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Research Paper

Acylation of arginine in goserelin-loaded PLGA microspheres

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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 was 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 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 and tyrosine 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 depend 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 in 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

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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 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.

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, $\geqslant$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 3000*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 5000*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%

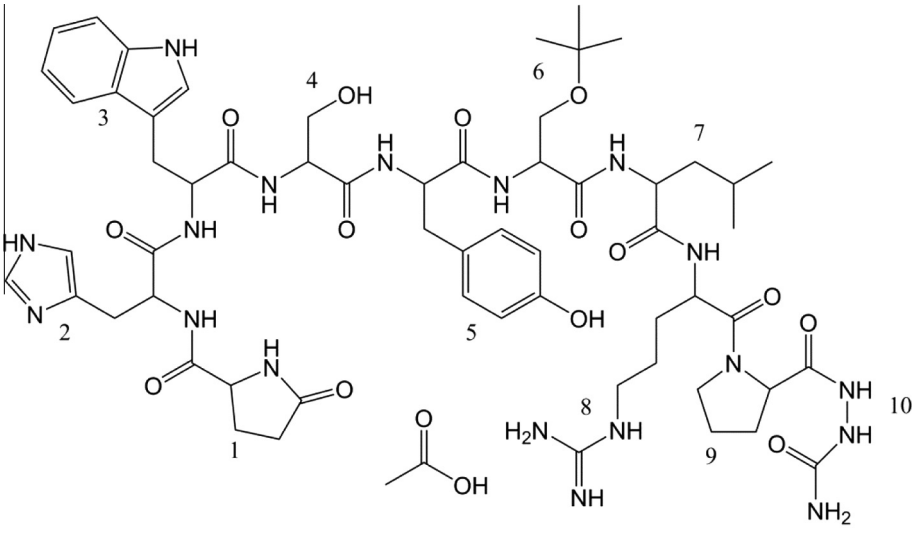

Fig. 1. Structural formula of goserelin acetate.

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 as follows: 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) was 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 (3000g, 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.

3.2. In vitro release of goserelin loaded microspheres

Fig. 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].

Fig. 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.

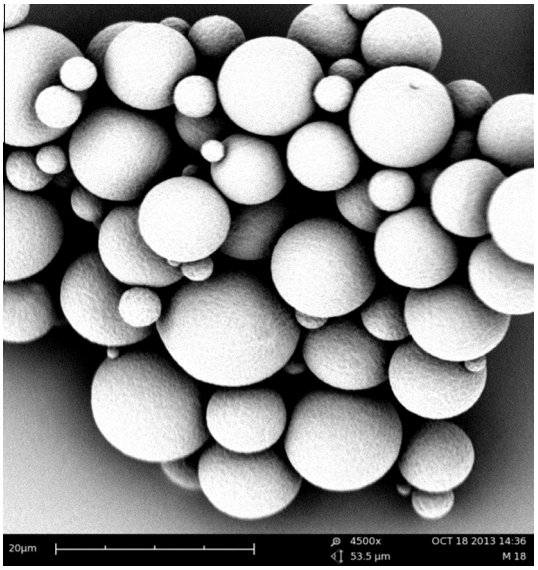

Fig. 2. SEM analysis of goserelin loaded PLGA microspheres.

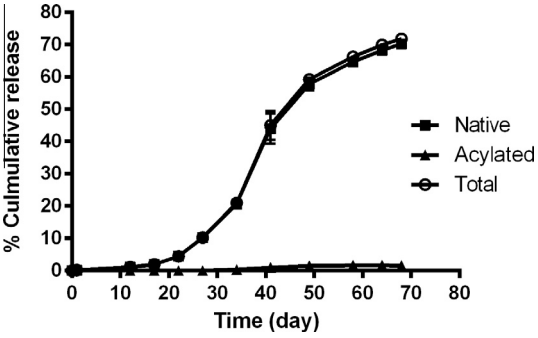

Fig. 3. *In vitro* release (n = 3) of goserelin from PLGA in PBS pH 7.4 at 37 °C: native goserelin (squares), acylated goserelin (triangles) and total goserelin (sum of native and acylated adducts, circles).

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). Fig. 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 and B), 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.

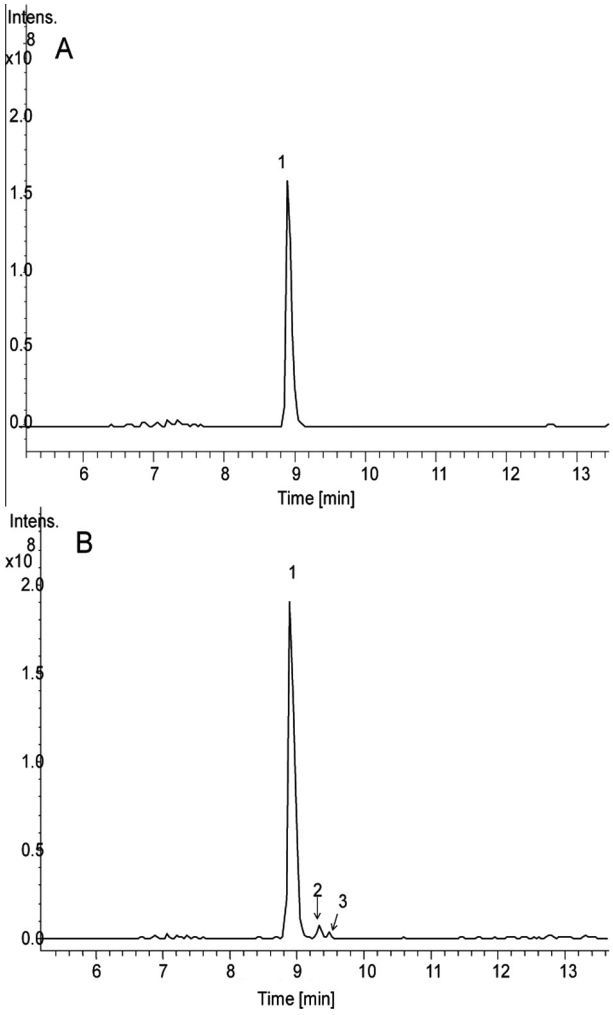

Fig. 4. HPLC chromatogram of (A) goserelin in PBS buffer, and (B) after 42 days released from PLGA microspheres.

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. Fig. 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 and tyrosine, in goserelin.

The total mass of the two released goserelin compounds that are modified at the arginine residue differs 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 NH_3 (−17 Da), i.e. resulting in 2-oxazolin-4-one (oxazolinone) and 5-methyl-2-oxazolin-4-one (methyl oxazolinone).

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

Table 1
Identification of observed peaks in Fig. 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)

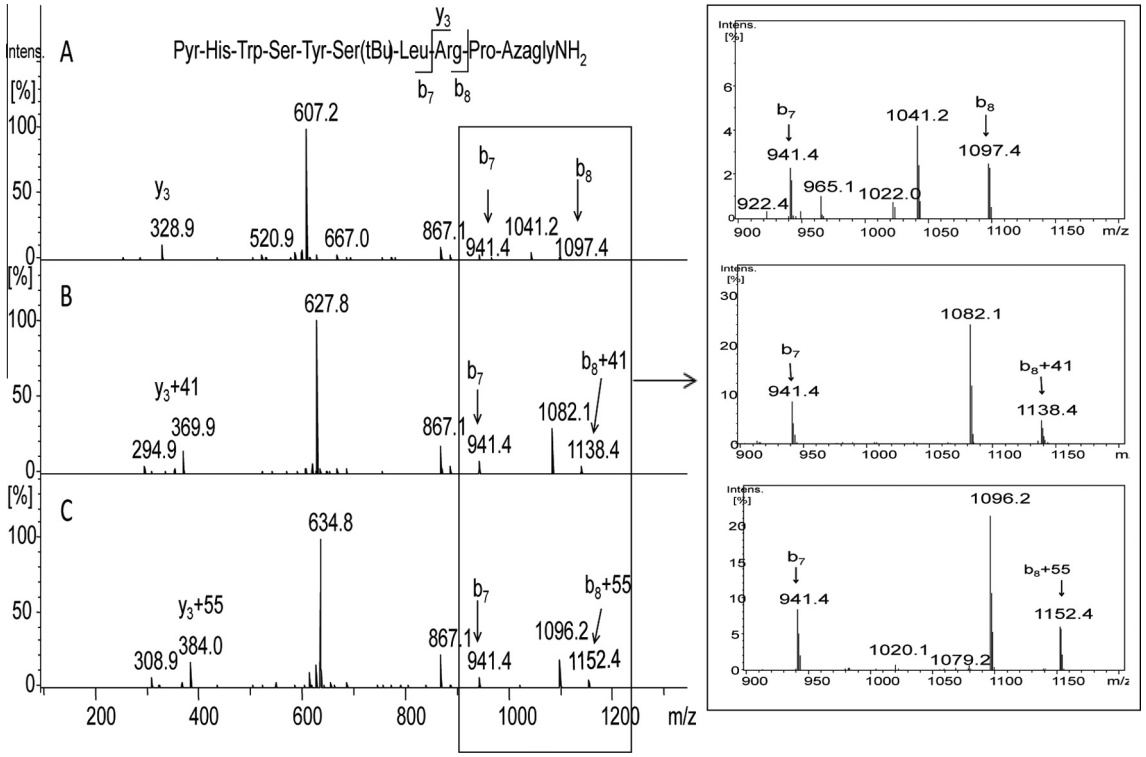

Fig. 5. MS/MS spectra of (A) native goserelin (precursor ion, $[M + 2H]^{2+}$ = 635.2), (B) modified goserelin with precursor ion $[M + 2H]^{2+}$ = 655.7 and (C) modified goserelin with precursor ion $[M + 2H]^{2+}$ = 662.7.

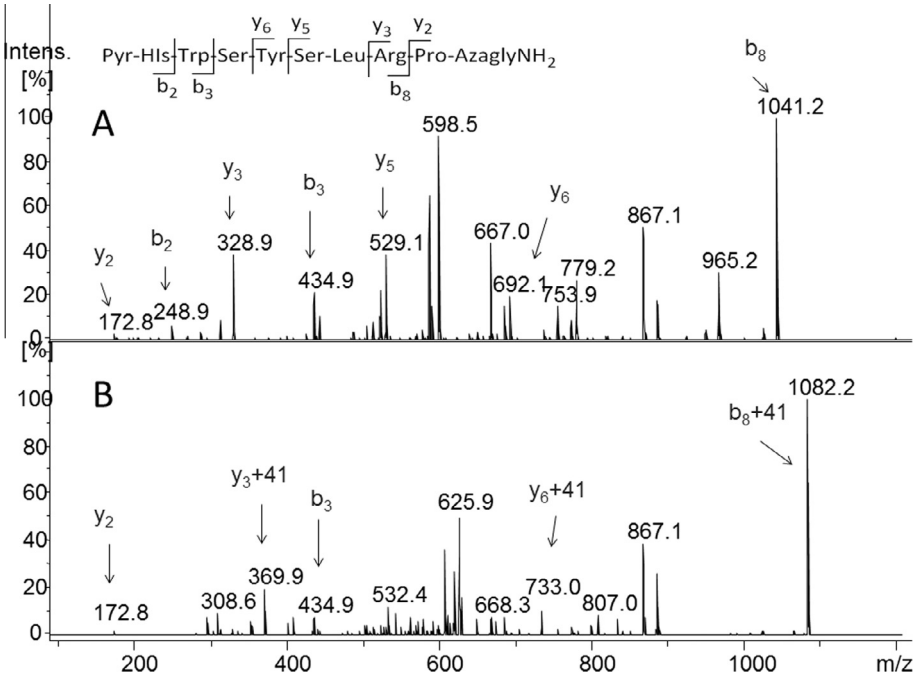

Fig. 6. MS^3 spectra of (A) goserelin (precursor ions 635.2 → 607.2) and (B) modified goserelin (precursor ions 655.7 → 627.7).

is formed in the peptide structure may act as a hapten due to its structural similarity to some reported haptens such as 4-ethoxyme thylene-2-phenyl-2-oxazoline-5-one [25] which might induce antibody formation [26,27]. Current strategies to inhibit the acylation such as the effects of pH-modifying excipients [28], water-soluble divalent cationic salts [29] and PEGylation of the peptide for inhibiting acylation [30] may also be applicable for prevention of arginine acylation.

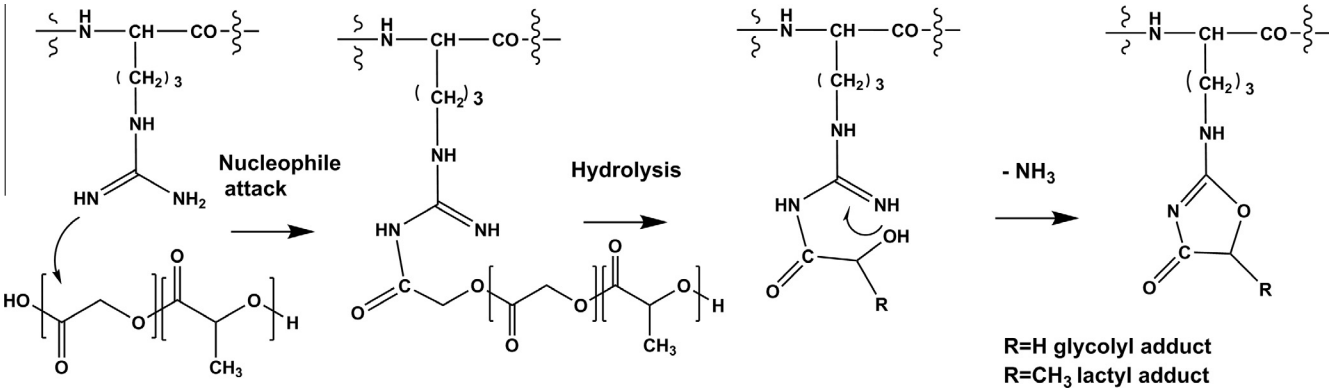

Fig. 7. Proposed mechanism of reaction between an arginine residue of a peptide and PLGA.

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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