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Impurity formation studies with peptide-loaded polymeric microspheres Part I. In vivo evaluation

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Abstract

The purpose of the present investigation was to assess the peptide related substances or impurities formed during incubation of drug loaded poly-(D,L-lactide-co-glycolide) (PLGA) and poly-(D,L-lactide) (PLA) microspheres under in vivo conditions. Sprague–Dawley rats were injected with separate batches of octreotide microspheres prepared by either an oil/water or oil/oil dispersion technique. At specified time points (days 14, 22, 30, and 41), animals were sacrificed and microsphere particles were recovered from the subcutaneous injection sites. The recovered particles were further extracted with 1:1 mixture of dimethylsulfoxide:dichloromethane for subsequent impurity analysis by HPLC and mass spectrometry. During incubation, the percentage purity of parent compound depended on the PLGA co-monomer ratio (e.g. 50:50, 85:15, and 100:0 glycolide:lactide ratios). After 41 days of incubation, for instance, octreotide area percentage by HPLC was determined to be ∼47% for PLGA 50:50 microspheres, ∼75% for PLGA 85:15 microspheres, and ∼87% for PLA microspheres. Spectral analysis of particle extracts revealed the presence of peptide related substances with 58 *m*/*z* and 72 *m*/*z* units higher than the parent peptide *m*/*z* value. This indicated the presence of glycoyl and lactoyl covalent substitutions on the drug compound, resulting from chemical interaction between peptide amine groups and PLGA or PLA ester groups.

Keywords: Somatostatin analogues; Octreotide acetate; PLA microspheres; PLGA microspheres; Peptide acylation; Peptide stability

1. Introduction

Currently, several products on the U.S. market utilize polylactide co-glycolide (PLGA) polymers for therapeutic delivery of drugs to humans. A prime example is the delivery of LHRH agonists (Lupron, Trel-

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star, and Zoladex) and somatostatin analogs (Sandostatin LAR) for the treatment of endocrine disorders. For the human and veterinary delivery of such products, however, chemical stability of drug compounds within the changing polymeric dosage form requires proper in vitro and in vivo characterization. Recently, in vitro evidence has been provided for the acylation and oxidation pathways of therapeutic peptides (i.e. octreotide, salmon calcitonin (sCT), and atrial natriuretic peptide (ANP)) formulated within dynamic matrices composed of either PLGA co-polymers, PLA homopolymers, or PEG-PLA block co-polymer matrices (Lucke et al., 2002a,b; Lucke and Gopferich, 2003; Murty, 2003a,b). Chemical instability was even reported during melt extrusion procedures for PLA implants containing another somatostatin analogue, RC-160 (Rothen-Weinhold et al., 2000). The formation of peptide impurities or related substances during manufacturing or in vitro incubation raises the issues of chemical potency, immunogenicity, and safety.

Microsphere delivery systems composed of either PLGA or PLA polymers undergo significant environmental changes when incubated in the presence of water. For instance, the solid polymeric materials hydrate resulting in hydrolytic degradation of polyester functional groups present in the polymer backbone. Consequently, the microspheres develop an internal acidic microenvironment with trapping of lactic and glycolic acid monomers released from hydrolysis (Fu et al., 2000). Consequently, as hydration and erosion of the polymeric dosage form simultaneously occur, chemical degradation of active agents within the environment requires extensive consideration.

For instance, Lucke et al. reported the formation of acylated peptides during incubation in PLA and PLGA microsphere dosage forms under in vitro conditions at pH 7.4 (Lucke et al., 2002a). The authors reported the chemical conjugation between glycolic and lactic acid monomers with ANP and sCT was catalyzed by local acidic microenvironment pH The proposed mechanism was the nucleophilic attack of either the N-terminus, lysine amino group, or serine alcohol group towards the electrophilic ester carbonyl on the PLGA backbone (Lucke et al., 2002a). Fig. 1 demonstrates the interaction of a reactive species (peptide) with the glycolic acid monomer in a PLGA polyester backbone.

Further evidence of acylation was provided with octreotide acetate, a somatostatin analogue (Fig. 2), formulated in both PLGA and PLA dosage forms (Murty et al., 2003b). The extent of impurity formation altered as a function of polymer co-monomer ratio and molecular weight utilized in the microsphere manufacturing process. In addition, analytical techniques such as FT–MS and LC–MS/MS provided structural confirmation of the related substances where glycoyl and lactoyl moieties were substituted on amine groups. These results concurred with the data provided by previous investigations in acylation pathways for peptide molecules embedded in polymeric matrices.

With literature reports presented thus far, the question remains on whether or not the same reaction mechanism occurs in vivo to a similar extent and under similar control factors (i.e. changing co-monomer ratio). Still another question is whether a change in formulation processing technique from oil/water (O/W) dispersion to oil/oil (O/O) dispersion (Fig. 3) would enhance peptide stability either during manufacturing or during incubation of the dosage form. Microspheres manufactured by the O/O dispersion technique resulted in enhanced erosion-phase release kinetics in vivo (Murty, 2003a). Herrmann et al. postulated that changing to a non-aqueous dispersion and solvent removal technique would alter drug stability and solubility issues inherent with the influx of water media during manufacturing (Hermmann and Bodmeier, 1998). Consequently, for octreotide acetate, the change to O/O processing allowed for enhanced entrapment in polymer matrix as opposed to porous regions for PLA microspheres potentially due to a peptide-polymer solubility phenomenon (Murty, 2003a; Murty et al., 2004).

The Sprague–Dawley rat animal model was selected for studying the questions identified above. Based on evidence provided under in vitro release testing conditions (phosphate buffered saline (pH 7.2) at 37 °C), the incubation of PLGA and PLA microspheres in the subcutaneous rat tissue would result in peptide acylation where polymers with higher lactide content (i.e. PLA) would result in a reduction of related substances formation. Further, the change in processing technique could enhance stability of peptide due to lack of aqueous environment during manufacturing. The proposed in vivo incubation experiments, as a result, would also provide direct translational evidence for therapeutic issues as-

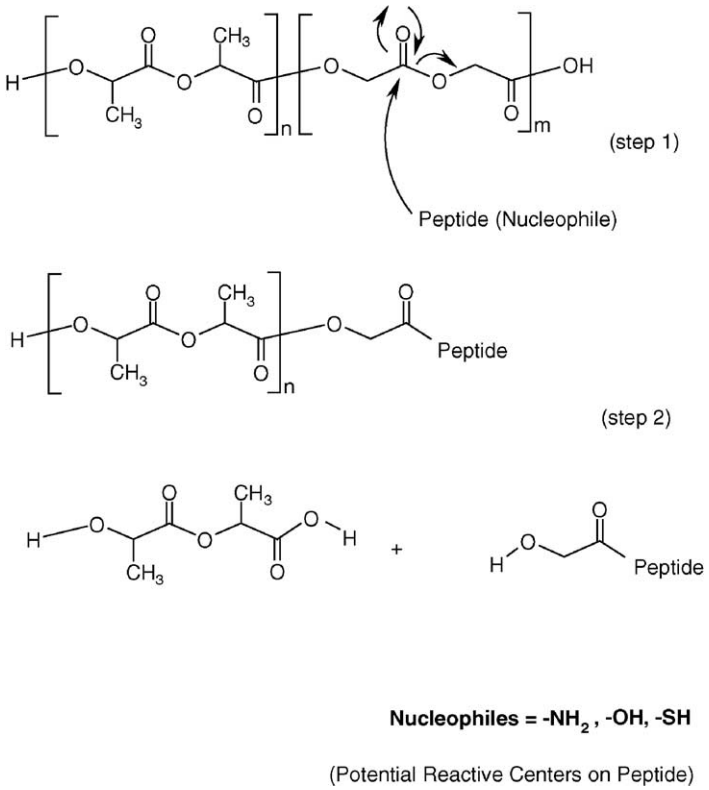

Fig. 1. Proposed mechanism of reaction between peptide and polymer (Lucke et al., 2002a; Lucke and Gopferich, 2003).

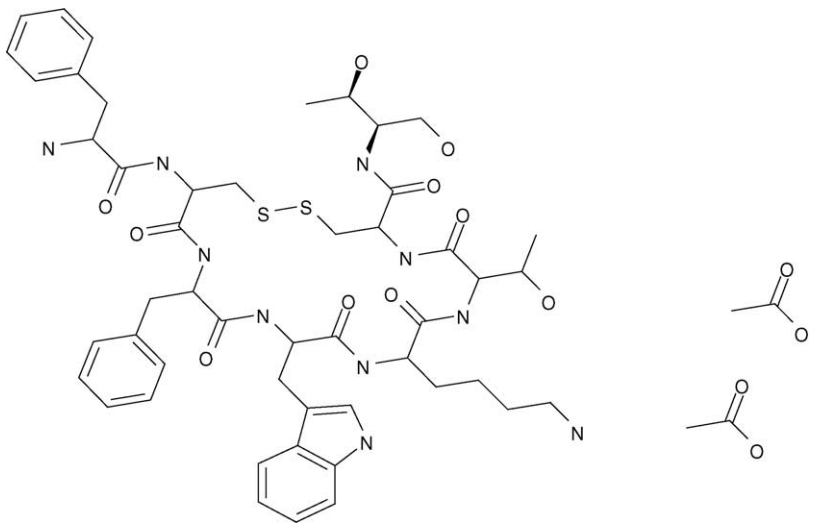

Fig. 2. Octreotide acetate.

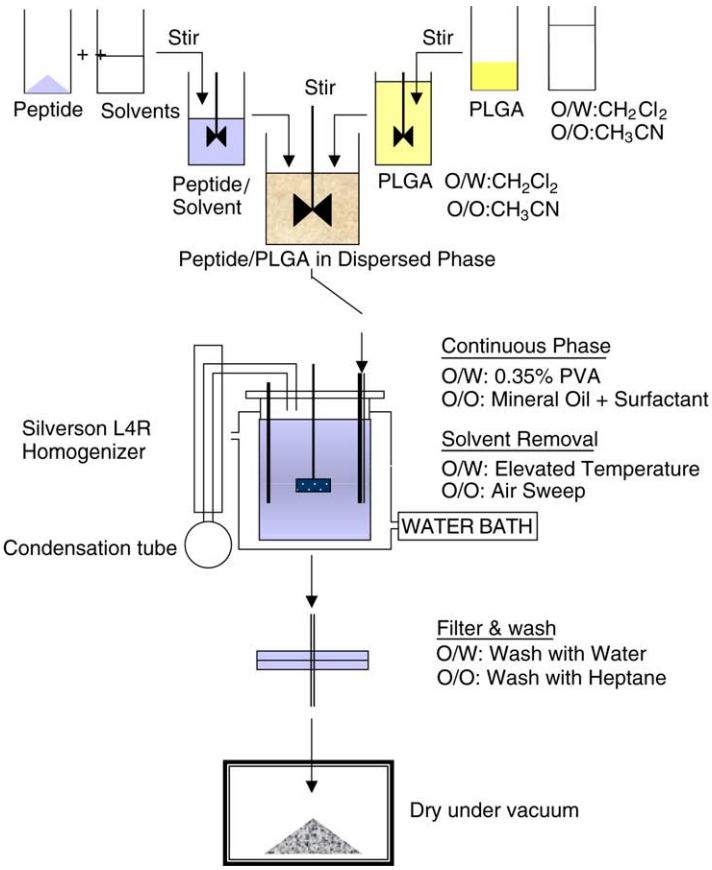

Fig. 3. Preparation of PLGA microspheres by either O/W or O/O dispersion methods.

sociated with Sandostatin LAR (octreotide acetate depot) delivery to human and veterinarian subjects.

2. Materials and methods

2.1. Materials

Octreotide acetate (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol; MW = 1018.4) was obtained from Bachem Inc. (Torrance, CA). Poly-(D,L-lactide-co-glycolide) 50:50 co-polymers were purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly-(D,L-lactide-co-glycolide) 85:15 co-polymer (MW = 9 kDa) and poly-(L-lactide) homopolymer (MW = 8 kDa) were purchased from Alkermes Inc. (Cincinnati, OH). Sandostatin LAR (Novartis; Basel, Switzerland) was commercially purchased for research and development efforts. All other chemicals used were of analytical reagent grade.

2.2. Microsphere preparation

Drug loaded (octreotide acetate) PLGA and PLA microspheres were prepared by both O/W and O/O dispersion methods with solvent extraction/evaporation as shown in Fig. 3 (Jeyanthi et al., 1996, 1997; Hermmann and Bodmeier, 1998; Thanoo and Murtagh, 1998, 2001). For the O/W method, the dispersed phase included methanol/methylene chloride solvent system and the continuous phase included 0.35% PVA aqueous solution. Octreotide acetate was dissolved in methanol and combined with the polymer solution dissolved in methylene chloride. This constituted the dispersed phase, which was slowly added to the continuous phase (0.35% (w/v) solution of PVA) while mixing. The

mixture was stirred at 5500 rpm with a Silverson™ L4R homogenizer (Silverson Machines Ltd., Waterside, England). Continuous stirring at 40 °C for 1 h along with continuous CP exchange and air sweep resulted in the extraction/evaporation of solvents from the formulation. Finally, hardened microspheres were recovered by vacuum filtration, followed by vacuum drying for 48 h at ambient temperature.

In contrast, the O/O method employed acetonitrile for solubilization of the polymer phase, which also included methanol and glacial acetic acid. Methanol helped to keep the drug solubilized in acetonitrile and acetic acid served to maintain a stable dispersion of microspheres without agglomerization. The drug–polymer solution was then dispersed in mineral oil containing surfactant. Solvent removal occurred at temperatures greater than 30 °C with continuous air sweeping for 12–24 h while stirring the dispersion. The microspheres were recovered by vacuum filtration and washed with heptane solvent.

2.3. Microsphere characterization

Microspheres were characterized by laser diffractometry using a Malvern 2600 laser sizer (Malvern, UK). Average particle sizes were reported in microns. Microspheres were also characterized for drug content and initial impurity content by an organic solvent extraction procedure using methylene chloride and buffer. The microspheres were dissolved in methylene chloride and the peptide was extracted with 0.1 M acetate buffer (pH 4.0). Peptide concentration was determined by HPLC.

2.4. Administration of microspheres to Sprague–Dawley rat animal model

Table 1 shows the injection regimen and particle recovery times for all groups of S–D rats involved in the study. Nine treatment groups were included with three receiving oil/water batches (PLGA 50:50, PLGA 85:15, and PLA 100), three more receiving oil/oil batches (PLGA 50:50, PLGA 85:15, and PLA 100), two receiving blank batches serving as controls (PLGA 50:50 and PLA 100), and a final group administered with Sandostatin LAR. All microsphere batches were reconstituted with vehicle diluent containing CMC, mannitol, and Tween-80 to make a uniform suspension prior to injection into rats. For each treatment group, eight animals received injections with varying doses of microspheres ($n = 2$ per dose) for the specified time point of recovery (e.g. 100 mg microspheres injected for 14 day recovery etc.). The purpose for injecting greater amounts of microspheres at later time points (i.e. 200 mg at 30 days and 250 mg at 40 days) was to account for mass loss phenomenon where sufficient material can be recovered at time of sacrifice.

With the eight treated rats from each group, two rats were sacrificed accordingly at the designated time point. After sacrifice, the subcutaneous tissue surrounding the injection site was excised from each rat. The recovered tissue samples were subsequently freeze-dried overnight to remove residual moisture and to preserve the embedded microparticles. Following Institutional Animal Care guidelines for the care and handling of animals species, all rats used for the study were

Table 1
Microsphere administration sequence with particle recovery at specified time points

Microsphere treatment groups ($n = 8$ per treatment group)	Particle-tissue recovery time point (days)	Amount of MS for administration (mg)
Group 1: PLGA 50:50 (O/W) Group 2: PLGA 85:15 (O/W) Group 3: PLA 100 (O/W)	14	100
Group 4: PLGA 50:50 (O/O) Group 5: PLGA 85:15 (O/O) Group 6: PLA 100 (O/O)	22	150
Group 7: Sandostatin LAR Group 8: PLGA 50:50 Blank Group 9: PLA 100 Blank	30	200
$n = 2$ Sacrificed per treatment group at each time point	40	250

housed in an animal care facility at the University of Kentucky with a controlled diet.

2.5. Microsphere recovery and extraction for peptide content

For extraction of peptide and peptide related substances, the freeze-dried particles embedded in tissue were first sliced with a scalpel for further treatment with 4 mL of a 1:1 mixture of dimethylsulfoxide/methylene chloride. After dissolution of polymeric material embedded within the tissue, 6 mL of 0.1 M acetate buffer (pH 4.0) was added to extract the peptide. The treated tissue material inside 15 mL polypropylene tubes was subsequently placed on a wrist shaker for 30 min and centrifuged at ∼2000 rpm for 20 min. Then 0.5 mL of supernatant from each tube was removed and treated with 0.5 mL 1% trifluoroacetic acid (TFA) for removal of interfering tissue proteins. A second centrifugation step was performed at 10,000 rpm. The final supernatant extract was removed and filtered through a 0.22 um syringe filter apparatus for HPLC and MALD-TOF MS instrumental analysis.

Extraction procedures were concurrently performed with a set of controls (Table 2) to determine the presence of interfering substances for the assay during peptide recovery. As shown in Table 2, excised subcutaneous tissue from untreated rats was blended with seven different mixtures containing various combinations from vehicle alone, peptide alone, blank microspheres, and drug-loaded microspheres. The controls were also subjected to treatment with 4 mL of 1:1 mixture of dimethylsulfoxide/methylene chloride and subsequent extraction with 0.1 M acetate buffer (pH 4.0) followed by identical extraction procedures as used above. The extraction procedures of recovered freeze-dried tissue from rats and the set of controls from Table 2 were not intended for absolute quantitation of peptide concentration for mass balance determinations. Rather, the extraction procedures employed were designed to assess the percentage peak purity by area percent of octreotide in each chromatogram generated by HPLC analysis. The major concern was the presence of interfering proteins or substances, which would appear in the HPLC chromatograms to superimpose with peptide peaks; hence, the controls were initiated.

2.6. Instrumental analyses

Buffer extracts were analyzed by RP-HPLC using an Alltech C18 column, 250 mm × 4.60 mm. A gradient elution method was utilized with mobile phase A (0.1% (v/v) trifluoroacetic acid in water) and mobile phase B (0.1% (v/v) trifluoroacetic acid in acetonitrile). The gradient was 80:20 (A:B) to 60:40 (A:B) over 25 min with a flow rate of 1.0 mL/min. UV absorbance was measured at 215 nm.

The buffer extracts were also analyzed by MALDI–TOF mass spectrometry (Bruker Daltronics). For the mass spectral analyses, all buffer extracts were placed under rotary vacuum conditions to evaporate the aqueous portions. Subsequently, the residual solid material was incorporated into a α-cyanohydroxy cinnamic acid (α-CHCA) matrix for crystallization.

3. Results

3.1. Microsphere characterization

Each of the drug-loaded batches manufactured by either the oil/water (O/W) or oil/oil (O/O) process was characterized for drug content and initial impurity content (Table 3). An insignificant reduction in initial impurity content was observed with the change in manufacturing process from O/W to O/O. In fact, with all

Table 2
Extraction controls employed for detection of interfering substances in assay[a]

Control #1	Excised tissue
Control #2	Excised tissue + 2.5 mg octreotide acetate
Control #3	Excised tissue + 25 mg blank PLGA 50:50 microspheres
Control #4	Excised tissue + 25 mg blank PLGA 50:50 microspheres + 2.5 mg octreotide acetate
Control #5	Excised tissue + 25 mg PLGA 50:50 (O/W) drug loaded microspheres

[a] All control materials were physically mixed with 0.5 g of excised subcutaneous tissue from untreated rats with the presence 0.3 mL vehicle.

Table 3
Characteristics of drug loaded microspheres

Microsphere characteristics	PLGA 50:50 (O/W)	PLGA 85:15 (O/W)	PLA 100 (O/W)	PLGA 50:50 (O/O)	PLGA 85:15 (O/O)	PLA 100 (O/O)
Polymer MW (kDa)	28	9	8	28	9	8
Manufacturing process	Oil/water	Oil/water	Oil/water	Oil/oil	Oil/oil	Oil/oil
Drug load (%)	8.60	11.5	9.20	6.19	7.36	7.82
Initial impurity content (percentage of drug load)	<1	<1	<1	<0.1	<0.1	<0.1

of the O/O batches, the initial drug content assay did not reveal the presence of any significant hydrophobic related peaks. With the O/W process, however, percentage impurities present on the chromatogram were ∼0.5% for all batches.

Although the reduction was minimal, the use of an O/O manufacturing process may have eliminated the influx of aqueous media into the dosage form during the initial dispersion process. Consequently, water could have played a role as a solvent mediator for initial acylation reactions occurring in manufacturing. For instance, in previous investigations with O/W batches, in vitro release testing performed in 0.1 M acetate buffer (pH 4.0) indicated 100% cumulative release (Murty et al., 2003b). With the superimposition of impurity release, however, total cumulative release was quantified to ∼107–109% for the respective batches. It was postulated from the proposed reaction mechanism in Fig. 1 that acyl transfer of the peptide amine (step 1) may have resulted during manufacturing and the hydrolysis of the conjugated peptide from the polymer backbone (step 2) could have occurred during incubation in aqueous media (i.e. in vitro release testing).

3.2. Peptide/impurity content from extraction of recovered tissue samples

The results of the extraction of tissue samples are presented in Table 4. At each time point, tissue particles were excised and were treated with the extraction procedure mentioned above. Supernatant samples were injected onto the HPLC to determine area percent of parent octreotide in relation with hydrophobic peptide related substances. According to Table 4, PLGA 50:50 (O/W batch) and the innovator formulation (Sandostatin LAR) displayed the lowest values for peptide purity; at 40 days, the tissue extracts contained 47.1 and 44.7% area percentage for remaining octreotide. Further, when comparing the PLGA 50:50 O/O batch with the PLGA 50:50 O/W batch, octreotide area percent was greater through 30 days for the batch prepared by non-aqueous dispersion. By day 40, however, the PLGA 50:50 O/O batch also displayed ∼50% purity for parent compound, which was comparable to both the Sandostatin LAR results as well as the O/W batch results for the same polymer.

Consequently, one could argue that indeed by altering the dispersion technique, initial stability of pep-

Table 4
Relative area percentage of octreotide from extracted rat tissue samples ($n = 2$ sacrificed per time point)

	PLGA 50:50 (O/W)	PLGA 85:15 (O/W)	PLA 100 (O/W)	PLGA (50:50) (O/O)	PLGA 85:15 (O/O)	PLA 100 (O/O)	Sandostatin LAR
Days[a]	Average (A.D.)[b]	Average (A.D.)	Average (A.D.)	Average (A.D.)	Average (A.D.)	Average (A.D.)	Average (A.D.)
0[c]	>99%	>99%	>99%	>99.9%	>99.9%	>99.9%	>99%
∼14	69.9 (1.08)	87.5 (0.460)	91.4 (2.15)	84.5 (0.388)	91.2 (0.328)	95.4 (0.908)	63.7 (0.222)
∼22	53.8 (0.385)	82.9 (1.06)	92.6 (0.0325)	74.0 (3.98)	83.9 (1.13)	92.2 (0.375)	59.5 (0.0800)
∼30	47.1 (0.648)	78.3 (2.00)	90.0 (2.24)	60.2 (0.547)	77.5 (0.498)	87.0 (0.268)	44.9 (1.83)
∼40	47.1 (4.22)	75.9 (0.605)	87.4 (1.13)	50.27 (0.220)	75.2 (1.10)	88.2 (1.50)	44.7 (0.850)

[a] Time points are ±24 h.
[b] Average deviation reported from the average of two independent tissue samples.
[c] Zero time assay from initial drug content determination prior to injection to rats.

tide may be enhanced either due to minimized acylation (step 1 of Fig. 1) during manufacturing or due to trapping of PLGA oligomers in the O/O dispersion. This trapping phenomenon could potentially result in the formation of ionic complexes between peptide and polymer to subsequently minimize chemical activity (Kostanski et al., 2000a; Kostanski and DeLuca, 2000b). The ionic complex theory arises from the previous investigation with PLA microspheres containing orntide acetate, which reportedly formed release plateau regions due to peptide–oligomer interactions thereby reducing physical activity of molecules. Further, the complex was postulated to occur between cationic amines with anionic carboxylic end groups at neutral pH values (Murty et al., 2001).

For both the PLGA 85:15 batches prepared by O/W and O/O dispersion, however, there appeared to be minimum difference in purity of parent octreotide from tissue samples collected through the time points. For instance, by 14 days, the O/W and O/O batches showed values of ∼87.5 and 91.2%, respectively. By 40 days, the values for the O/W and the O/O batches were ∼75.9 and ∼75.2%. As a result, the process change did not dramatically change the purity of octreotide at the respective time points. For the PLA 100 microspheres prepared by both dispersion techniques, area percentage values by day 40 were 87.4 and 88.2% for the O/W and O/O batches.

As reported previously, the polymer co-monomer ratio influences the extent of formation of acylated products under in vitro release testing conditions (Murty et al., 2003b). According to Table 4, the same phenomenon is observed after in vivo incubation in the subcutaneous tissue of live rats. The polymers with higher glycolide content in the co-monomer ratio appear to be more conducive to the formation of acylated peptide. Although absolute quantitation of peptide concentration was not possible in vivo, the area percent values were determined to reflect on purity of octreotide in each buffer extract. Fig. 4 shows area percent as a function of time for the three O/O batches. From the graph, one could assign the following rank order for peptide purity in microspheres made from polymers with varying co-monomer ratio:

PLA 100 > PLGA 85 : 15 > PLGA 50 : 50.

The co-monomer ratio effect has been attributed to the presence of the methyl substituent on the alpha carbon

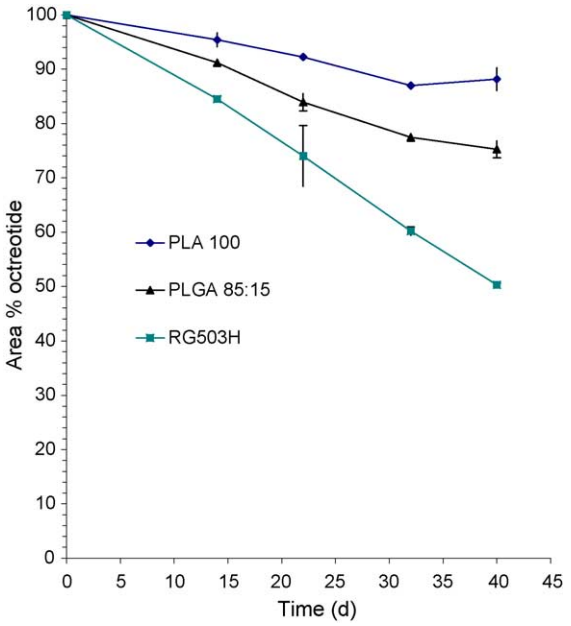

Fig. 4. Relative area percentage octreotide in extracted O/O microspheres.

present only in lactic acid monomers, which results in the inhibition of nucleophilic attack (Fig. 5). As a result, polymers with greater lactide content in the co-monomer ratio appear to produce fewer percentages of impurities.

3.3. Controls for extraction procedures

As previously stated, extraction of microspheres recovered from tissue administration sites was not intended for quantitation of peptide concentration for mass balance determinations in vivo. After administra-

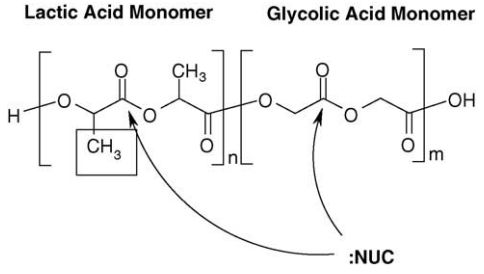

Fig. 5. Proposed explanation of reduced reactivity of nucleophilic species with lactic acid monomers.

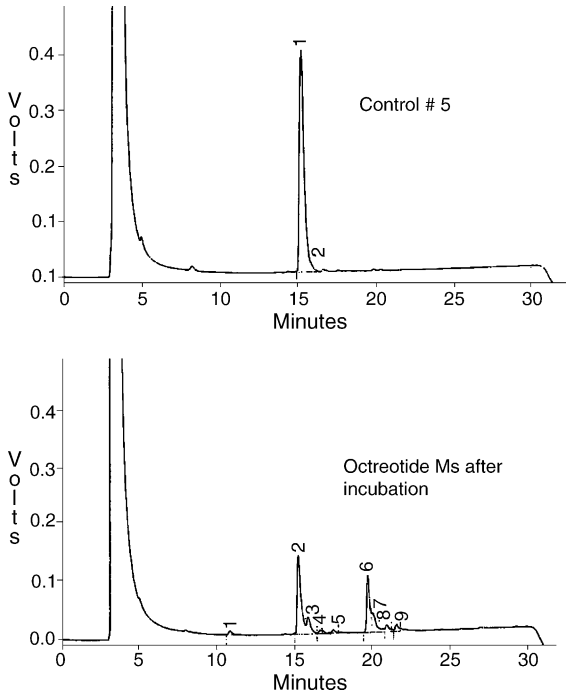

Fig. 6. Chromatograms of buffer extracts from control #5 (excised tissue with PLGA 50:50 octreotide MS) and PLGA 50:50 octreotide MS after 40 days incubation.

tion of microspheres, the injectable particulate matter was speculated to spread across the adipose tissue layers. In addition, increasing amounts of microspheres were administered for recovery at later time points (i.e. 250 mg at 40 days recovery) to account for mass loss phenomenon. As a result, the study was designed to assess percent peptide purity at each time point as opposed to peptide quantitation for mass balance relationships. With each extraction procedure, however, a determination of interfering tissue substances in the HPLC assay required further examination. Hence, the set of extraction controls detailed in Table 2 were implemented.

Fig. 6 shows chromatograms from the HPLC analysis of supernatant extracts from control #5 and PLGA 50:50 microspheres (incubated for 40 days). For each control, excised tissue from subcutaneous region of a sacrificed rat was physically mixed or homogenized with respective contents shown in Table 2. The chromatograms from controls #1–5 did not reveal any interfering peaks through the expected eluting time regions for the peptide molecules of interest (octreotide and hydrophobic related substances). As a result, the tissue-polymer interactions resulting from the incubation or extraction procedures, which would interfere with the HPLC assay, were assumed negligible. For instance, for control #4, with the addition of 2.5 mg octreotide to the tissue/polymer homogenized mixture, the expected peptide peak eluted at 15 min without the presence of interfering peaks. For PLGA 50:50 octreotide microspheres recovered after 40 days of incubation in live rats, hydrophobic related substances were apparent and eluted after the octreotide peak (∼15 min). The results concurred with previous LC–MS/MS data from where the glycoyl and lactoyl substituted peptide species were detected after the elution of pure octreotide on a C-18 separation column (Murty et al., 2003b).

Finally, the extraction of blank microspheres (PLGA 50:50 and PLA 100) incubated in live rats up to 40 days was performed as an additional control set. The chromatograms of the extracted blank microspheres from all time points (i.e. 14, 22, 30, and 40 days) also did not reveal the presence of any eluting peaks, similar to the results obtained with controls #1–4. The postulated acylation mechanism shown in Fig. 1, as a result, was suspected to be responsible for the creation of the lactoyl and glycoyl adducts during incubation of microspheres in vivo. To confirm the findings, MALDI–TOF mass spectrometry of all extracts from drug-loaded microspheres was performed.

3.4. Mass spectral analysis and results

Table 5 summarizes the results from the mass spectral analysis of supernatant extracts from O/O batches and Sandostatin LAR. Incidentally, similar spectral peaks were observed between the O/W and O/O batches, and hence the later set of data is presented. The MALDI–TOF analyses were purely qualitative due to crystallization and ionization procedures utilized with the matrix material cyanohydroxy cinnamic acid (CHCA matrix). Upon mixing the matrix solution with the sample, a crystallization step was performed on a sample plate. The crystallization step could not be precisely controlled and thus the lattice structure infinitely varied each time the procedure was employed. Consequently, upon contact of the laser beam with the crystal surface, ionization potentials of the compound of interest dramatically varied from sample to sample. From the previous investigation with in

Table 5
Major peaks identified from MALDI–TOF mass spectra from O/O batches and Sandostatin LAR

Recovery time point (days)	PLGA 50:50 (*m*/*z*)	PLGA 85:15 (*m*/*z*)	PLA 100 (*m*/*z*)	Sandostatin LAR
∼14	1041, 1099, 1157	1041, 1099, 1113	1041, 1113, 1185	1041, 1099, 1157, 1171
∼22	1041, 1099, 1113, 1157, 1215	1041, 1099, 1113, 1185	1041, 1113, 1185	1041, 1099, 1157, 1215
∼30	1041, 1099, 1157, 1171, 1185	1041, 1099, 1113, 1171, 1185	1041, 1113, 1185	1041, 1099, 1157, 1171, 1185
∼40[a]	1041, 1099	1041, 1099, 1113, 1171, 1185	1041, 1113, 1185	1041, 1099

[a] Note: low sample concentration resulted in difficulty for identification of all peaks at 40 days for PLGA 50:50 and the Sandostatin LAR.

vitro incubation of O/W microspheres in PBS (pH 7.4), potential adduct species were expected over the parent molecular weight of 1041 *m*/*z* (1018 *m*/*z* + 23 *m*/*z* to account for sodiated adducts) (Murty et al., 2003b).

Potential glycoyl adducts (for 50:50 and 85:15 microspheres only)

(1) Adduct 1: 1041 *m*/*z* + 58 *m*/*z* = 1099 *m*/*z*.
(2) Adduct 2: 1041 *m*/*z* + 58 *m*/*z* + 58 *m*/*z* = 1157 *m*/*z*.
(3) Adduct 3: 1041 *m*/*z* + 58 *m*/*z* + 58 *m*/*z* + 58 *m*/*z* = 1215 *m*/*z*.

Potential lactoyl adducts (for 50:50, 85:15, and PLA microspheres)

(4) Adduct 4: 1041 *m*/*z* + 72 *m*/*z* = 1113 *m*/*z*.
(5) Adduct 5: 1041 *m*/*z* + 72 *m*/*z* + 58 *m*/*z* = 1171 *m*/*z*.
(6) Adduct 5: 1041 *m*/*z* + 72 *m*/*z* + 72 *m*/*z* = 1185 *m*/*z*.

Thus, glycoyl adducts were expected with polymers only containing glycolic acid monomers and lactoyl adducts with all polymers employed in this study. When corroborating the expected adducts values with the data presented in Table 5, several possibilities were proposed for each *m*/*z* peak observed. In addition, the previous LC–MS/MS analysis provided evidence for only a mono-glycoyl substitution at the N-terminus. Thus, glycoyl-glycyl, lactoyl-lactyl, and mono-lactoyl substitutions were not detected at the N-terminus.

For instance, with the PLGA 50:50 microspheres at days 14 and 22, four major peaks were identified at 1099, 1113, 1157, and 1215 *m*/*z*, which could have represented the separate adduct species shown below (**G** = glycoyl and **L** =lactoyl).

(1) 1099 *m*/*z* = mono-glycoyl substitution at either the N-terminus or lysine group

H-[D-Phe + **G**]-Cys-Phe-D-Trp-Lys-Thr-Cys
-Thr-ol (1099 m/z)

or

H-D-Phe-Cys-Phe-D-Trp-[Lys + **G**]
-Thr-Cys-Thr-ol (1099 m/z)

(2) 1113 *m*/*z* = mono-lactoyl substitution at the lysine group only

H-D-Phe-Cys-Phe-D-Trp-[Lys + **L**]
-Thr-Cys-Thr-ol (1113 m/z)

(3) 1157 *m*/*z* = di-glycoyl substitution or glycoyl-glycyl substitution at lysine

H-[D-Phe + **G**]-Cys-Phe-D-Trp-[Lys + **G**]
-Thr-Cys-Thr-ol (1157 m/z)

or

H-D-Phe-Cys-Phe-D-Trp-[Lys + **G** + **G**]
-Thr-Cys-Thr-ol (1157 m/z)

(4) 1215 *m*/*z* = mono-glycoyl substitution at the N-terminus with glycoyl-glycl substitution at lysine or glycoyl-glycl-glycl substitution at lysine only

H-[D-Phe + **G**]-Cys-Phe-D-Trp-[Lys + **G** + **G**]
-Thr-Cys-Thr-ol (1215 m/z)

or

H-D-Phe-Cys-Phe-D-Trp-[Lys + **G** + **G** + **G**]
-Thr-Cys-Thr-ol (1215 m/z)

At day 30, two additional peaks appeared from PLGA 50:50 microspheres at 1171 *m*/*z* and 1185 *m*/*z* as shown in Fig. 7. These peaks could have represented the following adduct species:

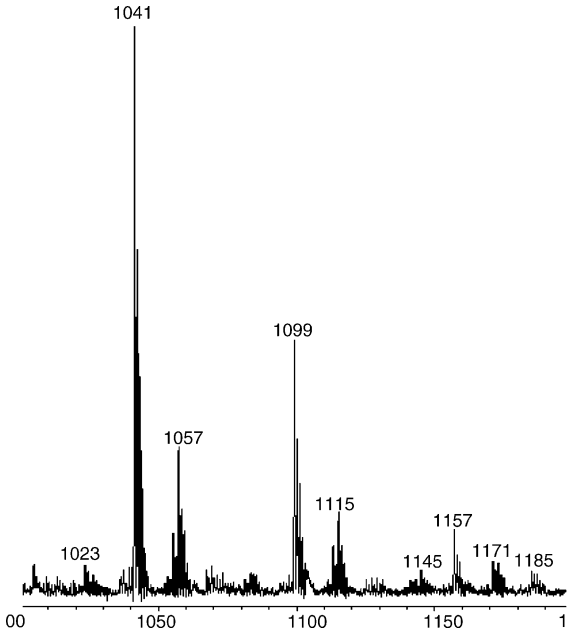

Fig. 7. Mass spectrum of extract from PLGA 50:50 microspheres incubated for 30 days.

(5) 1171 *m*/*z* = mono-glycoyl and mono-lactoyl substitutions at the N-terminus and lysine residue, respectively; or a lactoyl-glycyl substitution at the lysine residue

H-[D-Phe + **G**]-Cys-Phe-D-Trp-[Lys + **L**]

-Thr-Cys-Thr-ol (1171 m/z)

or

H-D-Phe-Cys-Phe-D-Trp-[Lys + **L** + **G**]

-Thr-Cys-Thr-ol (1171 m/z)

(6) 1185 *m*/*z* = lactoyl-lactyl substitution at the lysine residue

H-D-Phe-Cys-Phe-D-Trp-[Lys + **L** + **L**]

-Thr-Cys-Thr-ol (1185 m/z)

At day 40, mimimal sample concentration resulted in detection of only one adduct at 1099 *m*/*z* (glycoyl substitution). Hence, the MALDI–TOF mass spectrometer provided qualitative evidence for the presence of adduct species irrespective of concentration present in the buffer extract. As a result, adduct species may have been present at various time points with the possibility of being undetected by the instrument. These data contrast with the LC–MS/MS evidence where nine separate adduct species were detected after only 14 days of incubation in vitro (Murty et al., 2003b).

As for the PLGA 85:15 microspheres, two major related substances were present through the course of the study. At all time points, the 1099 *m*/*z* peak (mono-substituted glycoyl adduct) and the 1113 *m*/*z* peak (mono-substituted lactoyl adduct) were seen. In addition, minor peaks were observed at 1171 *m*/*z* and 1185 *m*/*z* at days 30 and 40. For the PLA microspheres, two major impurities were detected through the time points including the mono-substituted lactoyl adduct with *m*/*z* value of 1114 (otherwise seen as 1113 *m*/*z* on some spectra). In addition, the peak observed at 1186 *m*/*z* represented di-lactoyl substitutions as seen previously in both PLGA 50:50 and PLGA 8515 microspheres. Finally, the mass spectra for the Sandostatin LAR microspheres revealed the presence of peaks at 1099, 1157, 1171, 1185, and 1215 *m*/*z*. The spectral results were quite similar with the results obtained with PLGA 50:50 microspheres, which is logical considering the polymer utilized in the commercial formulation is also a 50:50 co-polymer.

4. Discussion

As observed during in vitro release testing and incubation experiments, the formation of hydrophobic related substances also occurred during in vivo incubation. The polymer co-monomer ratio played an important role in terms of extent of impurity formation where microspheres formulated with PLA homopolymers resulted in least detrimental stability effect. The in vivo evidence for the acylation reaction pathways during the time course of sustained drug delivery from PLGA microspheres raises important therapeutic issues for human and animal subjects. The instability of the peptide can result in decreased pharmacological activity, enhanced immunogenic properties, and increased safety concerns.

Although Sandostatin LAR has been proven safe and effective as a drug delivery dosage form, the question remains whether the octreotide depot formulation

can be improved for the above-mentioned reasons. For therapeutic delivery in a clinical setting, the most desired outcome is for the delivery of parent peptide molecule without chemical modification through the 30–40 day time course required for PLGA or PLA polymers to degrade and release at the subcutaneous or intramuscular injection sites. Hence, further research is required to identify methods to completely stabilize octreotide acetate formulated in biodegradable polymers.

Prior to the initiating further formulation efforts to eliminate acylation, however, the present investigations were extended for further study in an in vitro incubation model. The purpose was to understand the physical factors responsible for the formation of adduct species in a controlled environment where invasive parameters could be assessed. In essence, questions remained as to the role of polymer mass loss, polymer hydration, and microenvironmental pH during peptide degradation inside microspheres. These factors were difficult to quantitate in an in vivo environment.

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