Journal Pre-proofs

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PII: S0939-6411(20)30308-8
DOI: https://doi.org/10.1016/j.ejpb.2020.10.008
Reference: EJPB 13432

To appear in: *European Journal of Pharmaceutics and Biopharmaceutics*

Received Date: 22 October 2019
Revised Date: 12 September 2020
Accepted Date: 13 October 2020

Please cite this article as: T. Li, A. Chandrashekar, A. Beig, J. Walker, K. Yeung Hong, A. Benet, J. Kang, R. Ackermann, Y. Wang, B. Qin, A.S. Schwendeman, S.P. Schwendeman, Characterization of Attributes and *in vitro* Performance of Exenatide-loaded PLGA Long-acting Release Microspheres, *European Journal of Pharmaceutics and Biopharmaceutics* (2020), doi: https://doi.org/10.1016/j.ejpb.2020.10.008

Characterization of Attributes and *in vitro* Performance of Exenatide-loaded PLGA Long-acting Release Microspheres

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Abstract

Bydureon® (Bdn) is a once-weekly injectable long-acting release (LAR) product for adults with type 2 diabetes based on PLGA microspheres encapsulating glucagon-like peptide-1 (GLP-1) analog, exenatide. Despite its widespread use in type 2 diabetes treatment, little information has been published concerning the physical-chemical aspects and exenatide stability in this product. Here, we developed and validated methods to evaluate attributes and performance of Bdn such as particle size/size distribution and residual levels of moisture and organic solvent(s). The reverse engineering of the exenatide LAR was also described to identify and quantify principal components in the product. Stability-indicating UPLC and LC-MS methods were applied to determine the exenatide degradation (such as oxidation, deamidation and acylation products) during *in vitro* release evaluation. The 55-μm volume-median Bdn microspheres slowly release the exenatide *in vitro* over two months with a very low initial burst release to avoid unwanted side effects. Residual organic solvent levels (methylene chloride, ethanol, heptane, and silicon oil) also meet the USP criteria. Peptide acylation was the most prominent peptide reaction during both encapsulation and *in vitro* release, and the acylated peptide steadily increased during release relative to parent exenatide, becoming the most abundant peptide species extracted from microsphere at the later release stages. The presence of peptide impurities during the release period, which are not extractable in the polymer and likely insoluble in water, might be one potential cause for immunogenicity. Further evaluation will be needed to confirm this hypothesis. Release of peptide was minimal over the first 2 weeks before the microspheres steadily released peptide for more than 28 days. The rigorous technical approach discussed in this paper may provide critical information for both companies and FDA while developing generic exenatide-PLGA formulations and other important PLGA microsphere products.

1. Introduction

Exenatide is a glucagon-like peptide-1 receptor agonist, belonging to the group of incretin mimetics, approved in April 2005 for the treatment of diabetes mellitus type 2 [1–6]. It is a synthetic 39 amino acid peptide amide with the same amino acid sequence as exendin-4 which was originally isolated from a venomous lizard. This peptide shares 53% structural homology to glucagon-like peptide-1 (GLP-1) and can be used as a GLP-1 receptor agonist. Compared to native GLP-1, exenatide has a longer half-life due to its resistance to dipeptidyl peptidase-4 degradation and is mainly degraded and eliminated in the kidney after systemic absorption [1]. This peptide was first marketed as Byetta® in 2009 [7], which is administered twice daily as a subcutaneous injection in the abdomen, thigh, or arm, any time within 60 minutes before the first and last meals of the day. To achieve sustained plasma levels of exenatide and reduced injection frequency, various drug delivery systems including polymeric hydrogels, nanoparticles and microparticles have been tested in preclinical studies. To date, Bydureon® (Bdn) product, which is microspheres made of poly(lactic-*co*-glycolic acid) (PLGA), the most commonly used polymer of long acting release depots (LARs), is the only LAR containing exenatide approved by the FDA. Bdn is a once-weekly injection product for adults with type 2 diabetes [6,8–12]. Exenatide can be released from Bdn microspheres over several weeks, and the superposition of the release curves from each injection produces the therapeutic pharmacokinetic profile. Based on patent literature [13,14], the PLGA microspheres in Bdn are prepared by coacervation. Compared with Byetta®, the once-weekly administration of Bdn can help simplify patients' regimens and support long-term diabetes control.

Despite a large body of literature on PLGA microspheres, very little information has been published about the physical-chemical aspects of the exenatide and Bdn. It has been found that particle size/size distribution could influence the immune response towards PLGA particles, which implies a possibility that the release and erosion kinetics of microparticles along with peptide degradation products may also impact the immunogenicity of PLGA products. Additionally, there is a scientific gap between our current understanding of what happens when the LAR is manufactured and stored, and how they perform when administered to patients. This gap impacts companies attempting to develop generic LARs during research and development, set key specifications for regulatory filings, and trouble-shoot manufacturing for clinical trials and post marketing approval. Similarly, the knowledge gap also creates significant uncertainties for the FDA, when evaluating regulatory submissions. Therefore, the additional and more careful characterization methods are necessary to accurately assess this more complex PLGA-encapsulated peptide relative to other LARs.

In this paper, a rigorous technical approach was developed to characterize the physical-chemical characteristics of Bdn, which can provide critical information to both the FDA and generic companies developing Q1 (qualitatively)/Q2 (quantitatively) products mimicking Bdn. Such information could help those seeking to develop a generic Bdn formulation but also to identify potential issues regarding the safety and efficacy of exenatide-PLGA LARs. Finally, it is important for those developing the next generation of peptides and other large molecules in PLGA or other LARs to have specific information on Bdn, which encapsulates the largest and most complex peptide currently on the market.

2. Materials and Methods

2.1 Materials

Bydureon® (Bdn) was purchased from University of Michigan Health System Pharmacy Service. Exenatide was kindly provided from Amneal Pharmaceuticals Pvt. Ltd for these studies and was referenced to the peptide purchased from the United States Pharmacopeia. Acetone, trifluoroacetic acid (TFA), acetonitrile (ACN), tetrahydrofuran (THF), heptane, ethanol, methylene chloride, dimethyl terephthalate (DMT), silicone oil (viscosity 350 cSt) and Sucrose Fluorometric Assay Kit were purchased from Sigma-Aldrich. BODIPY FL dye was purchased from Thermo Fisher Scientific.

2.2 Microsphere size and morphology analysis

The volume weighted median diameter of the microsphere was determined by laser diffraction using a Malvern Mastersizer 2000. Dry microspheres were suspended in Bdn diluent (phosphate buffer, which also contains sodium chloride, sodium and polysorbate 20) at a concentration of 50 mg/mL and vortexed briefly before analysis. Samples were added dropwise to ddH_2O dispersant in the instrument until the desired obscuration level was reached (2-7% obscuration) at a constant stir speed of 2500 rpm. Measurements were conducted using a material refractive index of 1.59 and absorbance of 0.01. A general-purpose calculation model, with normal calculation sensitivity and irregular particle shape, was selected for size calculations.

Morphology of the surface and interior of the microspheres was determined by scanning electron microscopy (Tescan MIRA3 FEG SEM). In some cases, the microparticles were fractured using a blade for interior observation. Then, the surface and interior of the particles were coated with gold using a sputter coater and subject to SEM observation.

2.3 Determination of residual solvents and moisture

Based on the patent literature [13,14], residual solvents anticipated to be present in Bdn microsphere preparation (heptane, ethanol and methylene chloride) were determined by a Trace 1310 gas chromatograph (GC) (Thermo Fisher Scientific Inc., Waltham, MA, USA), with a TG-624 column. Samples of microspheres were weighed into a glass vial containing 1mL DMSO (anhydrous 99.9%) to make the final recorded concentration roughly 10 mg/ mL and the vial was sealed. The samples were applied to the GC by headspace injection, the GC conditions were as follows: nitrogen gas carrier solvent flow of 25 mL/min; air flow of 350 mL/min and hydrogen flow of 35 mL/min; the front detector temperature was 240°C and the front inlet flow was at 2 mL/min. Each sample was agitated for 20 min at 120°C and 1 mL of the headspace sample was injected into the front inlet with a temperature of 140°C, split flow of 40.0 mL/min, and a split ratio of 20. The GC column temperature was initially set at 40°C for 15 min, then increased at 10°C /min to 240°C and held at 240°C for 2 min. The standard curves were prepared by adding the desired solvent to DMSO. Silicone oil residual was determined by qNMR (Agilent 500 MHz NMR Spectrometer) from the peak at 0.068 ppm corresponding to methyl protons in the dimethylsiloxane. Various concentrations of silicone oil (viscosity 350 cSt) standards were used to generate the calibration curve.

Residual water content in microspheres was determined by Karl Fischer titration (KF, Mettler Toledo) [15]. Approximately 10 mg samples dissolved in 1.0 mL of anhydrous DMSO and sealed in a PTFE septum capped vial were briefly vortexed to fully extract the H_2O before injection. The H_2O content values were corrected for blank DMSO moisture, also determined by KF titration.

2.4 Determination of the content of peptide, sucrose, and PLGA in microspheres

The Bdn microspheres consist of exenatide, sucrose, and PLGA [16]. The total peptide and extractable peptide were determined by ultra-performance liquid chromatography (UPLC) and total nitrogen analysis. For total peptide content determination, 1-3 mg of microspheres were weighed into tin pans for nitrogen analysis. Samples of EDTA ranging in mass from 0.5 mg – 2.5 mg were used to calibrate the nitrogen analyzer (TruSpec Micro, Leco). The extractable exenatide was obtained from the PLGA microspheres by washing/centrifugation with acetone. To avoid the effects of peptide oxidation during extraction, acetone was purged with nitrogen for 10 min. 1.5 mL of acetone were added into the Eppendorf tube, which contained 5 mg Bdn microspheres. The tube was vortexed for 2 minutes and then centrifuged at 14000 rpm for 15 minutes at 4ºC. A portion of the supernatant (0.75 mL) was removed, replaced with the same amount of acetone, and then the tube was purged with nitrogen for another 10s. Extraction steps were repeated a total of five times. At the last step, 1 ml of supernatant was removed, and the remaining was dried in a vacuum oven for 1 h at room temperature. The remaining water-soluble material containing peptide was reconstituted in 0.5 mL of 50 mM sodium acetate buffer (pH 4.5) and quantitated by UPLC composed of an Acquity UPLC BEH C18 column (1.7μm, 2.1 × 100 mm) for analysis at 280 nm UV absorption wavelength. A linear gradient of 25-90% ACN with 0.1% (v/v) TFA over 3 min at a flow rate of 0.5 mL/min was used with the column heated at 40°C. To further separate the degradation products after extraction, a linear gradient of 25-90% ACN with 0.1% (v/v) TFA over 25 min at the same flow rate of 0.5 mL/min and column temperature 40°C was used. This provided an estimate of the parent peptide in Bdn microspheres.

The initial drug loading (L_0) in Bdn microspheres was calculated as:

$$L_0 = \frac{E_{D0}}{W_{0D0}} \times 100\ \%,$$

where E_{D0} is the weight of exenatide measured by UPLC after extraction from microspheres of net weight $W_{0D0.}$ To calculate the remaining exenatide content after incubation, the fractional microsphere mass loss after incubation (A_{Dt}) at time *t* was first determined as follows:

$$A_{Dt} = \frac{W_{0Dt} - W_{1Dt}}{W_{0Dt}},$$

where W_{0Dt} is the initial dry microspheres weight and W_{1Dt} is the weight after incubation for time *t*. The drug loading after incubation (L_t) was determined from:

$$L_t = \frac{E_{Dt}}{W'_{1Dt}} \times 100\ \%,$$

where E_{Dt} is the extracted exenatide weight from UPLC analysis and W'_{1Dt} is the weight of microspheres used for extraction. To account for mass loss, the corrected loading (L_{tcorr}) at time *t* was estimated as:

$$L_{tcorr} = L_t\,(1 - A_{Dt}).$$

Finally, the remaining peptide in the polymer was calculated from:

$$\text{Exenatide remaining} = \frac{L_{tcorr}}{L_0} \times 100\ \%.$$

The exenatide content according to N_2 content was similarly determined, except the amount of peptide from N_2 analysis was inserted in place of E_{Dt} before using the same expressions.

The sucrose level was determined in the reconstituted sediment after acetone extraction and performed by a fluorometric assay (Sucrose Fluorometric Assay Kit, Sigma MAK013). The amount of PLGA was determined by qNMR after dissolving the microspheres in $CDCl_3$ and adding dimethyl terephthalate (DMT) as the internal standard.

2.5 Determination of the PLGA molecular weight (MW) distribution, lactic/glycolic acid ratio, and end-capping

Gel permeation chromatography (GPC, Waters) with refractive index detection (2414 refractive index detector, Waters) was performed to determine the PLGA MW distribution by dissolving Bdn in tetrahydrofuran (THF), and using THF as the mobile phase at 1 mL/min with a Styragel® HR 5E and 1 THF 4.6x300nm tandem columns at 35°C. A standard curve was generated by dissolving polystyrene standards in THF from 4-70 kDa. NMR was used to investigate L/G ratio [17], and the acid number was determined by titration of the Bdn polymer, extracted in acetone, reconstituted in dehydrated THF:Acetone (50:50) with methanolic potassium hydroxide (0.01 M) to the end-point indicated by phenolphthalein (1.0 wt%) [18].

2.6 Release (retention) kinetics of exenatide from PLGA microspheres

Five mg of microspheres were placed in polypropylene tubes containing 1 mL of 10 mM phosphate buffered saline (PBS) + 0.02% Tween 80 + 0.02% NaN_3 (PBST), pH 7.4 at 37ºC under mild agitation. The supernatant was changed after centrifugation at 8,000 x g for 5 minutes at each time point up to 8 weeks (Days 1, 3, 7 and weekly thereafter) in order to maintain low levels of peptide and PLGA degradation products in the release media. On days of collection, microspheres were removed from the release experiment and vacuum dried before the UPLC and total nitrogen analysis to evaluate the peptide content. As the peptide is not fully stable in PBST [19], the kinetics of peptide retained in the microspheres was used to infer the release kinetics.

2.7 Kinetics of polymer degradation of PLGA microspheres

Bdn was incubated in the same manner including replacement of media as performed in the release study. After being collected from the release media, microspheres were washed with ddH_2O and

dried at room temperature under reduced pressure. THF was added to dissolve the microspheres to obtain approximately 2 mg/mL polymer concentration and then supernatants were subjected to GPC analysis as described above.

2.8 Kinetics of microsphere water uptake and mass loss

Microspheres were incubated with exchanges of media, as described above for release kinetics evaluation, and finally collected for analysis on pre-weighed nylon membrane filters under vacuum while washing off with ddH_2O. Then, the surface water was removed by further filtration under vacuum and the wet weight of the microspheres was immediately measured. The samples were dried at room temperature under reduced pressure to a constant weight and then the dry weight was recorded. To correct for the interparticle water, dry, unincubated Bdn microspheres were suspended in 4°C PBST for short time and the wet and dry weights were measured after filtering and drying, respectively, as described above. The detailed calculation for water uptake with the interparticle water correction assuming negligible water uptake in the 4°C microspheres was performed, as described previously [20].

2.9 Imaging of BODIPY uptake

In order to evaluate diffusion of the microsphere matrix as a function of release time, Bdn microspheres were incubated with BODIPY dye. This pH-insensitive dye is known to diffuse through solid PLGA matrix over reasonable time scales and is easily monitored by confocal microscopy [21]. In addition, this water-soluble dye can diffuse into aqueous pores inside microspheres, therefore the images show both polymer microstructure and pore interconnections inside the particles [22]. At each time point, the collected dry microspheres were incubated with BODIPY (5 μg/ml in PBST) at 37ºC for 3 days and evaluated by confocal microscopy (Nikon A1si confocal, inverted) using the 488 nm laser line to excite BODIPY 493/503 fluorescence and collect the narrow bandwidth of emitted light at 515 nm.

2.10 Characterization of encapsulated peptide degradation products before/during *in vitro* release

Agilent Q-TOF HPLC-MS (1290 Infinity) analysis was performed to provide identification of the degradation impurities extracted from Bdn microspheres after *in vitro* release. The analysis was performed using a C4 column (YMC America, 4.6 mm x 150 mm, 5μm, 30 nm) and a shallow gradient (from 30 to 45% A (A: ACN with 0.05% TFA, B: Water with 0.05% TFA) over 20 min at 40°C) allowing for separation and identification of major impurities.

3. Results and Discussion

3.1 Size and morphology of Bydureon®

The average volume median diameter (D[v, 0.5]) of Bdn was observed to be 55.30 ± 0.04 μm by laser diffraction, as shown in Figure 1A. The Span value was small (0.79), indicating a narrow size distribution of the microspheres. In Figure 1B representative SEM images of Bdn are displayed. As shown in the figure, the microspheres were not perfectly round which is consistent with the

extraction of organic solvent during coacervation. Microspheres were understandably slightly smaller when dry as seen by SEM, before they were hydrated as necessary for laser diffraction size analysis.

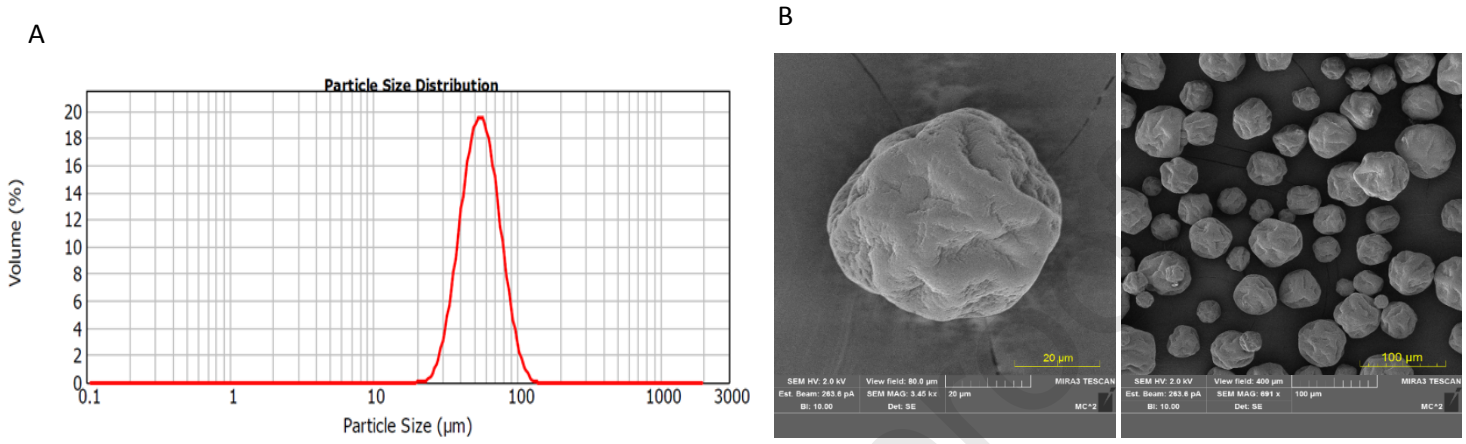


Figure 1. A) The particle size distribution of an aqueous suspension of Bdn was 55.30 µm by laser diffraction. B) SEM images of dry Bdn microspheres.

3.2 Residual solvents and moisture amount

Coacervation processes are known to result in significant residual solvents. The level of residual solvents can impact the Tg of the formulations, and typically Tg decreases as the amount of residual solvents increases. According to the patent literature, residual solvents in the microspheres described by the inventors of Bdn may include methylene chloride, ethanol, heptane, silicone oil and water. In USP 40 Chemical Tests (467) Residual Solvents [23] and the International Council for Harmonisation (ICH) Q3C(R6) guideline [24], methylene chloride belongs to Class 2 residual solvents. Ethanol and heptane belong to the Class 3 solvents, which are regarded as less toxic and lower risk to human health than Class 1 and 2 residual solvents. From the GC analysis, the average residual methylene chloride was determined as 0.11 ± 0.03% (w/w), heptane 1.39 ± 0.01% (w/w), and ethanol 0.30 ± 0.05% (w/w). The average residual H_2O was determined as 1.36 ± 0.04% (w/w). According to the ^{1}H-NMR, there was 0.036 ± 0.002% (w/w) silicone oil in the microspheres. The individual components of Bdn are summarized in Table 1. The total residual solvents of Bdn was also investigated by TGA. At 150 ℃, the weight loss was 1.35 ± 0.06% (w/w). At 200 ℃, the weight loss was 2.17 ± 0.07% (w/w) (Figure S1).

3.3 Determination of the content of major components and evaluation of the physical-chemical properties of PLGA in the microspheres

Before the analysis, the purity of exenatide used in this study was assessed by total N_2 analysis and UPLC relative to reference USP grade exenatide. According to the N_2 analysis, the exenatide powder contained 90.8% peptide. By comparing UPLC peak area, the exenatide as received was 87.7% relative to USP grade peptide. The amount of exenatide in the Bdn was determined by both total N_2 analysis and UPLC after extraction of the peptide. The peptide loading of Bdn was 4.89 ±

0.02% (w/w) (mean ± SEM, n = 3) and 4.47 ± 0.04% (w/w) (mean ± SEM, n = 3) total peptide, respectively, after reference purity correction. The values are close to the 5% loading indicated on the package insert [10,11]. The sucrose content was determined by glucose and sucrose colorimetric/fluorometric assay kit, and found to be 2.00 ± 0.02% (w/w) (mean ± SEM, n = 3) of sugar in Bdn, exactly as described in the package insert. The LA/GA ratio is an attribute of PLGA to control the duration of release. In Figure 2, qNMR was used to investigate the PLGA content in Bdn using DMT (3.95 ppm) as an internal standard. The sum of the masses of lactic acid (5.30 and 1.58 ppm) and glycolic acid (4.90 ppm) were used to calculate the content of polymer in Bdn to be 88.3 ± 0.6% (w/w) (mean ± SEM, n = 3). The L/G ratio of the polymer used in Bdn was 52.9/47.1, closely corresponding to the expected 50/50 ratio. The weight-averaged molecular weight (Mw) and number-averaged molecular weight (Mn) distribution of PLGA was determined by GPC as 49 kDa and 23 kDa, respectively with a PDI of 2.2. Increasing the molecular weight of PLGA was previously shown to lengthen the lag phase of release [9]. Based on the duration of the lag phase observed in the release study (Figure 3), the measured molecular weight is reasonable. With the acid-base titration, the acid number of the PLGA in Bdn was 81.31±1.84 µmol acid/g polymer (mean ± SEM, n = 3), indicating the acid end capped PLGA.

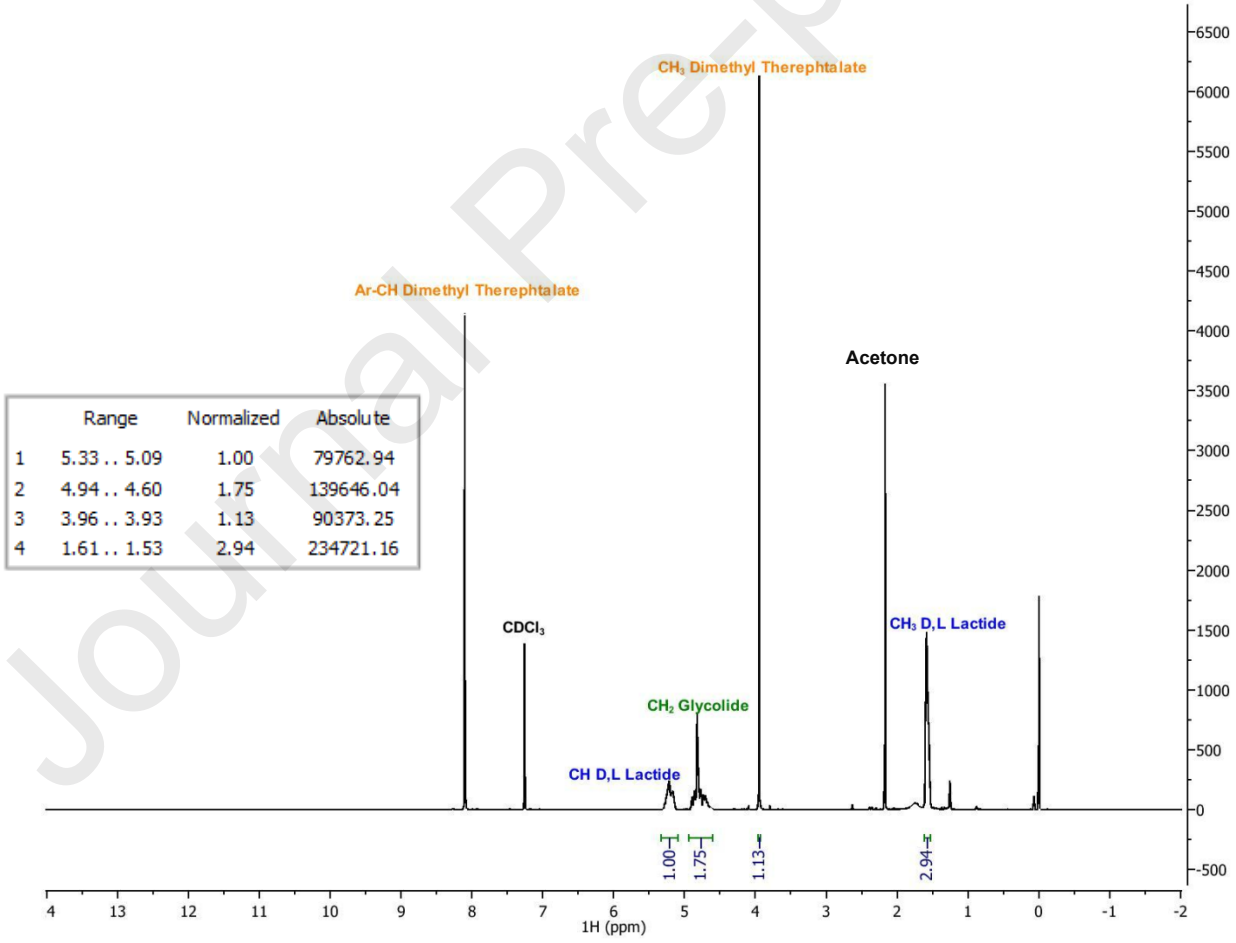


Figure 2. [1]HNMR spectrum of PLGA from the Bdn.

Table 1. Determination of the composition of Bdn.

Components	Characterization method	Content (% w/w)		
Total Peptide	Total nitrogen analysis and UPLC analysis	N_2 analysis	UPLC	
		4.89 ± 0.02	4.47 ± 0.04	
Parent Peptide	UPLC analysis	NA	4.34 ± 0.03	
Sucrose	Glucose and sucrose Fluorometric Assay	2.00 ± 0.02		
PLGA	NMR	88.3 ± 0.6		
Residual moisture	Karl Fischer Titration	1.36 ± 0.04		
Methylene chloride	GC	0.11 ± 0.03		
Heptane	GC	1.39 ± 0.01		
Ethanol	GC	0.30 ± 0.05		
Silicone Oil	NMR	0.036 ± 0.002		
Sum		98.39±0.77[a]	Total Peptide	97.97±0.79[b]
			Parent Peptide	97.84±0.78[c]

[a] The sum of the total peptide, determined by N_2 analysis, and the content of other components.
[b] The sum of the total peptide, determined by UPLC analysis, and the content of other components.
[c] The sum of the parent peptide, determined by UPLC analysis, and the content of other components.
Data represents mean ± SEM (n=3)

3.4 *In vitro* release and peptide degradation identification

As determined by both nitrogen analysis of, and peptide extraction from, the microspheres, exenatide was slowly released from Bdn with a low initial burst (Figure 3), which is essential for this peptide to reduce/avoid side effects (e.g., nausea) [16]. This was followed by a lag phase and more rapid release towards the end, with less than 20% peptide remaining at the end of 8 weeks. Compared with the single-dose exenatide extended-release trial on patients with type 2 diabetes [8], the *in vitro* release profile is quite reasonable. Besides the low initial burst, both *in vivo* pharmacokinetics and *in vitro* release showed multiphasic release behavior over an approximately 8-10-week period and rapid release after 4 weeks. The superposition of all the release curves from the weekly injections during normal use is required to achieve therapeutic concentrations of exenatide and to minimize initial burst side effects and deviations from non-zero order release. As exenatide is not stable in the release media owing to exenatide instability at neutral pH [19], the drug release was assayed by its retention in the microspheres. Total nitrogen analysis of the microspheres provides the content of all peptide species in the microspheres but cannot provide

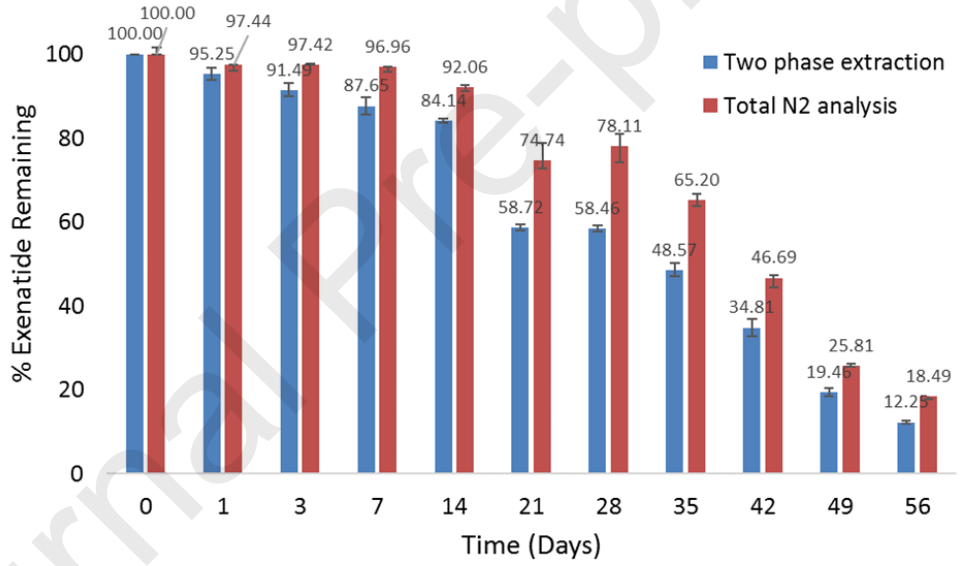
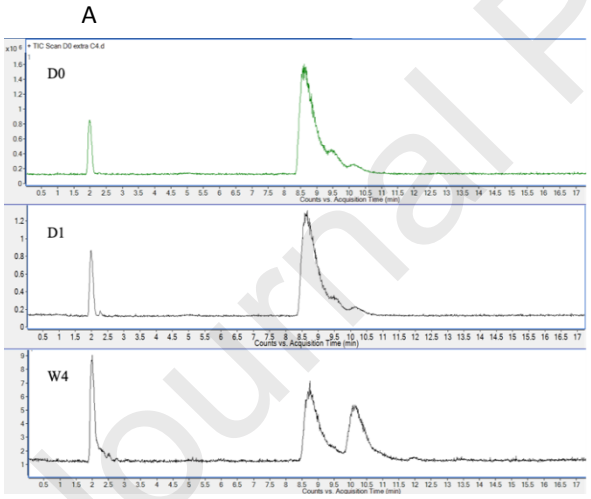


Figure 3. Kinetics of total exenatide remaining in Bdn microspheres during release incubation as determined by UPLC and N_2 analysis relative to initial values. Data represents mean ± SEM (n=3).

the necessary information about the content of degraded peptide, which can only be done by extraction assays. However, extraction assays can suffer from incomplete extraction. The amount of extractable exenatide obtained by two phase extraction was slightly lower (~9%) than that observed by total nitrogen analysis, which indicated the peptide might be lost during the extraction preparation, the peptide reacted with degraded polymer to form a peptide-polymer conjugate, or the peptide formed insoluble aggregated species. Additionally, fragments of the peptide may have formed without a sufficient concentration of aromatic residues for detection at 280 nm, and the extinction coefficients of all degradation products may not be in an acceptably narrow range. The slight separation of the two curves in Figure 3 may have been caused by some of these factors.

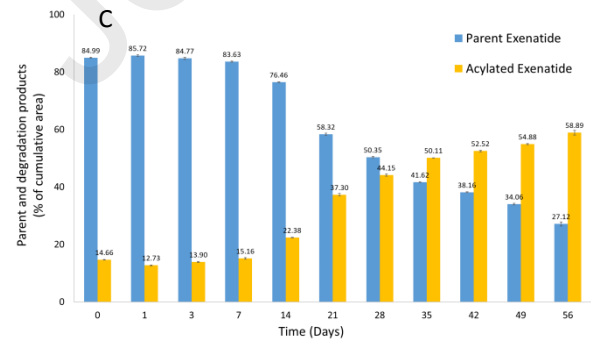
HPLC-qTOF analysis was then used to identify the degradation impurities extracted from the microspheres. Over the release period, the amounts and number of degradation products increased, as seen by the relative peak areas, and the acylated product became the predominant species towards the end of the release interval. According to the peptide sequence (Figure S2), several potential degradation sites on exenatide were expected to change the peptide mass. The peptide has two potential oxidation sites (Methionine **M** and Tryptophan **W**), and two deamidation sites (Asparagine **N** and Glutamine **Q**). Deamidation is a common protein post-translational modification that arises spontaneously and non-enzymatically. The amino acid sequence, -NG- or -AsnGly-, is most prone to deamidation with a half-life around 24 h under physiological conditions. The intermediate of deamidation, which localized to an -NG- motif, is stable at mildly acidic condition than at higher pH [25–27]. Additionally, exenatide has 3 positively charged amino acids (Lysine 12, 27**K** and Histidine 1**H**) to react with the side chain of PLGA to form acylated peptide [19,28,29].

In Figures 4A and B, the presence of probable oxidized (RT 5.2 and 6.0) and acylated peaks (RT 6.0, 9.5 and 10.2) indicated the physical stresses during encapsulation can result in peptide degradation. The unknown +40 peak is consistent with potassium ion but requires further analysis for verification. After 2-3 weeks of incubation, deamidated products were formed likely due to expected neutral to mildly basic conditions exposed to the encapsulated peptide. More degradation peaks formed and the amount of acylated exenatide increased with time of incubation in the release media (Figures 4C and D).



B

HPLC peak retention time, min	Observed molecular mass	Expected Structure
5.2	4202	Oxidation
6.0	4260	+GA & Oxidation
8.7	4186	Parent Exenatide
9.5	4187 4244	Deamidation +GA
10.2	4244	+GA
10.9	4259	+LA & Deamidation
11.9	4169 4245	Succinimide intermediate +GA & Deamidation
13.4	4227	Deamidation



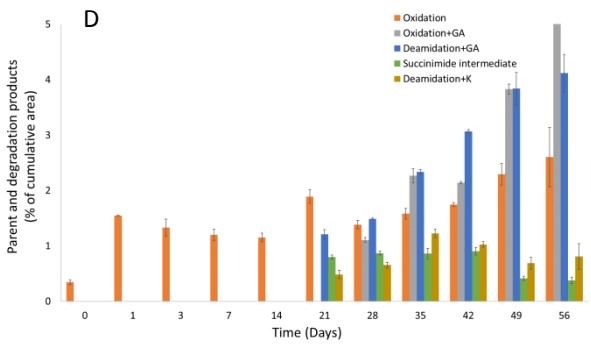


Figure 4. (A, B) HPLC-qTOF analysis of exenatide extracted from Bdn after D0, D1 and W4 incubation. (C, D) Acylated exenatide and other degradation product areas as a percentage of the total peptide peak area in microspheres extracted from Bdn over 8 weeks *in vitro* release. Data represents mean ± SEM (n=3).

3.5 PLGA microspheres performance during incubation

Drug release mechanisms from PLGA microspheres are typically governed by an interplay of water absorption, hydrolysis, erosion, and diffusion through the PLGA polymer matrix. In this study, to begin the mechanistic approach, each of the above indicators were examined.

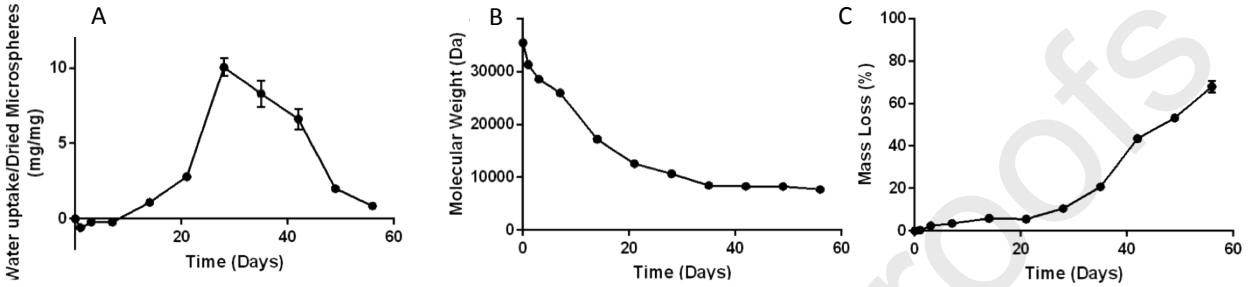


Figure 5. (A) Kinetics of water uptake into Bdn. (B) Kinetics of Mw decline of Bdn incubated in PBST 7.4. (C) Kinetics of mass loss of Bdn. Water uptake was corrected with interparticle water. Data represents mean ± SEM (n=3).

As seen in Figure 5A, the polymer microspheres took up very little water during the first week, before beginning to swell significantly on day 14. A very large swelling increase occurred starting around week 3 with a maximal swelling in the 4-6-week interval, before losing most of the water by week 8. It is noted that the swelling measurement is imperfect owing to the necessary correction for interparticle water (see Methods). As expected, the polymer molecular weight declined steadily and sharply over the first 3 weeks (Figure 5B) before slowing down to a virtual halt by day 35 where oligomeric PLGA remained. As shown in Figure 5C, the polymer lost very little mass over the first 3 weeks, consistent with its medium Mw (~35 kDa) expected to have an induction period as well as the low initial drug release and low porosity resulting from preparation by the coacervation method. A more in-depth investigation of the later release time periods could potentially provide interesting details regarding the very slow molecular decline with steady mass loss during this time. The kinetics of water uptake into Bdn is consistent with the data of Mw decline and mass loss. Water uptake steadily increased during polymer degradation until 28 days before significant drug release and mass loss. When the polymer degradation slowed, water left the polymer possibly owing to salt formation of linear PLGA oligomers and peptide in the microspheres [30].

Along with the PLGA polymer degradation, acidic monomers and oligomers were produced, which likely lowered the pH of microenvironment and the release media. The accumulation of acidic products in the polymer likely resulted in autocatalysis of the hydrolysis reaction. It is important to note that even though the release media was changed at each time point during the investigation of erosion behavior depicted in Figure 5, the pH of the buffer decreased to a minimum value slightly below pH 6 (Figure S3) during the active erosion phase of weeks 4-6. Note that common PBS with its low buffering capacity is susceptible to this issue, which depends on the initial concentration of the microspheres in the release media and the frequency of media exchange. Variations in pH in the media and more importantly within the microclimate pH of the polymer

(i.e., in the aqueous pores) are anticipated to have an important influence on the stability of the encapsulated peptide [31,32].

3.6 BODIPY uptake

To image the changes in polymer microstructure during the release interval and to develop a qualitative understanding of pore access to the outside, PLGA microspheres were incubated with BODIPY fluorescence dye. BODIPY FL is an interesting molecule for this purpose as it is capable of rapid diffusion through pores yet also preferentially partitions into the PLGA where it very slowly diffuses in the polymer solid state [21,33]. Therefore, very bright regions of the polymer matrix imaged by the dye represent regions of solid polymer where the dye heavily partitioned and has had access to on significant time-scales to allow its diffusion. At each release collection time point, after fully drying, the particles were incubated with BODIPY in PBST media at 37°C over 3 days to help facilitate extensive dye entry into the polymer microspheres. As shown in Figure 6, Day 0 and Day 1, the green outlines indicated the PLGA microspheres had sufficiently low surface porosity to strongly inhibit penetration of the dye beyond the first few microns of the surface, even with an extra 3 days BODIPY solution incubation. These results were consistent with the very low initial burst release. At Day 7, small pores appeared on the surface of the particles. Inside the microsphere, one spot was brighter than the rest of area due to the formation of a pore channel network (i.e., a crack), meanwhile, the rest polymer phase still remained relatively intact. After week 2, the pores on the particle surface became larger and the number of inner pores were increased. Compared with other time points, PLGA microspheres at week 4 had the largest space of pores, which corresponded to the moment when water uptake was the highest. With longer incubation, the inside of the particles became hollow at the later stage. The particle size decreased, then eventually collapsed (at weeks 5-8). The confocal images supported the results of kinetics of water uptake, polymer MW decline, mass loss and peptide release, which have been discussed above.

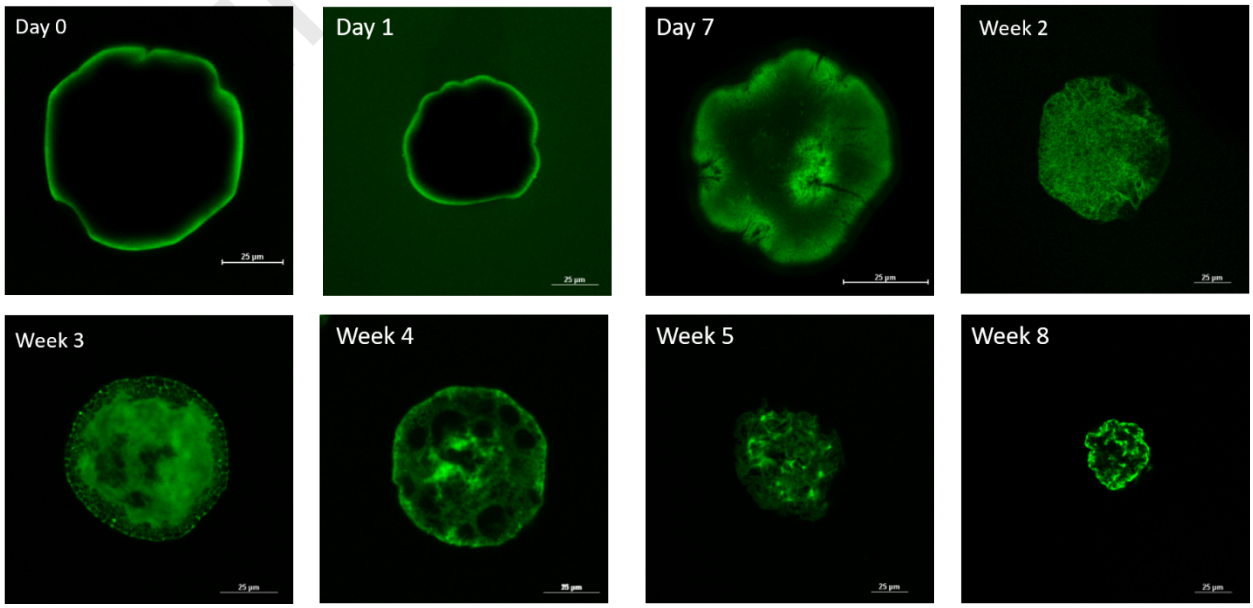


Figure 6. Confocal microscopic images of Bdn incubated with 5 µg/ml BODIPY in PBST at 37ºC over 3 days after various indicated times of release incubation.

3.7. Analysis of peptide stability and release data.

The UPLC assay indicated roughly 3% of impurity (Table 1) in the extracted peptide at time 0, which likely was formed during manufacturing of the product (e.g., during encapsulation). HPLC-qTOF analysis of the extracts from Bdn showed that the initially encapsulated peptide goes through several degradation pathways over the release period, such as acylation, oxidation and deamidation. Peptide acylation was the most prominent peptide reaction during release, and the glycolic acid adduct with the PLGA steadily increased relative to the parent peptide, becoming the most abundant peptide species extracted after day 35 of release. The only other peptide impurity detectable in the encapsulated peptide and early release period was a single oxidation addition, probably caused by the single methionine residue in exenatide. Identifying the precise location of the modification and quantitation of each species would require further analysis. Corresponding to the period of significant water uptake, there appeared to be a marked increase in peptide degradation in the polymer at around day 21, including: (a) an accelerated increase in peptide acylation (Fig. 4C); (b) the first appearance of deamidation and likely cyclic imide formation (Fig. 4D), and (c) importantly the level of total extractable peptide recorded by N_2 analysis became much higher than that recorded in the UPLC detected extract, pointing to the likelihood that some amount of peptide may be insoluble in water or could not be efficiently extracted for other reasons. We have confirmed in control experiments by size-exclusion chromatography that there is clearly a growth of aggregate peptide at later stages of release (*data not shown*). This analysis will be published in a follow-on paper in the near future. Collectively, these data suggest that a higher microclimate pH in the later release time points, as each of these trends are expected at neutral pH and inhibited at slightly acidic pH. This insoluble peptide might be one potential cause for immunogenicity [34]. The multiple release mechanisms of the encapsulated peptide after days 14-21 (e.g., osmotic-mediated pore formation and polymer mass loss) accelerated peptide release until most peptide was released by day 56. Note that the dose of exenatide in Bdn far exceeds that expected by the equivalent 14 doses of immediate release peptide in Byetta. The relative bioavailability of exenatide released from Bdn compared to Byetta is approximately 63% in rat and 23% in monkey [16]. The lower bioavailability of Bdn compared with Byetta may be caused by significant levels of exenatide released from Bdn that is metabolized or degraded before entering the systemic circulation. One final point is that negatively charged phosphate buffering ions (mono- and di-basic phosphate) are not expected to partition into the PLGA phase like neutral BODIPY does. The microclimate pH in PLGA is well known to most often differ from the outside pH as a result. Therefore, we assume that that uptake of buffering species is limited until percolating pore networks are formed with the surface of the polymer.

4. Conclusion

To our knowledge, this is the first detailed report of the physical-chemical product attributes and *in vitro* performance of Bydureon® long-acting release formulation. The reverse engineering of the exenatide LAR was performed to identify and quantify the principal components, including the peptide, polymer, sugar, and residual solvents in the product. Low contents of residual organic solvents meet the USP criteria. The 55-μm volume-median Bdn microspheres slowly release

exenatide *in vitro* over two months with a very low initial burst release. Stability-indicating UPLC and LC-MS methods were applied to determine the exenatide degradation products during *in vitro* release evaluation. Peptide acylation is by far the most prominent peptide reaction during both encapsulation and release. Some of the initially encapsulated peptide includes the glycolic-acid adduct with PLGA, and the acylated peptide steadily increases during release relative to the parent peptide and becomes the most abundant extractable peptide species by the later release periods. The presence of a significant level of non-extractable peptide, some of which is likely insoluble in water, raises potential concerns about the immunogenicity of the encapsulated peptide, and warrants further investigation. The analysis described in this paper may be useful for future generic exenatide microspheres development as well as other PLGA-based long-acting release formulations.

5. Funding

This research was funded by FDA contract HHSF223201810187C. This paper reflects the views of the authors and should not be construed to represent FDA's views or policies.

6. Supplementary Information

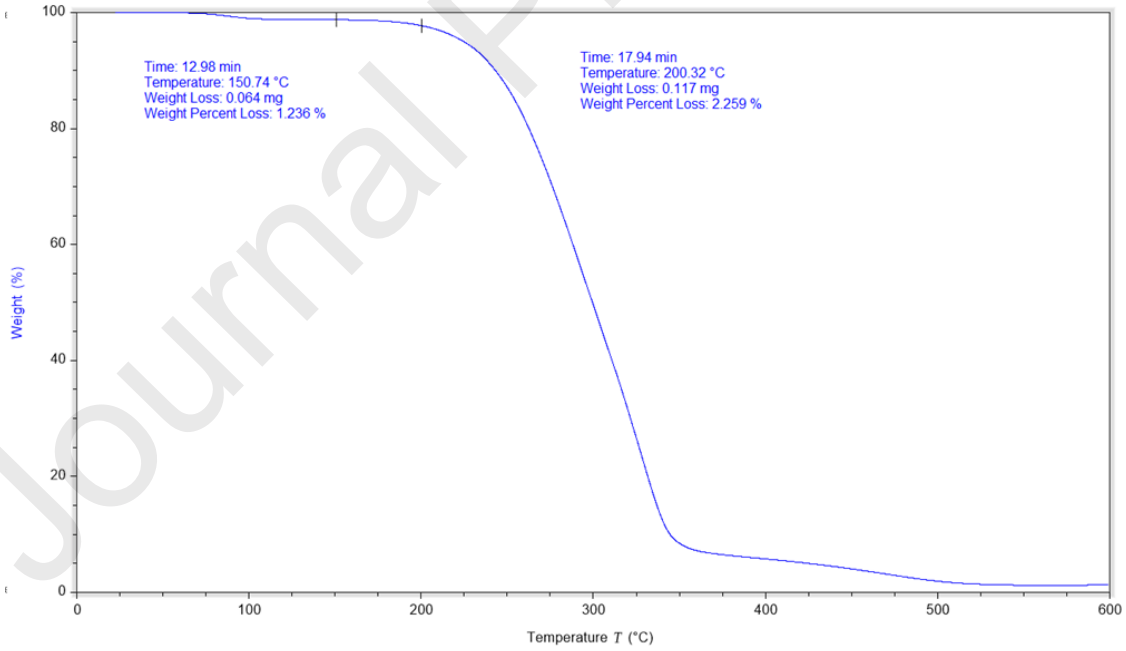


Figure S1. TGA thermogram of Bdn. At 150℃, the weight loss was 1.35±0.06% (w/w). At 200℃, the weight loss was 2.17±0.07% (w/w).

1 5 10 15 20 25 30 35

HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS

M (Methionine) and W (Tryptophan): 2 potential oxidation sites (+16 each)

N (Asparagine) and Q (Glutamine): 2 potential deamidation sites (+1 each)

(NG is a deamidation hotspot)

Succinimide intermediate during deamidation (-17)

K (Lysine) and H (Histidine): potential acylation sites (+58 for each glycolic addition)

Figure S2. Sequence of exenatide

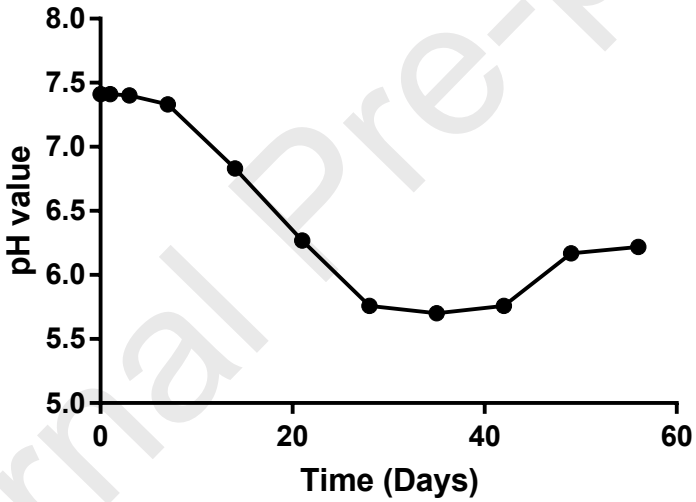


Figure S3. Media pH kinetics during *in vitro* release from Bdn.

References

[1] R. Göke, H.C. Fehmann, T. Linn, H. Schmidt, M. Krause, J. Eng, B. Göke, Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells, J. Biol. Chem. 268 (1993) 19650–19655.

[2] J.W. Neidigh, R.M. Fesinmeyer, K.S. Prickett, N.H. Andersen, Exendin-4 and Glucagon-like-peptide-1: NMR Structural Comparisons in the Solution and Micelle-Associated States, Biochemistry. 40 (2001) 13188–13200. https://doi.org/10.1021/bi010902s.

[3] A. Thum, K. Hupe-Sodmann, R. Göke, K. Voigt, B. Göke, G. McGregor, Endoproteolysis by isolated membrane peptidases reveal metabolic stability of glucagon-like peptide-1 analogs, exendins-3 and -4, Exp Clin Endocrinol Diabetes. 110 (2002) 113–118. https://doi.org/10.1055/s-2002-29087.

[4] L. Simonsen, J.J. Holst, C.F. Deacon, Exendin-4, but not glucagon-like peptide-1, is cleared exclusively by glomerular filtration in anaesthetised pigs, Diabetologia. 49 (2006) 706–712. https://doi.org/10.1007/s00125-005-0128-9.

[5] J.G. Lee, J.H. Ryu, S.-M. Kim, M.-Y. Park, S.-H. Kim, Y.G. Shin, J.-W. Sohn, H.H. Kim, Z.-Y. Park, J.Y. Seong, J.I. Kim, Replacement of the C-terminal Trp-cage of exendin-4 with a fatty acid improves therapeutic utility, Biochemical Pharmacology. 151 (2018) 59–68. https://doi.org/10.1016/j.bcp.2018.03.004.

[6] M. Yu, M.M. Benjamin, S. Srinivasan, E.E. Morin, E.I. Shishatskaya, S.P. Schwendeman, A. Schwendeman, Battle of GLP-1 delivery technologies, Advanced Drug Delivery Reviews. 130 (2018) 113–130. https://doi.org/10.1016/j.addr.2018.07.009.

[7] AstraZeneca Pharmaceuticals LP, Byetta® (exenatide) injection: Highlights of Prescribing Information, (2015).

[8] M. Fineman, S. Flanagan, K. Taylor, M. Aisporna, L.Z. Shen, K.F. Mace, B. Walsh, M. Diamant, B. Cirincione, P. Kothare, W.-I. Li, L. MacConell, Pharmacokinetics and Pharmacodynamics of Exenatide Extended-Release After Single and Multiple Dosing:, Clinical Pharmacokinetics. 50 (2011) 65–74. https://doi.org/10.2165/11585880-000000000-00000.

[9] M.B. DeYoung, L. MacConell, V. Sarin, M. Trautmann, P. Herbert, Encapsulation of Exenatide in Poly-(D,L-Lactide-Co-Glycolide) Microspheres Produced an Investigational Long-Acting Once-Weekly Formulation for Type 2 Diabetes, Diabetes Technology & Therapeutics. 13 (2011) 1145–1154. https://doi.org/10.1089/dia.2011.0050.

[10] AstraZeneca Pharmaceuticals LP, Bydureon BCISE®(exenatide sustained-release) for injectable suspension: Highlights of Prescribing Information, (2017).

[11] AstraZeneca Pharmaceuticals LP, Bydureon®(exenatide sustained-release) for injectable suspension: Highlights of Prescribing Information, (2015).

[12] R.R. Holman, M.A. Bethel, R.J. Mentz, V.P. Thompson, Y. Lokhnygina, J.B. Buse, J.C. Chan, J. Choi, S.M. Gustavson, N. Iqbal, A.P. Maggioni, S.P. Marso, P. Öhman, N.J. Pagidipati, N. Poulter, A. Ramachandran, B. Zinman, A.F. Hernandez, Effects of Once-Weekly Exenatide on Cardiovascular Outcomes in Type 2 Diabetes, New England Journal of Medicine. 377 (2017) 1228–1239. https://doi.org/10.1056/NEJMoa1612917.

[13] S.G. Wright, T. Christensen, T. Yeoh, M.E. Rickey, J.M. Hotz, R. Kumar, H.R. Costantino, Polymer-based sustained release device, US8431685B2, 2013.

[14] S.G. Wright, T. Christenson, T.Y. Yeoh, M.E. Rickey, J.M. Hotz, R. Kumar, H.R. Costantino, C. Smith, D. Lokensgard, J. Ong, Polymer-based sustained release device, EP1734971B1, 2011.
[15] H.R. Costantino, R. Langer, A.M. Klibanov, Moisture-Induced Aggregation of Lyophilized Insulin, Pharm Res. 11 (1994) 21–29. https://doi.org/10.1023/A:1018981208076.
[16] AstraZeneca Canada Inc., Bydureon® Product Monograph Including Patient Medication Information, (2020).
[17] T. Rundlöf, M. Mathiasson, S. Bekiroglu, B. Hakkarainen, T. Bowden, T. Arvidsson, Survey and qualification of internal standards for quantification by 1H NMR spectroscopy, Journal of Pharmaceutical and Biomedical Analysis. 52 (2010) 645–651. https://doi.org/10.1016/j.jpba.2010.02.007.
[18] Y. Zhang, A.M. Sophocleous, S.P. Schwendeman, Inhibition of Peptide Acylation in PLGA Microspheres with Water-soluble Divalent Cationic Salts, Pharm Res. 26 (2009) 1986–1994. https://doi.org/10.1007/s11095-009-9914-2.
[19] R. Liang, R. Zhang, X. Li, A. Wang, D. Chen, K. Sun, W. Liu, Y. Li, Stability of exenatide in poly(D,L-lactide-co-glycolide) solutions: A simplified investigation on the peptide degradation by the polymer, European Journal of Pharmaceutical Sciences. 50 (2013) 502–510. https://doi.org/10.1016/j.ejps.2013.08.014.
[20] K. Hirota, A.C. Doty, R. Ackermann, J. Zhou, K.F. Olsen, M.R. Feng, Y. Wang, S. Choi, W. Qu, A.S. Schwendeman, S.P. Schwendeman, Characterizing release mechanisms of leuprolide acetate-loaded PLGA microspheres for IVIVC development I: In vitro evaluation, Journal of Controlled Release. 244 (2016) 302–313. https://doi.org/10.1016/j.jconrel.2016.08.023.
[21] J. Kang, S.P. Schwendeman, Determination of Diffusion Coefficient of a Small Hydrophobic Probe in Poly(lactide-co-glycolide) Microparticles by Laser Scanning Confocal Microscopy, Macromolecules. 36 (2003) 1324–1330. https://doi.org/10.1021/ma021036+.
[22] A.C. Doty, D.G. Weinstein, K. Hirota, K.F. Olsen, R. Ackermann, Y. Wang, S. Choi, S.P. Schwendeman, Mechanisms of in vivo release of triamcinolone acetonide from PLGA microspheres, Journal of Controlled Release. 256 (2017) 19–25. https://doi.org/10.1016/j.jconrel.2017.03.031.
[23] USP-NF General Chapter <467> Residual Solvents, (2019).
[24] Impurities: Guideline for Residual Solvents Q3C(R6), (2016).
[25] T. Geiger, S. Clarke, Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation., Journal of Biological Chemistry. 262 (1987) 785–794.
[26] S. Liu, K.R. Moulton, J.R. Auclair, Z.S. Zhou, Mildly acidic conditions eliminate deamidation artifact during proteolysis: digestion with endoprotease Glu-C at pH 4.5, Amino Acids. 48 (2016) 1059–1067. https://doi.org/10.1007/s00726-015-2166-z.
[27] M.L. Houchin, K. Heppert, E.M. Topp, Deamidation, acylation and proteolysis of a model peptide in PLGA films, Journal of Controlled Release. 112 (2006) 111–119. https://doi.org/10.1016/j.jconrel.2006.01.018.
[28] R. Liang, X. Li, Y. Shi, A. Wang, K. Sun, W. Liu, Y. Li, Effect of water on exenatide acylation in poly(lactide-co-glycolide) microspheres, International Journal of Pharmaceutics. 454 (2013) 344–353. https://doi.org/10.1016/j.ijpharm.2013.07.012.

[29] R. Liang, X. Li, R. Zhang, Y. Shi, A. Wang, D. Chen, K. Sun, W. Liu, Y. Li, Acylation of Exenatide by Glycolic Acid and its Anti-Diabetic Activities in db/db Mice, Pharm Res. 31 (2014) 1958–1966. https://doi.org/10.1007/s11095-014-1298-2.
[30] J. Wang, B.M. Wang, S.P. Schwendeman, Characterization of the initial burst release of a model peptide from poly(D,L-lactide-co-glycolide) microspheres, Journal of Controlled Release. 82 (2002) 289–307. https://doi.org/10.1016/S0168-3659(02)00137-2.
[31] Y. Liu, S.P. Schwendeman, Mapping Microclimate pH Distribution inside Protein-Encapsulated PLGA Microspheres Using Confocal Laser Scanning Microscopy, Mol. Pharmaceutics. 9 (2012) 1342–1350. https://doi.org/10.1021/mp200608y.
[32] A.G. Ding, A. Shenderova, S.P. Schwendeman, Prediction of Microclimate pH in Poly(lactic-co-glycolic Acid) Films, J. Am. Chem. Soc. 128 (2006) 5384–5390. https://doi.org/10.1021/ja055287k.
[33] A.C. Doty, Y. Zhang, D.G. Weinstein, Y. Wang, S. Choi, W. Qu, S. Mittal, S.P. Schwendeman, Mechanistic analysis of triamcinolone acetonide release from PLGA microspheres as a function of varying in vitro release conditions, European Journal of Pharmaceutics and Biopharmaceutics. 113 (2017) 24–33. https://doi.org/10.1016/j.ejpb.2016.11.008.
[34] S. Hermeling, D.J.A. Crommelin, H. Schellekens, W. Jiskoot, Structure-Immunogenicity Relationships of Therapeutic Proteins, Pharm Res. 21 (2004) 897–903. https://doi.org/10.1023/B:PHAM.0000029275.41323.a6.