

Direct Determination of the Peptide Content in Microspheres by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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A quantitative determination of peptides incorporated into poly(D,L-lactide-co-glycolide) microspheres by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was accomplished in a single step without pretreatment for extracting the peptide from the microsphere. The conventional extraction methods often underestimate the actual amount of peptide because of incomplete extraction from the microspheres or loss during the procedures. In this study, the microspheres dissolved in acetonitrile containing 0.1% trifluoroacetic acid were mixed with matrix solution containing the internal standard, and the peptide content was directly determined by MALDI-TOF MS. The drug content values determined by MALDI-TOF MS in both the leuprolide- and salmon calcitonin-incorporated microspheres were closer to the theoretical contents than those determined by the conventional extraction method. This method using MALDI-TOF MS could be a good alternative to time-consuming and less-accurate conventional methods.

In the development of a microsphere formulation for long-term controlled delivery of peptides and proteins, an accurate and precise determination of drug amount incorporated into microspheres is essential for designing the microsphere preparation process, testing drug release under in vitro experimental conditions, and dosing the targeted amount for in vivo studies.¹ Until now, several methods have been employed, including amino acid analysis, radioactivity measurement, colorimetric assay, and HPLC.^{2–5} However, these methods necessitate troublesome pretreatment steps, such as hydrolysis of microsphere by 6 N HCl or 1 N NaOH, or extraction using acetonitrile, alkaline sodium dodecyl sulfate (SDS) solution, dimethyl sulfoxide, or methylene chloride. These treatments also give variable results with microsphere formulations of differing polymer types, release characteristics, excipients or stabilizers, and physical characteristics.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a soft ionization method and has become a rapid and convenient method for the characterization of biomolecules such as proteins and peptides in various research fields.^{6–9} In general, it has been used to provide the molecular weight and qualitative information about sample composition. The quantitation of peptides and proteins by MALDI-TOF MS is difficult because significant variations in ion signals can occur from shot to shot and spot to spot within a given sample.^{10,11} These variations in signal intensity are due primarily to the heterogeneous nature of the cosolidified analyte and matrix mixture, fluctuations in laser energy, and changes in detector response. Nevertheless, the use of appropriate internal standards to normalize instrument responses minimizes these problems, allowing useful quantitative information to be obtained.^{12,13} Recently, there have been reports on quantitative determinations with MALDI-TOF MS employing internal standards.^{14,15}

MALDI-TOF MS has been shown to be useful for the characterization of proteins adsorbed directly onto biomaterials.^{16,17} These studies suggested that proteins adsorbed onto polymeric biomaterials, such as polyurethane, poly(vinylidene fluoride), and poly(ethylene terephthalate), could be analyzed by MALDI-TOF MS. A distinct advantage of MALDI-TOF MS for characterizing biomolecules is the possibility of probing proteins in a nonaqueous condition. This has been demonstrated in analyses of proteins electroblotted into polymeric membranes, small peptides encapsulated into polymeric silica, and DNA strands covalently attached

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to a solid surface.^{18–20} These studies suggest the possibility of application of MALDI-TOF MS for characterization and quantitation of peptides and proteins incorporated into microspheres.

The purpose of this study was to evaluate whether MALDI-TOF MS could directly detect the peptides incorporated into microspheres and quantitate the peptide content in an accurate and convenient manner. The conventional extraction methods often underestimate the actual amount of peptide/protein because of incomplete extraction from the microspheres or loss during the procedures.¹ Direct determination without extraction procedure may eliminate or minimize these problems. For the determination of drug content in the microspheres by MALDI-TOF MS, an internal standard addition method was used.¹² Leuprolide- and salmon calcitonin (sCT)-incorporated poly(D,L-lactide-co-glycolide) (PLGA) microspheres were prepared and tested as the model microspheres for this study.

EXPERIMENTAL SECTION

Materials. PLGA (50:50) with free carboxyl end groups, Resomer RG503H (MW 28 000), was obtained from Boehringer Ingelheim (Ingelheim, Germany). Leuprolide, triptorelin, salmon calcitonin (sCT), and human parathyroid hormone (hPTH) (1–34) were obtained from Bachem (Torrance, CA). α -Cyano-4-hydroxycinnamic acid (α -CHCA) was purchased from Sigma (St. Louis, MO). Acetonitrile and methylene chloride (HPLC grade) were purchased from J. T. Baker (Phillipsburg, NJ). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL). All other chemicals were of analytical grade and were obtained commercially.

Preparation of Microspheres. PLGA microspheres containing leuprolide or sCT were prepared by a dispersion method followed by solvent extraction and evaporation.^{21,22} The target loads of leuprolide and sCT microspheres were 18% and 5%, respectively. Briefly, a solution of peptide in methanol was combined with a solution of PLGA in methylene chloride and stirred using a magnetic stirrer for \sim 10 min. The clear solution was then slowly dispersed in 0.35% (w/v) solution of poly(vinyl alcohol) and stirred at 3500 rpm with a Silverson L4R homogenizer (Silverson Machines Inc., East Longmeadow, MA). The temperature of the reactor was maintained at 25 °C for 30 min during extraction of the solvents from the microspheres and after that at 40 °C for 60 min using a circulating water bath to facilitate evaporation. Once the microspheres were hardened, the contents of the reactor were passed through an 0.8- μ m membrane filter (Gelman Sciences, Ann Arbor, MI), and the recovered microspheres were washed with water and dried under reduced pressure for 48 h at room temperature.

Direct Peptide Detection in the Microspheres by MALDI-TOF MS. MALDI-TOF MS was performed using a Voyager Biospectrometry Workstation (PerSeptive Biosystem, MA). The matrix solution for MALDI-TOF MS was a saturated solution of

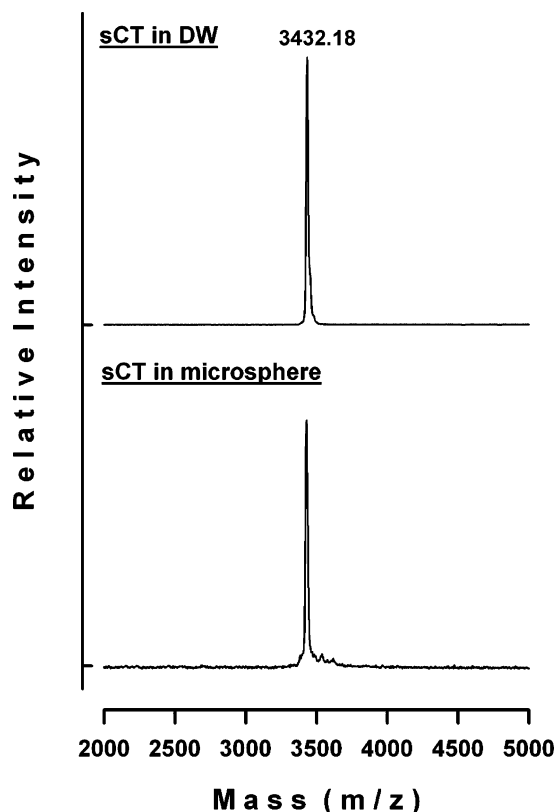


Figure 1. Direct detection of sCT in the microspheres by MALDI-TOF MS.

α -CHCA in 50:50 water/acetonitrile with 0.1% TFA. Approximately 1 mg of sCT microspheres was deposited onto the MALDI-TOF MS sample plate and 1 μ L of α -CHCA solution was added. The samples were dried by vacuum evaporation prior to MALDI-TOF MS. Data for 2-ns pulses of the 337-nm nitrogen laser were averaged for each spectrum in a linear mode, and a positive ion TOF detection was performed using an accelerating voltage of 25 kV. Spectra were smoothed with a 19-point Savitzky-Golay filter, and the external calibration was performed using Mass Standard Kit 1 (PerSeptive Biosystems, MA), a mixture of angiotensin I, ACTH (1–17), ACTH (18–39), ACTH (7–38), and bovine insulin.

Adsorption of Peptide to the Microspheres. Blank microspheres were prepared by the same method described in Preparation of Microspheres without peptide. Approximately 2 mg of blank microspheres was added to 2 mL of 50 μ g/mL sCT solution in 10 mM phosphate buffer (pH 7.4). The vials were mounted on a rotary wheel vertically spinning at a speed of 18 cycles/min. After incubation for 1 day at 25 °C, the vials were centrifuged at 12 000 rpm for 10 min, and the supernatant was analyzed by reversed-phase HPLC for determining the amount of sCT adsorbed to the microspheres.

Determination of Peptide Content in the Microspheres by MALDI-TOF MS. The calibration curves were constructed in the range of 20–500 μ g/mL for leuprolide and 5–200 μ g/mL for sCT. Precision and accuracy were determined using five replicates of each of three concentrations: 40, 80, and 160 μ g/mL. Within-run precision and accuracy were calculated from all of the replicates of each of the three concentrations run in 1 day. Between-run precision and accuracy were calculated from all replicates of the same concentrations on three separate days.

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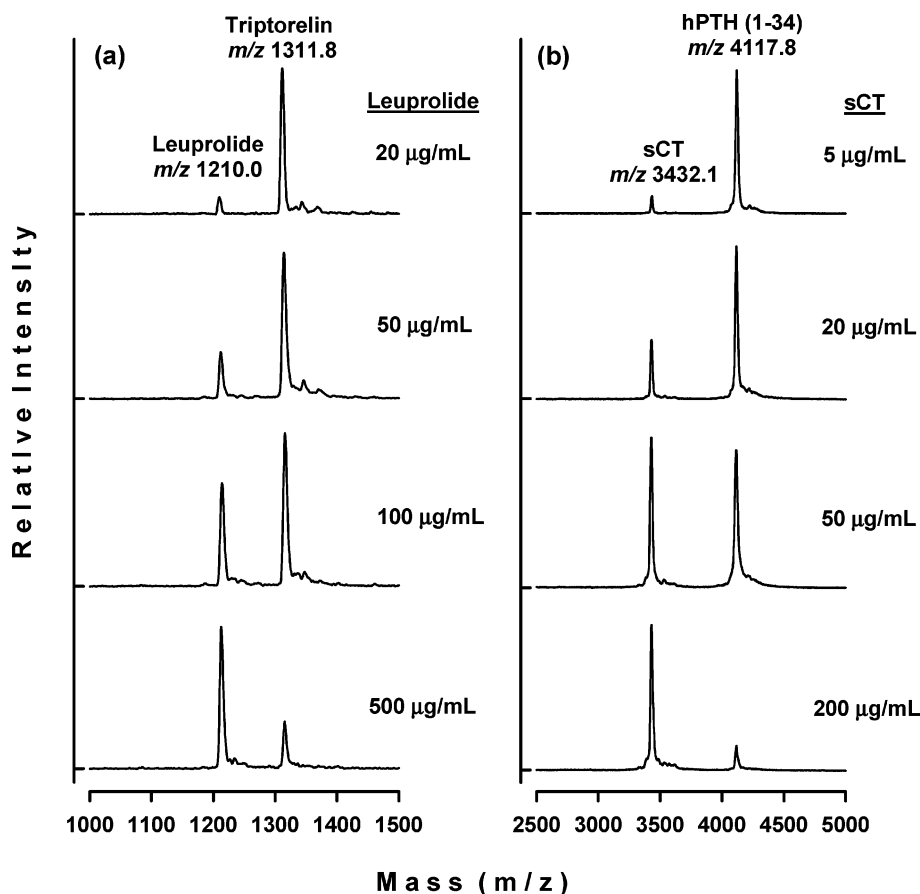


Figure 2. MALDI-TOF mass spectra of target peptides obtained by addition of a constant amount of internal standard (a, leuprolides by addition of triptorelin; b, sCTs by addition of hPTH (1–34)).

Approximately 5 mg of peptide-loaded microspheres was added to an appropriate amount of acetonitrile containing 0.1% TFA. Each 1 μL of microspheres suspended in acetonitrile and each internal standard (100 $\mu\text{g}/\text{mL}$ of triptorelin for leuprolide and hPTH (1–34) for sCT) was mixed with 1 μL of α -CHCA solution and spotted into a well of the MALDI sample plate. The samples were dried by vacuum evaporation prior to MALDI-TOF MS. The acquisition of MALDI-TOF MS spectrum was performed by a series of 256 laser shots. Other conditions were the same as described above.

Determination of Peptide Content by Conventional Extraction Method. Peptide content in the microspheres was determined by reversed-phase HPLC after dissolving the microspheres in methylene chloride (MC) and extracting the peptide with 0.1 M acetate buffer (pH 4.0), described previously.^{21,22} Briefly, 10 mg of microspheres was dissolved in 2 mL of methylene chloride, and the peptide was extracted with 10 mL of 0.1 M acetate buffer by agitation for 1 h. The leuprolide or sCT in the aqueous phase was determined by HPLC using a LiChrospher RP 18 column (4.0 \times 125 mm, 5 μm , Merck, Germany) with a mobile phase of 0.1% TFA in distilled water (buffer A) and acetonitrile containing 0.1% TFA (buffer B). The mobile phase was run with a linear gradient from 20 to 80% buffer B for 15 min at a flow rate of 1.0 mL/min, and the effluent was monitored at UV 215 nm. The injection volume was 20 μL .

RESULTS AND DISCUSSION

Figure 1 displays MALDI-TOF MS spectra of sCTs in water and from PLGA microspheres. When the microsphere particles

containing sCT were placed on a sample plate and crystallized by adding the matrix solution of α -CHCA, the result of MALDI-TOF MS yielded a characteristic $[\text{M} + \text{H}]^+$ signal at m/z 3432, which is consistent with the mass of the standard sCT in water. This implies that the presence of peptide incorporated into microspheres could be detected by MALDI-TOF MS without any pretreatment, such as extraction. The peaks that may derive from PLGA polymers were not observed in the mass range from 1000 to 9000. With this evidence, the approach to directly determine the content of peptide incorporated into the microspheres using MALDI-TOF MS was attempted.

In a previous study, the MALDI-TOF MS was successfully applied for monitoring the acylation reaction of peptides in the degrading PLGA microspheres,⁹ which has been recently regarded as another stability issue in the peptide microspheres.^{23,24} The direct detection capacity of MALDI-TOF MS for peptide in the microsphere suggests that it can be applied for determining the acylation of peptide in the microspheres caused by the presence of moisture during storage. The procedure of MALDI-TOF MS can be performed in \sim 10 to 20 min from sample preparation to data acquisition. The detection of peptide incorporated into microspheres has been mostly accomplished by reversed-phase HPLC after the extraction and separation of peptide from the PLGA polymer, because the polymer may interfere with the peptide detection or deteriorate the HPLC column due to

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incomplete solubilization in the HPLC solvent. To extract sufficiently the peptide from the microspheres, an appropriate extraction method should be developed according to each peptide drug. MALDI-TOF MS will eliminate the tedious steps.

For quantitation using MALDI-TOF MS, selection of an appropriate internal standard is important. When proteins or peptides similar to the target molecule were used as internal standards, a linear correlation between the peak height ratio and the sample loading was found.¹³ In this study, the internal standards selected for quantitating leuprolide (nonapeptide, MW 1209) and sCT (32 amino acids, MW 3432 Da) from microspheres were triptorelin (decapeptide, MW 1311.5) and hPTH (1–34) (34 amino acids, MW 4118 Da). Triptorelin is a synthetic analogue of the naturally occurring gonadotropin-releasing hormone and has a sequence of eight amino acids identical to that of the leuprolide. The hPTH (1–34) is an active fragment of PTH, which plays a major role in regulating blood calcium concentration with calcitonin, and both have been used for the treatment of osteoporosis.²⁵

Figure 2a represents the MALDI-TOF mass spectra generated by addition of the constant amount of internal standard triptorelin (100 $\mu\text{g}/\text{mL}$ in distilled water containing 0.1% TFA) to leuprolide solutions with various concentrations. The spectra have been normalized to the triptorelin molecular ion signal. As the triptorelin was added to the higher concentration of leuprolide, the molecular ion signals of leuprolide increased relative to the triptorelin signal. A good linear relationship (correlation $R^2 > 0.999$) was obtained between the normalized signal intensity and the concentration of leuprolide (20–500 $\mu\text{g}/\text{mL}$) present in the solution. The calibration curve was constructed by plotting the leuprolide concentrations vs the signal ratio (leuprolide/triptorelin) and was used to determine the leuprolide content incorporated into the PLGA microsphere. The accuracy and precision were in the range of 1.5–2.8% relative standard deviation. When the constant amount of hPTH (1–34) (100 $\mu\text{g}/\text{mL}$ in distilled water containing 0.1% TFA) was added to various concentrations of sCT, a good linear relationship (correlation $R^2 > 0.999$) was also obtained between the normalized signal intensity and the various concentrations of sCT (5–200 $\mu\text{g}/\text{mL}$) present in the solution (Figure 2b). The accuracy and precision were in the range of 2.2–4.5% relative standard deviation. Although the quantitation limit of sCT was higher than that of leuprolide with the same concentration of internal standard, the sensitivity of leuprolide could be improved to 5 $\mu\text{g}/\text{mL}$ when 50 $\mu\text{g}/\text{mL}$ triptorelin was used. However, the calibration curve constructed with 100 $\mu\text{g}/\text{mL}$ triptorelin was suitable for the quantitation of a leuprolide content at a target load of 18% in the microspheres.

To investigate if MALDI-TOF MS could directly determine the actual content of peptide incorporated into the microspheres, the sCT was adsorbed to blank PLGA microspheres²⁶ and analyzed by MALDI-TOF MS (Figure 3). When the sCT was incubated with blank PLGA microspheres for 1 day at room temperature, >95% of the peptide was adsorbed to the surface of the microspheres, as determined by reversed-phase HPLC analysis of the supernatant following centrifugation. In Figure 3b, the MALDI-TOF MS spectrum shows the residual amount of sCT in the supernatant after incubation of 1 day. However, when the microspheres

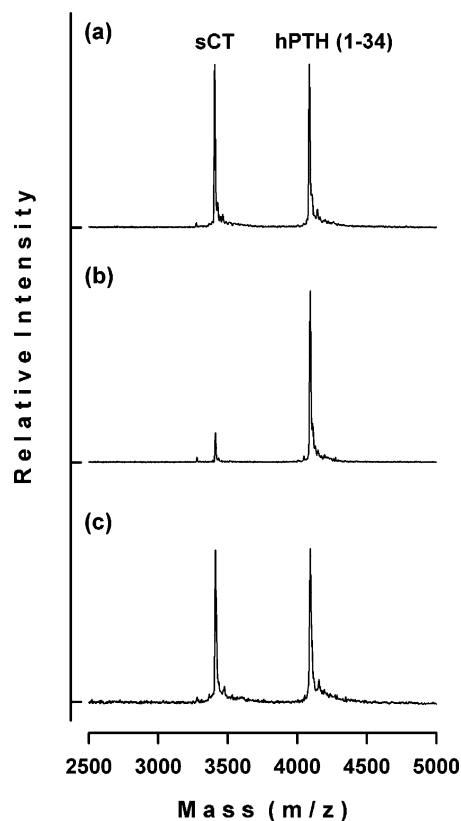


Figure 3. MALDI-TOF MS spectra before adsorption of sCT to PLGA (a), of supernatant after centrifugation of sCT incubated with PLGA polymers for 1 day (b), and of suspension without centrifugation of sCT incubated with PLGA polymers for 1 day (c). As the internal standard, hPTH (1–34) of 100 $\mu\text{g}/\text{mL}$ in distilled water was used.

Table 1. Determination of Peptide Contents in Microspheres

microsphere	batch	target load, %	MALDI-TOF MS, %	extraction method, ^a %
leuprolide microsphere	1	18	16.2 \pm 0.9 ^b	15.8 \pm 0.6 ^b
	2	18	15.5 \pm 1.0	15.0 \pm 0.8
	3	18	16.1 \pm 0.8	15.4 \pm 0.6
sCT microsphere	1	5	4.6 \pm 0.5	4.1 \pm 0.3
	2	5	4.5 \pm 0.4	3.9 \pm 0.4
	3	5	4.5 \pm 0.4	3.8 \pm 0.3

^a Extraction method was performed by MC and acetate buffer extraction followed by HPLC analysis. ^b The values are means of peptide loadings \pm standard deviation of triplicate measurements.

suspended without centrifugation were mixed with acetonitrile containing 0.1% TFA and analyzed by MALDI-TOF MS, the overall content of added sCT could be determined, as shown in Figure 3c. This indicates that MALDI-TOF MS can directly determine peptide content incorporated into the microspheres without any prior centrifugation or extraction procedures.

The drug contents of leuprolide- and sCT-loaded PLGA microspheres were determined by MALDI-TOF MS, and the results were compared with the conventional extraction method (Table 1). In the determination using MALDI-TOF MS, the microsphere particles were added to MALDI matrix solutions containing internal standard and then vacuum-dried. In the conventional extraction method, the microspheres were dissolved in methylene chloride, and the peptide was extracted with 0.1 M

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acetate buffer (pH 4.0). The supernatants were recovered after centrifugation and analyzed by reversed-phase HPLC. The amounts determined by MALDI-TOF MS were slightly higher than those by the conventional extraction method, and the differences were more significant with the microspheres containing sCT. The underestimation with the conventional extraction method compared to MALDI-TOF MS may be attributed to the incomplete extraction of the peptide from the microspheres¹ and the adsorption of peptide to the polymer during the extraction process.^{27,28} This study suggests that direct peptide determination using MALDI-TOF MS may eliminate the problem of underestimation by the extraction procedures.

CONCLUSIONS

MALDI-TOF MS could be successfully applied to directly determine the peptide content of PLGA microspheres with an

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internal standard addition method. Direct determination of MALDI-TOF MS eliminates troublesome procedures such as extraction or hydrolysis of the conventional methods. In addition to the merits of MALDI-TOF MS of speed analysis and easy operation, this technique is very tolerant of sample contaminants, such as salts and detergents, and could probe the samples in a nonaqueous condition. These properties will allow it to easily assess the peptide stability inside microspheres, such as acylation by PLGA polymer. MALDI-TOF MS is also expected to be conveniently applied to in vitro peptide release study from microspheres.

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