in the Supporting Information. OctdiSIP is quite stable in pH 5 (Figure 2A) while the conversion into OctmonoSIP, native octreotide, and the other byproducts occurs at elevated pH (7.4 and 9.0; Figure 2B and C, respectively). These figures also show that the amount of the monoprotected intermediate products first increases and then decreases over time because these intermediates are 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 C). Table 2 shows the rate constants of the degradation of OctdiSIP ($c = 75 \mu\text{g/mL}$ 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 OctdiSIP in pH 5.0 at 37 °C is estimated to be 1050 h in comparison with 63 h 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 the general principle of ester hydrolysis with the minimum rate constant at $\sim\text{pH}$ 4.5.^{39,40}

It must be stressed that the values derived from Figure 2 are the rate constants for hydrolysis in buffer/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.⁴¹ They have found higher k_{obs} values at higher dielectric constant of the medium ranging from $\epsilon = 47.7$ to 68.3. The dielectric constant of buffer/acetonitrile (2:1) that we used in our studies was 64.8 according to the formula $\epsilon = \epsilon_{\text{acetonitrile}} \times$

Scheme 3. Scheme of Proposed Degradation Pathway for OctdiSIP in Neutral or Basic Media



Scheme 4. Proposed Mechanism of Intramolecular Aminolysis



● native octreotide ■ OctmonoSIP ▲ OctdiSIP
 ▼ acetylated octreotide ◆ acetylated OctmonoSIP

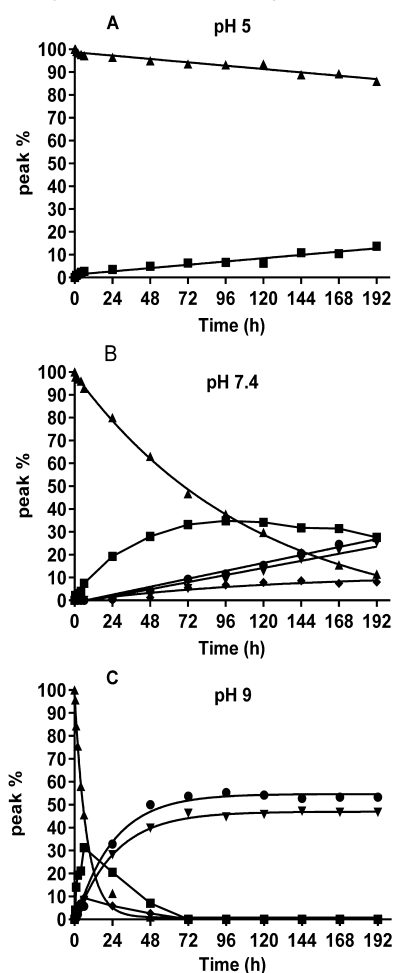


Figure 2. Degradation profile of OctdiSIP (75 µg/mL buffer/ acetonitrile (2:1)) at (A) pH 5, (B) pH 7.4, and (C) pH 9.

$f_{\text{acetonitrile}} + \epsilon_{\text{water}} \times f_{\text{water}}$, with f being the volume fractions and $\epsilon_{\text{acetonitrile}} = 37.5$ and $\epsilon_{\text{water}} = 78.5$. Assuming the same dependence of ϵ on the degradation rate constant as found by de Jong et al.⁴¹ for oligo(lactic acid) hydrolysis, we anticipate that the rate constant of OctdiSIP is 6.3-fold higher in buffer

Table 2. Reaction Rate Constants of Degradation of OctdiSIP ($c = 75 \mu\text{g/mL}$) at 37 °C at Different pH Values

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

than in buffer/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 OctdiSIP to be around 10 h at physiological pH in water.

Importantly, the pH value inside degrading PLGA microspheres was reported to be approximately 4.^{42–44} For successful application of OctdiSIP to minimize acylation of peptide inside degrading PLGA microspheres, the stability of the protecting group at lower pH (Figure 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 OctdiSIP in pH 7.4 is around 63 h 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,⁴⁵ 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. 4.2 µg of the esterase (or 0.02 units) was added to 15 µg (0.01 µmol) of OctdiSIP in 1 mL PBS buffer pH 7.4 containing 3% acetonitrile, an amount of enzyme that in principle is suitable to hydrolyze the two protecting groups in octreotide in 1 min (one unit of enzyme is able to hydrolyze one µmol ester bonds per minute, so 0.02 unit which is also a physiologically relevant concentration⁴⁶ is able to hydrolyze 0.02 µmol ester bonds per minute). Figure 3 shows the relative peak areas in the UPLC chromatogram of OctdiSIP 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

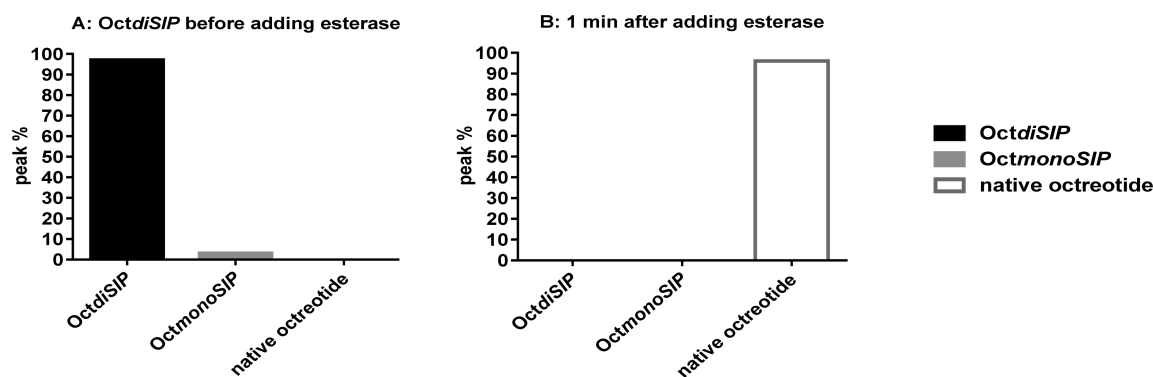


Figure 3. Relative peptide content (A) before adding esterase and (B) 1 min after adding esterase to a solution of OctdiSIP in PBS pH 7.4 containing 3% acetonitrile.

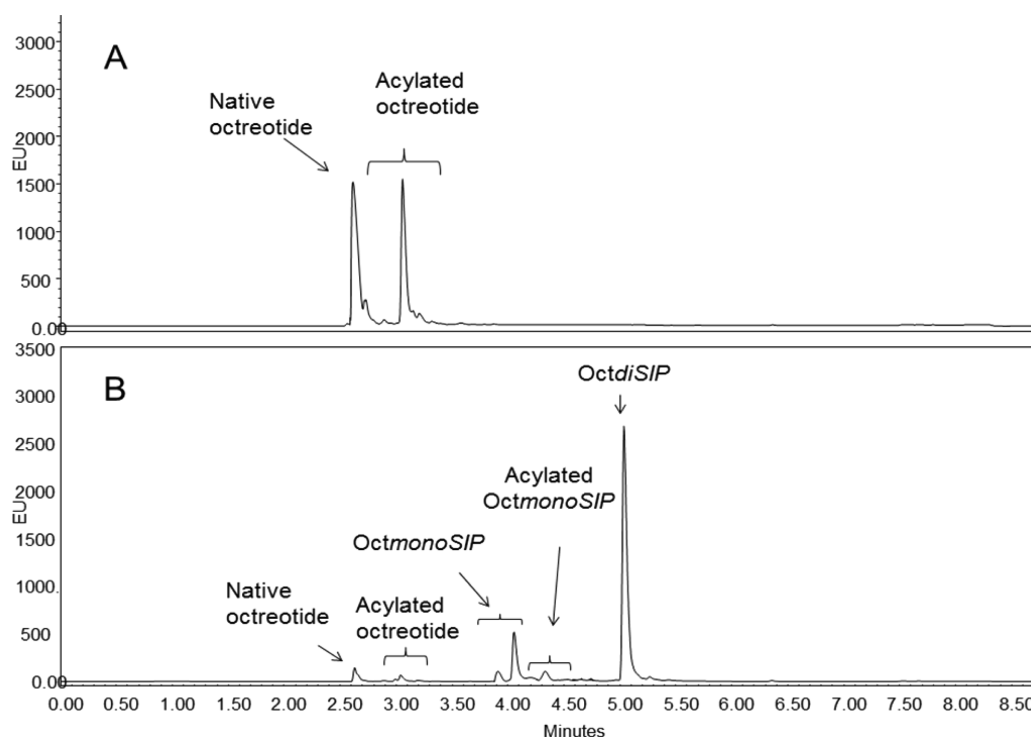


Figure 4. UPLC chromatograms of extracted peptides from PLGA microspheres after 50 days incubation in PBS buffer pH 7.4 at 37 °C: (A) Octreotide and (B) OctdiSIP loaded microspheres.

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 body⁴⁶ the conversion is expected to be completely to native octreotide.

Preparation and Characterization of Microspheres.

PLGA microspheres loaded with octreotide or OctdiSIP were prepared using a double emulsion/solvent evaporation technique. The average particle sizes of octreotide and OctdiSIP loaded microspheres were $23.5 \pm 0.6 \mu\text{m}$ and $46.9 \pm 11.2 \mu\text{m}$, respectively. The OctdiSIP 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 $86.5 \pm 2.4\%$ and $61.3 \pm 6.7\%$ and loading capacities were $3.9 \pm 0.1\%$ and $3.0 \pm 0.3\%$ for octreotide and OctdiSIP, respectively ($n = 3$).

Stability of OctdiSIP in the PLGA Microspheres. To determine the stability of OctdiSIP inside the PLGA micro-

spheres, 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. Figure 4A,B shows the chromatograms of extracted peptides that were still present in the PLGA microspheres after 50 days, for microspheres loaded with native and protected peptide, respectively. Figure 4A shows a main peak eluting after approximately 2.6 min which corresponds to native octreotide (m/z 1020), while extra peaks with longer retention times emerged that were attributed by MS analysis to acylated octreotide (glycolyl and lactyl adducts or both, mainly glycol duct at m/z 1078). Figure 4B shows the main peak eluting after approximately 5 min that corresponds to remaining OctdiSIP (m/z 1404), while some conversion to OctmonoSIP (around 4 min, m/z 1212) and native octreotide (2.6 min, m/z 1020) had occurred. 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, m/z 1078) and 4.3 min (glycolyl OctmonoSIP, m/z 1270) were observed. Importantly, no acetylated compounds (like those that emerged in the hydrolysis study presented above) could be detected.

Figure 5 shows the relative peak areas of the peptides that were extracted from degrading PLGA microspheres loaded with

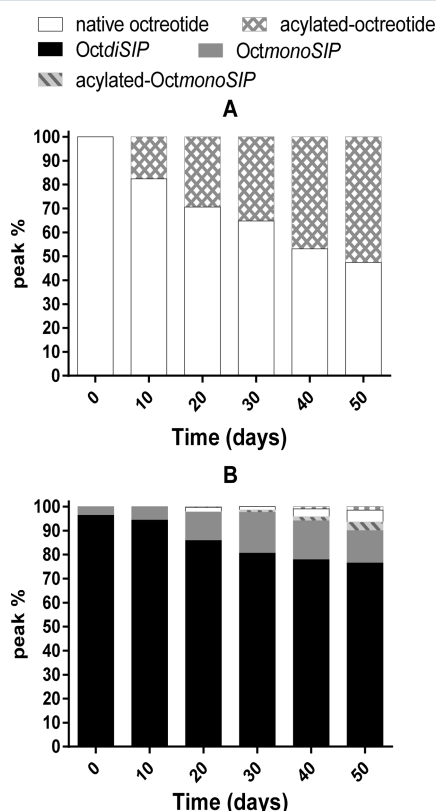


Figure 5. Change of relative peptide content in time, of PLGA microspheres loaded with (A) octreotide and (B) OctdiSIP, during incubation in PBS 7.4 at 37 °C. The contents are expressed as relative peak areas from UPLC analysis.

OctdiSIP, 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 OctmonoSIP). 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 OctdiSIP 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.

CONCLUSION

Octreotide was successfully protected by a self-immolative group at the two amine functionalities 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 OctdiSIP deprotection was investigated in buffer/acetonitrile mixtures of different pH and in the presence of an esterase. The protecting group was stable at the acidic pH inside degrading microspheres and decomposed 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 the action of an esterase, which triggered the elimination of the protecting groups. The self-immolative protecting group presented here was successfully designed and can be 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 OctdiSIP from PLGA microspheres should be studied further.

MATERIALS AND METHODS

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). Poly(vinyl alcohol) (PVA; MW 30 000–70 000; 88% hydrolyzed) and esterase from porcine liver were 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, $\geq 99.5\%$) 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.

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 1H NMR (400 MHz) and ^{13}C NMR (100 MHz) analysis measured on an Agilent 400-MR NMR spectrometer (Agilent Technologies, Santa Clara, USA).

4-Acetoxybenzyl alcohol (3). 4-Hydroxybenzyl alcohol **2** (0.62 g, 5 mmol) and triethylamine (0.4 mL, 3 mmol, 0.6 equiv) 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 equiv) followed by triethylamine (0.4 mL, 3 mmol, 0.6 equiv) were added dropwise during 3 min. The mixture was stirred at 0 °C under nitrogen atmosphere until 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. 1H NMR (CD_3CN): δ = 2.28 (s, 3H, OC(O)-CH₃), 4.61 (s, 2H, CH₂O), 7.09 (d, 2H, -C₆H₄), 7.41 (d, 2H,

-C₆H₄) (Figure S1, Supporting Information); ¹³C NMR (CD₃CN): δ = 169.77, 149.94, 139.69, 127.81, 121.64, 63.24, 20.33 (Figure S2).

O-4-Nitrophenyl-O'-4-acetoxybenzyl carbonate (5). 4-Nitrophenyl chloroformate **4** (1.1 g, 5.5 mmol, 1.1 equiv) dissolved in 3 mL dry acetonitrile was added dropwise 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 equiv) 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 \times 15 mL). The organic layer was dried over anhydrous MgSO₄, filtered, and evaporated to yield the crude product as a white solid, which was recrystallized from ethyl acetate/petroleum ether in 60% yield.⁴⁷ Melting point was determined at 114.7 °C using differential scanning calorimetry (DSC) Q2000 apparatus (TA Instruments, New Castle, DE, USA), (Figure S5). ¹H NMR (CD₃CN): δ = 2.27 (s, 3H, OC(O)-CH₃), 5.30 (s, 2H, -CH₂OC(O)O), 7.16 (d, 2H, -C₆H₄OAc), 7.46 (d, 2H, -C₆H₄OAc), 7.51 (d, 2H, -C₆H₄NO₂), 8.30 (d, 2H, -C₆H₄NO₂) (Figure S3); ¹³C NMR (CD₃CN): 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).

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 dropwise 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 \times 150 mm, 5 μ m, 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 (OctdiSIP), was collected, freeze-dried, and molecular weight measured by mass spectrometry (Figure S6B). The yield was 21%. The purity of the obtained OctdiSIP was calculated by UPLC analysis from the areas under the curve of the UPLC chromatogram (Figure S6A).

Hydrolytic and Enzymatic Deprotection of OctdiSIP. The hydrolytic deprotection of OctdiSIP 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 OctdiSIP (75 μ g/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 OctdiSIP concentration versus time.

To evaluate the instability of the protecting group in the presence of an esterase, OctdiSIP was incubated at pH 7.4 with porcine liver esterase. In detail, 4.2 μ L from a stock solution of 1 mg/mL of porcine esterase in PBS 7.4 was added to 1 mL of a solution of 15 μ g/mL of OctdiSIP in PBS pH 7.4 that also contained 3% acetonitrile to solubilize the peptide. After 1 min of incubation at 37 °C the solution was analyzed by UPLC measurement. Prior to investigation of the esterase-catalyzed conversion of OctdiSIP, 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 μ mol *p*-nitrophenol per min in pH 7.4 at 37 °C.⁴⁸

Preparation and Characterization of PLGA Microspheres Loaded with OctdiSIP or Octreotide. PLGA microspheres loaded with octreotide or OctdiSIP were prepared by a double emulsion (W/O/W) solvent evaporation technique.¹⁸ Briefly, 10 mg of an octreotide solution in 50 μ L Milli-Q water was emulsified in 500 μ L 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 OctdiSIP, which has poor solubility in water due to the hydrophobic protecting groups, 10 mg of OctdiSIP was dissolved in 50 μ L of 50:50 (v/v) water/DMF prior to emulsification in the organic solution. Next, 500 μ L 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 3000 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 OctdiSIP 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 5000 g for 2 min. The octreotide or OctdiSIP content in the supernatant was measured by ultraperformance 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.

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 being gently shaken. At different time points, three tubes were taken, and the microspheres were collected after centrifugation at $3000 \times g$ 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.

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 \times 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 $\mu\text{g/mL}$, 7 μL injection volume) were used for calibration, and detection was done using fluorescence 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 as follows: capillary voltage, 2 kV; nebulizer pressure, 60 psi; dry gas flow, 11 L/min; dry gas temperature, 350 °C; scan range, m/z 50–2000 Da.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00598.

Copies of all NMR spectra, UPLC chromatogram, and MS spectrum of Oct*di*SIP (PDF)

■ AUTHOR INFORMATION

Corresponding Author

* E-mail: C.F.vanNostrum@uu.nl. Phone: (+31) 620274607.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Iranian ministry of Health and Medical Education for financial support of this project.

■ ABBREVIATIONS

Oct*di*SIP octreotide with two self-immolative protecting groups; Oct*mono*SIP octreotide with one self-immolative protecting group; PLGA poly(D,L-lactide-co-glycolide); Acylglycolyl or lactyl; acylated octreotide octreotide with a glycolyl or lactyl adduct ($\Delta m + 58$ or $+72$ Da); acetylated octreotide octreotide

with an acetyl adduct ($\Delta m + 42$ Da); UPLCultra performance liquid chromatography

■ REFERENCES

- (1) Ye, M., Kim, S., and Park, K. (2010) Issues in long-term protein delivery using biodegradable microparticles. *J. Controlled Release* 146, 241–260.
- (2) White, L. J., Kirby, G. T. S., Cox, H. C., Qodratnama, R., Qutachi, O., Rose, F. R. A. J., and Shakesheff, K. M. (2013) Accelerating protein release from microparticles for regenerative medicine applications. *Mater. Sci. Eng., C* 33, 2578–2583.
- (3) Jain, A., Jain, A., Gulbake, A., Shilpi, S., Hurkat, P., and Jain, S. K. (2013) Peptide and protein delivery using new drug delivery systems. *Crit. Rev. Ther. Drug Carrier Syst.* 30, 293–329.
- (4) Zhang, N., Chittasupho, C., Duangrat, C., Siahaan, T. J., and Berkland, C. (2008) PLGA nanoparticle-peptide conjugate effectively targets intercellular cell-adhesion molecule-1. *Bioconjugate Chem.* 19, 145–152.
- (5) van de Weert, M., Hennink, W. E., and Jiskoot, W. (2000) Protein instability in poly(lactic-co-glycolic acid) microparticles. *Pharm. Res.* 17, 1159–1167.
- (6) Houchin, M. L., and Topp, E. M. (2008) Chemical degradation of peptides and proteins in PLGA: A review of reactions and mechanisms. *J. Pharm. Sci.* 97, 2395–2404.
- (7) Mohammadi-Samani, S., and Taghipour, B. (2015) PLGA micro and nanoparticles in delivery of peptides and proteins; problems and approaches. *Pharm. Dev. Technol.* 20, 385–393.
- (8) Lucke, A., Kiermaier, J., and Gopferich, A. (2002) Peptide acylation by poly(alpha-hydroxy esters). *Pharm. Res.* 19, 175–181.
- (9) Murty, S. B., Goodman, J., Thanoo, B. C., and DeLuca, P. P. (2003) Identification of chemically modified peptide from poly(D,L-lactide-co-glycolide) microspheres under in vitro release conditions. *AAPS PharmSciTech* 4, 392–405.
- (10) Na, D. H., Lee, J. E., Jang, S. W., and Lee, K. C. (2007) Formation of acylated growth hormone-releasing peptide-6 by poly(lactide-co-glycolide) and its biological activity. *AAPS PharmSciTech* 8, E105–E109.
- (11) Lucke, A., Fustella, E., Tessmar, J., Gazzaniga, A., and Gopferich, A. (2002) The effect of poly(ethylene glycol)-poly(D,L-lactic acid) diblock copolymers on peptide acylation. *J. Controlled Release* 80, 157–168.
- (12) Houchin, M. L., Neuenswander, S. A., and Topp, E. M. (2007) Effect of excipients on PLGA film degradation and the stability of an incorporated peptide. *J. Controlled Release* 117, 413–420.
- (13) Sophocleous, A. M., Zhang, Y., and Schwendeman, S. P. (2009) A new class of inhibitors of peptide sorption and acylation in PLGA. *J. Controlled Release* 137, 179–184.
- (14) Sophocleous, A. M., Desai, K. H., Mazzara, J. M., Tong, L., Cheng, J., Olsen, K. F., and Schwendeman, S. P. (2013) The nature of peptide interactions with acid end-group PLGAs and facile aqueous-based microencapsulation of therapeutic peptides. *J. Controlled Release* 172, 662–670.
- (15) Zhang, Y., Sophocleous, A. M., and Schwendeman, S. P. (2009) Inhibition of peptide acylation in PLGA microspheres with water-soluble divalent cationic salts. *Pharm. Res.* 26, 1986–1994.
- (16) Zhang, Y., and Schwendeman, S. P. (2012) Minimizing acylation of peptides in PLGA microspheres. *J. Controlled Release* 162, 119–126.
- (17) Qi, F., Yang, L., Wu, J., Ma, G., and Su, Z. (2015) Microcosmic mechanism of dication for inhibiting acylation of acidic peptide. *Pharm. Res.* 32, 2310–2317.
- (18) Shirangi, M., Hennink, W. E., Somsen, G. W., and van Nostrum, C. F. (2015) Identification and assessment of octreotide acylation in polyester microspheres by LC-MS/MS. *Pharm. Res.* 32, 3044–3054.
- (19) Ghassemi, A. H., van Steenberg, M. J., Barendregt, A., Talsma, H., Kok, R. J., van Nostrum, C. F., Crommelin, D. J., and Hennink, W. E. (2012) Controlled release of octreotide and assessment of peptide acylation from poly(D,L-lactide-co-hydroxymethyl glycolide) compared to PLGA microspheres. *Pharm. Res.* 29, 110–120.

- (20) Vaishya, R. D., Mandal, A., Patel, S., and Mitra, A. K. (2015) Extended release microparticle-in-gel formulation of octreotide: Effect of polymer type on acylation of peptide during in vitro release. *Int. J. Pharm.* 496, 676.
- (21) Pfister, D., and Morbidelli, M. (2014) Process for protein PEGylation. *J. Controlled Release* 180, 134–149.
- (22) Roberts, M. J., Bentley, M. D., and Harris, J. M. (2012) Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev.* 64, 116–127.
- (23) Pasut, G., and Veronese, F. M. (2012) State of the art in PEGylation: The great versatility achieved after forty years of research. *J. Controlled Release* 161, 461–472.
- (24) Gong, Y., Leroux, J., and Gauthier, M. A. (2015) Releasable conjugation of polymers to proteins. *Bioconjugate Chem.* 26, 1172–1181.
- (25) Peleg-Shulman, T., Tsubery, H., Mironchik, M., Fridkin, M., Schreiber, G., and Shechter, Y. (2004) Reversible PEGylation: A novel technology to release native interferon alpha 2 over a prolonged time period. *J. Med. Chem.* 47, 4897–4904.
- (26) Erfani-Jabarian, L., Dinarvand, R., Rouini, M. R., Atyabi, F., Amini, M., Mohammadhosseini, N., Shafiee, A., and Foroumadi, A. (2012) PEGylation of octreotide using an alpha, beta-unsaturated-beta'-mono-sulfone functionalized PEG reagent. *Iran J. Pharm. Res.* 11, 747–753.
- (27) Na, D. H., and DeLuca, P. P. (2005) PEGylation of octreotide: I. separation of positional isomers and stability against acylation by poly(D,L-lactide-co-glycolide). *Pharm. Res.* 22, 736–742.
- (28) Na, D. H., Lee, K. C., and DeLuca, P. P. (2005) PEGylation of octreotide: II. effect of N-terminal mono-PEGylation on biological activity and pharmacokinetics. *Pharm. Res.* 22, 743–749.
- (29) Hermanson, G. (2008) *Bioconjugate techniques*, London.
- (30) Liu, Y., Ghassemi, A. H., Hennink, W. E., and Schwendeman, S. P. (2012) The microclimate pH in poly(D,L-lactide-co-hydroxymethyl glycolide) microspheres during biodegradation. *Biomaterials* 33, 7584–7593.
- (31) Fu, K., Pack, D., Klibanov, A., and Langer, R. (2000) Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres. *Pharm. Res.* 17, 100–106.
- (32) Ahn, J. H., Park, E. J., Lee, H. S., Lee, K. C., and Na, D. H. (2011) Reversible blocking of amino groups of octreotide for the inhibition of formation of acylated peptide impurities in poly(lactide-co-glycolide) delivery systems. *AAPS PharmSciTech* 12, 1220–1226.
- (33) Vaishya, R. D., Mandal, A., Gokulgandhi, M., Patel, S., and Mitra, A. K. (2015) Reversible hydrophobic ion-pairing complex strategy to minimize acylation of octreotide during long-term delivery from PLGA microparticles. *Int. J. Pharm.* 489, 237–45.
- (34) Kratz, F., Muller, I. A., Ryppa, C., and Warnecke, A. (2008) Prodrug strategies in anticancer chemotherapy. *ChemMedChem* 3, 20–53.
- (35) Blencowe, C. A., Russell, A. T., Greco, F., Hayes, W., and Thornthwaite, D. W. (2011) Self-immolative linkers in polymeric delivery systems. *Polym. Chem.* 2, 773–790.
- (36) Wang, W., and Alexander, C. (2008) Self-immolative polymers. *Angew. Chem., Int. Ed.* 47, 7804–7806.
- (37) Roth, M. E., Green, O., Gnaim, S., and Shabat, D. (2015) Dendritic, oligomeric, and polymeric self-immolative molecular amplification. *Chem. Rev.* 150910115924009.
- (38) Lim, E., Kang, H., Jung, H., and Park, E. (2007) Anti-angiogenic, anti-inflammatory and anti-nociceptive activity of 4-hydroxybenzyl alcohol. *J. Pharm. Pharmacol.* 59, 1235–1240.
- (39) Euranto, E. K. (1969) Esterification and ester hydrolysis. In *Carboxylic Acids and Esters*, pp 505–588, John Wiley & Sons, Ltd.
- (40) Reubsat, J. L. E., Beijnen, J. H., Bult, A., Vanderhouwen, O. A. G. J., Teeuwssen, J., Koster, E. H. M., and Underberg, W. J. M. (1995) Degradation kinetics of antagonist [Arg6, D-Trp7,9, MePhe8]-substance-P {6–11} in aqueous solutions. *Anal. Biochem.* 227, 334–341.
- (41) de Jong, S. J., Arias, E., Rijkers, D.T. S., van Nostrum, C. F., Kettenes-van den Bosch, J., and Hennink, W. E. (2001) New insights into the hydrolytic degradation of poly(lactic acid): Participation of the alcohol terminus. *Polymer* 42, 2795–2802.
- (42) Brunner, A., Mader, K., and Gopferich, A. (1999) pH and osmotic pressure inside biodegradable microspheres during erosion. *Pharm. Res.* 16, 847–853.
- (43) Li, L., and Schwendeman, S. (2005) Mapping neutral microclimate pH in PLGA microspheres. *J. Controlled Release* 101, 163–173.
- (44) Shenderova, A., Burke, T. G., and Schwendeman, S. P. (1999) The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. *Pharm. Res.* 16, 241–248.
- (45) Satoh, T., and Hosokawa, M. (2006) Structure, function and regulation of carboxylesterases. *Chem.-Biol. Interact.* 162, 195–211.
- (46) Rudakova, E. V., Boltneva, N. P., and Makhaeva, G. F. (2011) Comparative analysis of esterase activities of human, mouse, and rat blood. *Bull. Exp. Biol. Med.* 152, 73–75.
- (47) Sonkaria, S., Boucher, G., Florez-Olvarez, J., Said, B., Hussain, S., Ostler, E. L., Gul, S., Thomas, E. W., Resmini, M., Gallacher, G., and Brocklehurst, K. (2004) Evidence for 'lock and key' character in an anti-phosphonate hydrolytic antibody catalytic site augmented by non-reaction centre recognition: Variation in substrate selectivity between an anti-phosphonate antibody, an anti-phosphate antibody and two hydrolytic enzymes. *Biochem. J.* 381, 125–130.
- (48) Anderson, J., Byrne, T., Woelfel, K. J., Meany, J. E., Spyridis, G. T., and Pocker, Y. (1994) The hydrolysis of p-nitrophenyl acetate - a versatile reaction to study enzyme-kinetics. *J. Chem. Educ.* 71, 715–718.

■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was originally published to the Web on January 19, 2016. The supporting information file was updated, and the paper was re-published to the Web on January 27, 2016.