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Effect of Manufacturing Variables and Raw Materials on the Composition-Equivalent PLGA Microspheres for 1-Month Controlled Release of Leuprolide

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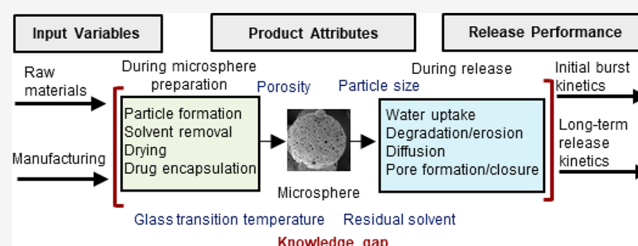
ABSTRACT: The 1-month Lupron Depot (LD) is a 75/25 acid-capped poly(lactic-*co*-glycolic acid) (PLGA) microsphere product encapsulating water-soluble leuprolide acetate with no generic products available in the U.S. Composition-equivalent PLGA microsphere formulations to the LD as a function of raw material and manufacturing variables were developed by using the solvent evaporation encapsulation method. The following variables were adjusted: polymer supplier/polymerization type, gelatin supplier/bloom number, polymer concentration, first homogenization speed and time, volume of primary water phase, second homogenization time, volume of secondary water phase, and stirring rate. The loading and encapsulation efficiency (EE) of leuprolide and gelatin were determined to identify a large number of composition-equivalent formulations within a ±10% specification of the LD. Key physical−chemical properties of the formulations (e.g., morphology, particle size distribution, glass transition temperature (T_g), residual moisture and solvent, and porosity) were characterized to determine the effect of manufacturing variables on the product attributes. The EE of gelatin across all formulations prepared (101 ± 1%) was observed to be much higher than the EE of leuprolide (57 ± 1%). Judicious adjustment of polymer concentration, second homogenization time, and volume of second water phase was key to achieving high EE of leuprolide, although EE higher than 70% was not easily achievable owing to the difficulty of emulsifying highly viscous primary emulsion into homogeneous small droplets that could prevent peptide loss during the second homogenization under the conditions and equipment used. The in vitro release kinetics of the formulations was highly similar to the LD in a zero-order manner after ∼20% initial burst release, indicating a critical role of the composition on peptide release in this formulation. The characterization of composition-equivalent formulations described here could be useful for further development of generic leuprolide PLGA microspheres and for guiding decisions on the influence of process variables on product physicochemical attributes and release performance.



KEYWORDS: *PLGA microspheres, leuprolide, Lupron Depot, composition-equivalent formulation, generic drugs*

1. INTRODUCTION

Polymer based long acting release (LAR) formulations have been widely used in peptide/protein delivery systems to increase bioavailability and reduce dosing frequency. Commonly used biodegradable and biocompatible polymers include poly(lactic-*co*-glycolic acid) (PLGA) and poly(lactic acid) (PLA).[1,2] Several PLGA/PLA based microsphere products have gained commercial success and thus are more likely to be selected as reference drugs for generic development. However, the intricate preparation process may impede the development of PLGA-based generic drug products and increase the difficulties of regulation by the U.S. Food and Drug Administration (FDA). Moreover, the composition-equivalent formulations developed under different manufacturing conditions may have different attributes and release performance, which may further influence the drug bioavailability and in turn drug safety and efficacy. Despite of the effort on investigating effects of variables on some product properties, the relationship between raw materials/manufacturing parameters and products attributes/release performance from the scope of composition-equivalent formulations has not been fully studied.

The Lupron Depot (LD) is a group of PLGA/PLA based LARs loaded with water-soluble nonapeptide, leuprolide acetate, for 1-, 3-, 4-, 6-month administration.[3] As a highly active luteinizing hormone-releasing hormone (LH-RH) agonist, leuprolide is able to inhibit the secretion of

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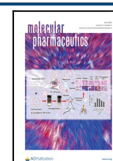

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gonadotropin after continuous administration in therapeutics doses.[4,5] Hence leuprolide is used in the treatment for the hormone sensitive cancer or disorder, like breast and prostate cancer and endometriosis.[4] It has been a top LH-RH agonist[6] holding a significant market share in the global peptide pharmaceutical market for decades.[7] Despite expiration of patent coverage, no generic product for the LD has been approved in the United States. The 1-month Lupron Depot is a PLGA microsphere product launched on the U.S. market in 1989 designed for monthly delivery of leuprolide. The LD marketed in the U.S. consists of PLGA microspheres loaded with leuprolide and gelatin (7.5 mg of leuprolide acetate, 1.3 mg of gelatin, and 66.2 mg of PLGA), which are prepared by double emulsion-solvent evaporation method. D-Mannitol (13.2 mg) is added before lyophilization to prevent aggregation of microspheres.[8,9] Therefore, to mimic the LD product, one must consider both the complexity of the manufacturing process and an extensive list of product ingredients.[1]

In previous work from our group, we reverse-engineered the LD to establish the relevant analytical methods and determine the raw materials, composition, characteristic properties, and release kinetics of LD.[10] On the basis of the published literature (including multiple patents) and our reverse engineering results, we identified or confirmed the raw materials of inactive ingredients used in preparation of LD microspheres as Wako 7515 PLGA polymer produced by direct condensation (DC) method[11,12] and beMatrix low endotoxin type B gelatin with bloom number 300 produced by Nitta Gelatin, Inc. First, those raw materials were used in preliminary pilot studies with the manufacturing variables regulated to establish empirically a "standard" condition that could produce microspheres with desirable attributes and performance (e.g., equivalent loading as LD, high EE, spherical shape, suitable particle size, low initial burst release, and continuous long-term in vitro release). Then, a formulation table was established by creating multiple levels of the variables in the standard conditions and different formulations were generated by changing one variable at a time from the standard condition. An equivalent PLGA synthesized by ring-opening (RO) polymerization of cyclic D,L-lactide and glycolide and various type B gelatins with different bloom numbers were employed to study the influence of raw materials on the leuprolide/PLGA formulations. Finally, sameness, key product attributes, and release performance were studied to determine the causes of the possible differences between composition-equivalent formulations and the reference product.

We describe the development of composition-equivalent PLGA microsphere formulations to the 1-month LD. The characterization of composition-equivalent formulations described here could be useful for further development of generic or new LAR microsphere products and for guiding decisions on the influence of manufacturing process variables on product attributes and release performance.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents. Leuprolide acetate with purity more than 99% by high-performance liquid chromatography (HPLC) analysis was purchased from Bachem Americas Inc. (Torrance, CA, USA). This leuprolide acetate was detected by UV absorbance at 280 nm wavelength on an ultraperformance liquid chromatography (UPLC) instrument and confirmed to be within 101 ± 3% (mean ± SEM, $n = 3$) of the USP standard (USP 36 NF 31; catalog number 1358503; lot I0M442) in the range of 0−600 μg/mL. Type B gelatin derived from porcine skin with bloom number 300 (beMatrix low endotoxin gelatin LS-W) was purchased from Nitta Gelatin Inc. (Osaka, Japan). Type B gelatins derived from bovine skin with bloom numbers 75 and 225 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Wako 7515 PLGA polymer was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Resomer 752H polymer was purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly(vinyl alcohol) (PVA) (GOHSENOL EG-40P) was purchased from Soarus L.L.C. (Arlington Heights, IL, USA). The AccQ·Tag chemistry kit was purchased from Waters (Waters Corporation, Milford, MA, USA). The 7.5 mg leuprolide dose for 1-month administration Lupron Depot (LD, AbbVie Inc., North Chicago, IL, USA) was purchased from the Hospital Pharmacy at University of Michigan Health System. All solvents used were HPLC grade and were purchased from Fisher Scientific.

2.2. Preparation of PLGA Microspheres Loaded with Leuprolide. Leuprolide acetate and gelatin were loaded into PLGA microspheres by solvent evaporation method. The raw materials used in the LD were identified in the previous reverse engineering studies[10] and published references by the originators.[8,9,13] Particularly, the polymer produced by Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) by direct condensation method with average molecular weight of 14 000 and LA/GA ratio of 75/25 and type B gelatin derived from porcine skin with bloom number 300 (beMatrix low endotoxin gelatin LS-W, Nitta Gelatin Inc., Osaka, Japan) were employed in the procedure. Pilot studies were conducted to establish a "standard" condition that could produce microspheres with desirable attributes and performance (e.g., equivalent loading as LD, high EE, spherical shape, suitable particle size, low initial burst release, continuous long-term release, etc.). The standard condition consisted of dissolving Wako PLGA (500 mg) in 1 mL of methylene chloride (DCM). Gelatin (bloom number 300) (10.6 mg) and leuprolide acetate (100 mg) were dissolved in 120 μL of ddH_2O at 60 °C. The oil phase was transferred to the water phase and immediately vortexed for 20 s followed by emulsification using a VirTis Tempest IQ^2 homogenizer (SP Scientific Inc., Warminster, PA, USA) at speed 10 000 rpm for 2 min to form a W1/O emulsion. The obtained W1/O emulsion was cooled in an ice bath for 2 min to increase the viscosity of the primary emulsion. 2 mL of aqueous 0.25% PVA solution was mixed with the cooled primary emulsion by vortex for 20 s and homogenization at 15 000 rpm for 30 s to form the secondary emulsion, i.e., a W1/O/W2 emulsion. The W1/O/W2 emulsion was transferred into 200 mL of 0.25% PVA solution and stirred with an overhead stirrer (IKA Eurostar 60 digital constant-speed mixer, IKA Works, Inc., Staufen im Breisgau, Germany) at 750 rpm for 3 h to evaporate the DCM and solidify the polymer phase. The microsphere suspensions were passed through a 75 μm opening sieve and washed with at least 1 L of water to rinse off the unencapsulated drug and PVA and remove the large microspheres. The microspheres were collected by centrifugation at 4000 rpm for 7 min at 4 °C and then mixed with a suitable amount of D-mannitol. The microspheres were freeze-dried under vacuum for 48 h.

2.3. Establishment of the Formulation Table. To prepare a series of formulations with the same composition as the LD as a function of raw materials and manufacturing variables, a formulation table was created based on the

Table 1. Formulation Table

no.	parameter	level 1	level 2 (standard condition)	level 3
raw materials				
1	polymer products/polymerization type	Resomer/RO	Wako/DC	
2	gelatin suppliers/bloom number	Sigma/225	Nitta/300	Sigma/75
primary emulsion				
3	concentration of PLGA in DCM (mg/mL)	400	500	600
4	W1/O phase volume ratio (v/v)	100 μL/1 mL	120 μL/1 mL	150 μL/1 mL
5	1st homogenization speed (rpm)	8000	10000	12000
6	1st homogenization time (min)	1	2	3
secondary emulsion				
7	2nd homogenization time (s)	10	30	45
8	O/W2 phase volume ratio (v/v)	1/1	1/2	1/4
in-liquid drying conditions				
9	stir rate (rpm)	450	750	900

standard condition. The selected manufacturing variables included polymer supplier/polymerization type, gelatin supplier/bloom number, polymer concentration, first homogenization speed and time, volume of primary water phase, second homogenization time, volume of secondary water phase, and stirring rate. The polymers synthesized by ring-opening (RO) polymerization of cyclic D,L-lactide and glycolide with the brand name Resomer 752H were chosen as the second source of polymer. Another two type B gelatin products with lower bloom numbers from Sigma-Aldrich were used in the corresponding formulations. For the other microencapsulation variables, the values in the standard condition served as medium level (level 2) and based on which low/high levels (level 1/level 3) were created to form the formulation table (Table 1). Different formulations were generated by changing one variable at a time from the standard condition with the constant theoretical loading of leuprolide at 16.4% and gelatin at 1.73%. The theoretical loading was calculated by

$$\text{theoretical loading} = \frac{\text{mass of peptide or gelatin}}{\text{mass of PLGA} + \text{peptide} + \text{gelatin}} \times 100\% \quad (1)$$

2.4. Determination of the PLGA Weight-Average Molecular Weight (Mw). Raw polymer was dissolved in dehydrated tetrahydrofuran (THF) at 4 mg/mL. The samples were subjected to gel-permeation chromatography (GPC) installed with two styragel columns (HR 1 and HR 5E columns, Waters, Milford, MA, USA) and a refractive index detector (2414 refractive index detector, Waters, Milford, MA, USA). Polystyrene standards with Mw ranging from 1000 Da to 50 000 Da were dissolved in the dehydrated THF. The Mw of PLGA was calculated by Breeze software (Waters, Milford, MA, USA).

2.5. Determination of Leuprolide Acetate Loading. The loading of leuprolide acetate was determined by single extraction and amino acid analysis (AAA).[10] In the single extraction method, 5 mg of formulation was dissolved in 1 mL of DCM and then mixed with 2 mL of 1/30 M sodium phosphate buffer (pH 6.0) by vortex for 5 min.[8,14] The supernatant of the aqueous phase was obtained after centrifugation (4000 rpm, 5 min) at room temperature. The content of leuprolide acetate in the aqueous phase was determined by UPLC. The UPLC system consisted of an Acquity Quaternary Solvent Manager, Sample Manager-FTN, Column Manager, and TUV detector (Waters, Milford, MA, USA). The separation of leuprolide was carried out using an Acquity UPLC peptide BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters, Milford, MA, USA) and a gradient elution of acetonitrile with 0.1% TFA (solvent A) and water with 0.1% TFA (solvent B) at a flow rate of 0.5 mL/min as follows: 0 min (25% A), 2 min (35% A), and 2.5 min (25% A), followed by 1 min recovery with initial conditions. The concentration of leuprolide was detected by UV absorbance at 215 nm wavelength, and its peak appeared around a retention time of 2.2 min. The loading of peptide and encapsulation efficiency (EE) were determined by the following:

$$\text{peptide loading} = \frac{\text{mass of peptide loaded}}{\text{mass of microspheres}} \times 100\% \quad (2)$$

$$\text{EE (\%)} = \frac{\text{leuprolide loading}}{\text{theoretical loading}} \times 100 \quad (3)$$

Amino acid analysis was used as the second method to determine the content of leuprolide acetate in the microspheres.[10] Leuprolide contains nine amino acids, and tyrosine (Tyr) and histidine (His) are the specific amino acids that do not exist in the gelatin.[9,15] Histidine content was used to determine the content of leuprolide as its peak was better resolved in the chromatographic separation than tyrosine and therefore provided more accurate results. About 25 mg of leuprolide loaded PLGA formulations or 5 mg of leuprolide acetate was weighed into hydrolysis tubes, and 1.0 mL of 6 N constant boiling HCl (Fisher Chemical, Fair Lawn, NJ, USA) was added. The tubes were purged under nitrogen, sealed under light vacuum, and incubated at 110 °C for 24 h. After incubation, the solution was frozen with liquid nitrogen and lyophilized under vacuum at room temperature. Then, 400 μL of 20 mM HCl was added into each tube to reconstitute the samples. Standard solutions of leuprolide acetate were prepared by dilution of the hydrolyzed leuprolide samples. Derivatization and analysis were performed by using Waters AccQ·Tag chemistry kit. Briefly, hydrolyzed amino acids were derivatized using the borate buffer (<5% sodium tetraborate in water) with the Waters AccQ·Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate). Norleucine was added to the samples during the derivatization and used as the internal standard. The derivatized samples were separated by reverse phase UPLC using a C18 column (AccQ·Tag Ultra C18, 1.7 μm (Millipore Corporation, Milford, MA, USA)) and a gradient elution of solvent A (5% solution of Waters AccQ·

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Tag eluent A concentrate in 19 wt % sodium acetate, 6−7 wt % phosphoric acid, and 1−2 wt % triethylamine) and solvent B (2% formic acid in acetonitrile solution) at a flow rate of 0.5 mL/min as follows: 0 min (99.9% A), 1 min (98.5% A), 11.5 min (78% A), 13.5 min (40% A), and 15 min (99.9% A), followed by a 2 min recovery with initial conditions. The urea derivatives yielded during the derivatization were detected by fluorescence (excitation−emission, 250−395 nm).

2.6. Determination of Gelatin Loading. Amino acid analysis was performed in the same way as described in the previous section. Standard solutions of gelatin were prepared by dilution of the hydrolyzed Nitta B 300 gelatin samples. Gelatin has several specific amino acids such as alanine (Ala), asparagine and aspartic acid (Asx), hydroxylproline (OH-Pro), and valine (Val), which do not exist in the nonapeptide sequence of leuprolide.[9,15] The second abundant amino acid in the gelatin, alanine, was used to determine the gelatin content in the formulation.[10] The experiment was performed in triplicate. The EE of gelatin was calculated in the similar way as in eq 3.

2.7. Particle Size Distribution. The median diameter of the microspheres was determined using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, U.K.). About 30−40 mg of microspheres was suspended in 1 mL of water and vortexed vigorously before adding to the instrument sample dispersion unit. The refractive indices for sample and dispersant were set at 1.59 and 1.33, respectively.[16] Three measurements were performed per sample at a stirring speed of 2500 rpm and sampling time of 15 s.

2.8. Surface Morphology. The surface morphology of microspheres was examined using a TESCAN MIRA3 FEG scanning electron microscope (SEM) (Kohoutovice, Czech Republic). The microspheres were fixed on a brass stub using double-sided carbon adhesive tape, and the samples were prepared electrically conductive by coating with a thin layer of gold for 60 s at 18 mA under vacuum.[17] Images were taken at an excitation voltage of 2 kV.

2.9. Glass Transition Temperature. The T_g of microspheres was determined with a modulated differential scanning calorimeter (mDSC) (TA Instruments, New Castle, DE, USA). Microspheres (3−5 mg) were crimped in DSC aluminum pans. Temperatures were ramped between −20 and 90 °C at 3 °C/min. All samples were subjected to a heat/cool/heat cycle. The results were analyzed by using TA TRIOS software, and T_g was taken as the midpoint of the reversing heat event.

2.10. Residual Moisture. Residual water content in the microspheres was determined by Karl Fischer (KF) titration. An amount of 80 mg of formulation was weighed into a vial and sealed with a septum cap. Anhydrous dimethyl sulfoxide (DMSO) was added to make the final concentration at 10 mg/mL, and the sample was sonicated for 10 min before injection into the KF for titration. The moisture in the blank DMSO was also determined and subtracted from the total moisture in the microspheres/DMSO mixture to obtain the moisture level in the microspheres.

2.11. Residual Solvent. Residual solvent (DCM) in the microspheres was determined by a Trace 1310 gas chromatograph (GC) (Thermo Fisher Scientific Inc., Waltham, MA, USA). The microspheres were added into a glass vial containing anhydrous DMSO to make the final concentration at ∼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 was used as the carrier solvent at a flow of 25 mL/min; air flow was 350 mL/min, and hydrogen flow was 35 mL/min; the front detector temperature was 240 °C, and the front inlet pressure was a constant flow at 2 mL/min. Each sample was agitated for 20 min at 80 °C, and 1 mL of the headspace sample was injected into the front inlet with the 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. A standard curve was prepared by adding methylene chloride to DMSO at 1, 10, 50, 100, 250, and 500 ppm.

2.12. Porosity. The porosity of microspheres was determined by mercury intrusion porosimetry (AutoPore V series, Micromeritics, Norcross, GA, USA). An amount of about 80−150 mg of microspheres was weighed into 3 cc powder penetrometers to make the final used penetrometer stem volume in the range of 25−80%. Analysis was performed over low and high pressure ranging from 0.5 psia to 61 000 psia with an equilibration of 10 s at each pressure. The curve of the cumulative intrusion volume per gram (mL/g) vs filling pressure was reported. Interstitial void volume between the particles was filled before the mercury intrusion into the pores and subtracted from the total intrusion volume. The completion of interparticle space filling was indicated by an abrupt change in filling rate on the intrusion volume curve, and the starting point of the corrected intrusion volume used for porosity calculation was determined at the inflection point.[18] The porosity was calculated by the percentage of intrusion volume in the bulk volume.

2.13. Release Kinetics of Leuprolide from PLGA Microspheres. Drug release of microspheres was carried out using a sample-and-separate method in release medium PBST (phosphate buffered saline (PBS) + 0.02% Tween 80 + 0.02% NaN_3, pH 7.4). Microspheres (∼10 mg) were suspended in 1 mL of medium and shaken mildly at 37 °C. At each time point (1, 3, 7 days and weekly thereafter), the medium was completely collected after centrifugation at 8000 rpm for 5 min and replaced with fresh PBST buffer. The concentration of leuprolide in the supernatant was determined by UPLC as described in the section Determination of Leuprolide Acetate Loading.

2.14. Kinetics of Erosion. Microspheres (weight = W_0) were incubated in release medium at 37 °C under mild agitation. At each time point, the microspheres were washed by ddH_2O and retrieved on 0.20 μm nylon filter paper under reduced pressure. The collected microspheres and filter paper were transferred into preweighed tubes and then dried under reduced pressure at room temperature for about 2 days.[21] Dried microspheres were weighed ($W_1(t)$), and the mass loss was calculated by

$$\text{mass loss (\%)} = \frac{W_0 - W_1(t)}{W_0} \times 100 \quad (4)$$

The mass loss after initial burst was plotted against the release after initial burst, which was calculated by

$$\text{release after initial burst (\%)} = \frac{A(t) - A_1}{A_L - A_1} \times 100 \quad (5)$$

where $A(t)$ is the amount of peptide released at time t, A_1 is the amount of peptide released on the first day, and A_L is the amount of peptide loaded in the microspheres.

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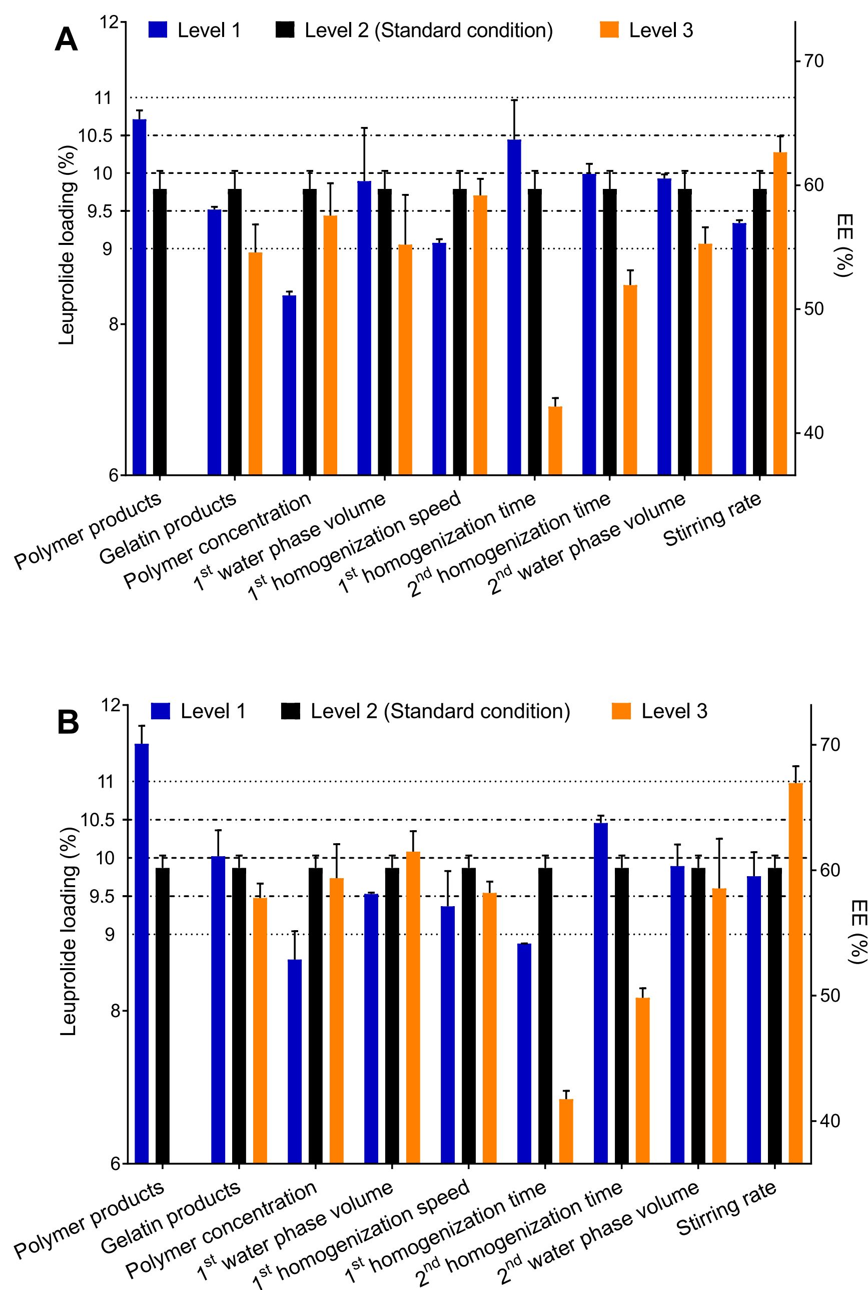

Figure 1. Loading of leuprolide determined by one-time extraction (A) and AAA methods (B). All values present as mean ± SEM (n = 3). The dash lines indicate the desired loading of 10% w/w (— — —) ± 5% (-·-·-) or 10% (···) specification.

2.15. Statistical Analysis. All data were expressed as mean ± SEM (n = 3 or specifically indicated). Statistical analyses were carried out using GraphPad Prism 8.0.2 software. An ANOVA test was performed to determine the significance. The level of significance was established at the 95% confidence interval (α = 0.05).

3. RESULTS

3.1. Effect of Manufacturing Parameters on EE of Leuprolide. In most formulations, the encapsulation efficiency of leuprolide was above 50% (Figure 1). The loading determined by one time extraction (Figure 1A) was slightly lower than the results determined by AAA (Figure 1B) due to the decreased recovery caused by peptide−polymer interaction.[10,19] Most formulations were within 5−10% of the desired loading of 10%, observed in the LD after removal of surface mannitol.[10,20] The effect of manufacturing parameters on the EE of leuprolide included the following: the substitution of ring-opening polymerized PLGA (Mw = 15 140, 75/25, acid-capped) in place of polycondensation PLGA (Mw = 13 887, 75/25, acid-capped) slightly increased the EE of leuprolide; when the gelatin was replaced by low bloom number gelatin, the EE was decreased while the substitution of similar bloom number gelatin did not significantly change the EE. To achieve a higher EE of leuprolide, higher polymer concentration, higher first homogenization speed, shorter homogenization time, lower second water phase volume, and higher stirring rate were preferred.

3.2. Effect of Manufacturing Parameters on EE of Gelatin. During development of composition equivalent formulations, the EE of gelatin (101 ± 1%) in the formulations

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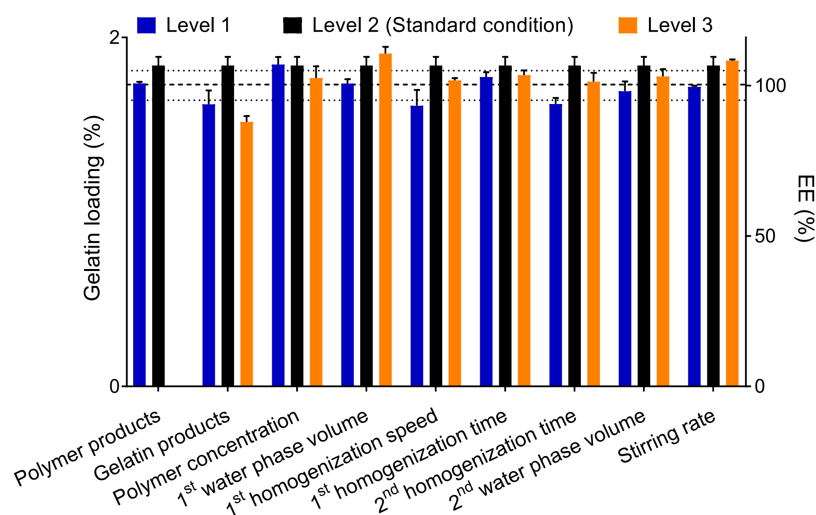

Figure 2. Loading of gelatin. All values are presented as the mean ± SEM ($n = 3$). The dash lines indicate the desired loading of 10% w/w (− − −) ± 5% (· · ·) specification.

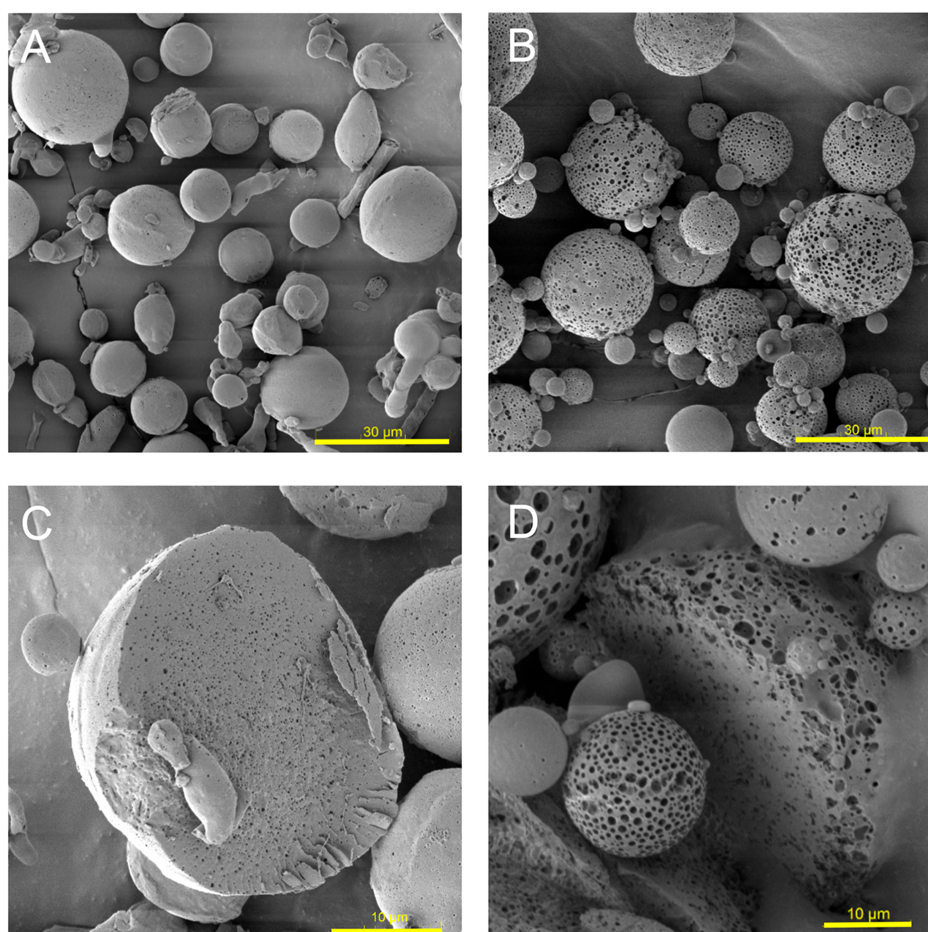

Figure 3. SEM micrographs of (A) LD microspheres, (B) standard condition microspheres, (C) sectioned LD microspheres, and (D) sectioned standard condition microspheres.

loaded with gelatins with relative high bloom numbers (225 or 300) was observed to be much higher than the EE of LUP. A majority of the formulations had desirable loading of 1.73% w/w gelatin (Figure 2), as in the LD. When the gelatin was replaced with the lower bloom Sigma 75, the EE was decreased.

3.3. Effect of Manufacturing Parameters on Product Attributes. *3.3.1. Morphology.* SEM micrographs indicated the LD microspheres (Figure 3A) were fine and small particles with smooth surface. The formulation made under the standard condition (Figure 3B) exhibited higher surface porosity compared to the LD. The figures of sectioned microspheres showed that the LD have small pores distributed

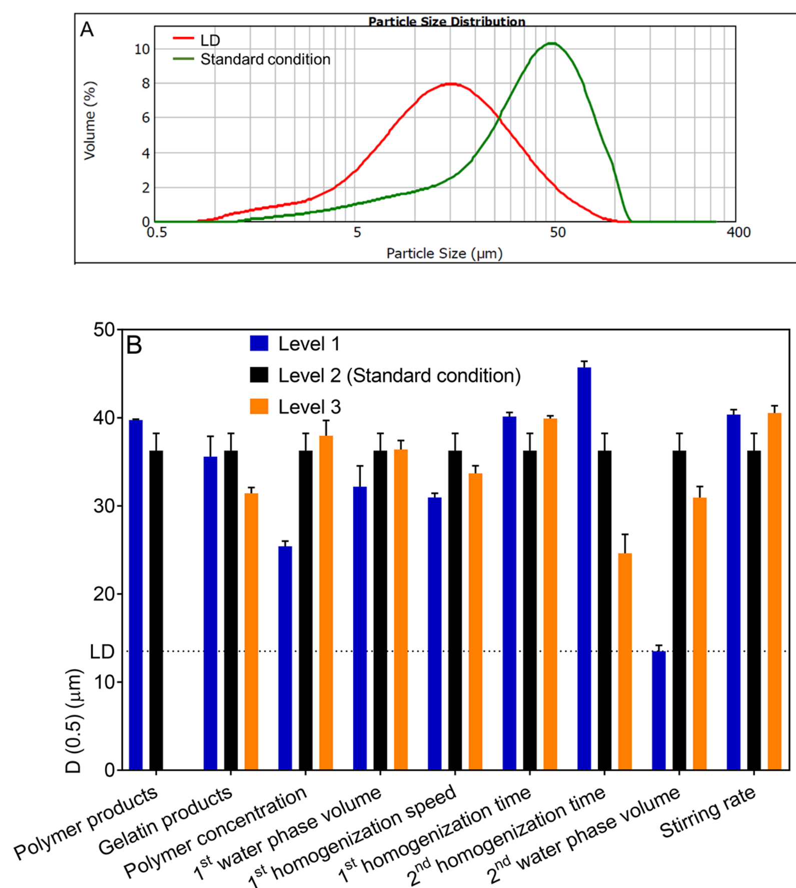

Figure 4. (A) Particle size distribution of LD and standard condition formulation and (B) median diameters (D (0.5)) of the formulations. The columns indicate the mean ± SEM (n = 3).

homogeneously inside the microspheres (Figure 3C). The standard condition microspheres had a denser polymer core under the porous surface (Figure 3D).

3.3.2. Particle Size Distribution. The particle size distribution of LD was narrow with a volume median diameter (D (0.5)) (13.5 ± 0.29 μm) (mean ± SEM, n = 3) and the standard condition formulation showed a left skewed particle size distribution with a D (0.5) at 36.38 ± 1.95 μm (Figure 4A). The effect of manufacturing parameters on the particle size included the following (Figure 4B): replacement of polymer with Resomer polymer increased the particle size; using gelatin with low bloom number decreased the size; when the polymer concentration, primary water phase volume, first homogenization speed, and second water phase volume were decreased, the particle size was decreased; and increasing second homogenization time significantly decreased the particle size.

3.3.3. Glass Transition Temperature (T_g). Figure 5 displays T_g for raw polymer, LD, and formulations. The T_g of the raw Wako polymer was observed to be 39.6 ± 0.3 °C and 49.5 ± 0.1 °C for the standard condition formulation (w/mannitol) (Figure 5A,B), consistent with the well-known PLGA–leuprolide interaction upon microspheres form.[9] The T_g of LD microspheres (w/mannitol) (49.4 ± 0.2 °C) and standard condition formulation (w/mannitol) were not significantly different (p > 0.05). The standard condition formulation (without mannitol) exhibited a T_g of 49.4 ± 0.2 °C, indicating that the presence of mannitol in the formulations did not significantly affect the T_g (p > 0.05) (Figure 5B). In addition, the measured T_g values for all other formulations prepared with the Wako polymer were very close to that of commercial product and standard condition formulation (Figure 5C). The T_g of raw Resomer polymer was measured as 46.2 ± 0.1 °C, and the microspheres prepared by this polymer had a T_g of 51.5 ± 0.1 °C. In the formulations prepared by Wako polymer, the loading of peptide varied in a relatively narrow range and showed no significant difference from the LD. The T_g of Resomer polymer was 6.6 °C higher than that of Wako, but the increased T_g in microspheres relative to raw polymer was only ∼5 °C.

3.3.4. Residual Moisture, Solvent, and Porosity. The water content of the LD was determined by Karl Fischer titration to be 0.14 ± 0.06%. The prepared formulations showed higher residual water content, and the majority showed values around 1−2% (Figure 6). The highest residual moisture was observed in the formulation prepared with low first water phase volume and shortest second homogenization time. The residual content of DCM in LD was lower than 1 ppm. The prepared formulations showed residual DCM in the range of 0.02−0.2% (Figure 7). We note that our formulations were not annealed, which would be expected to further reduce residual solvent. The porosity of the LD was 11.6% (n = 2), while the porosity of the standard condition formulation was observed to be 47% (n = 2). The other prepared formulations also showed a higher level of porosity compared to LD, and no significant difference was observed in the group of formulations prepared with different second homogenization time (Figure 8).

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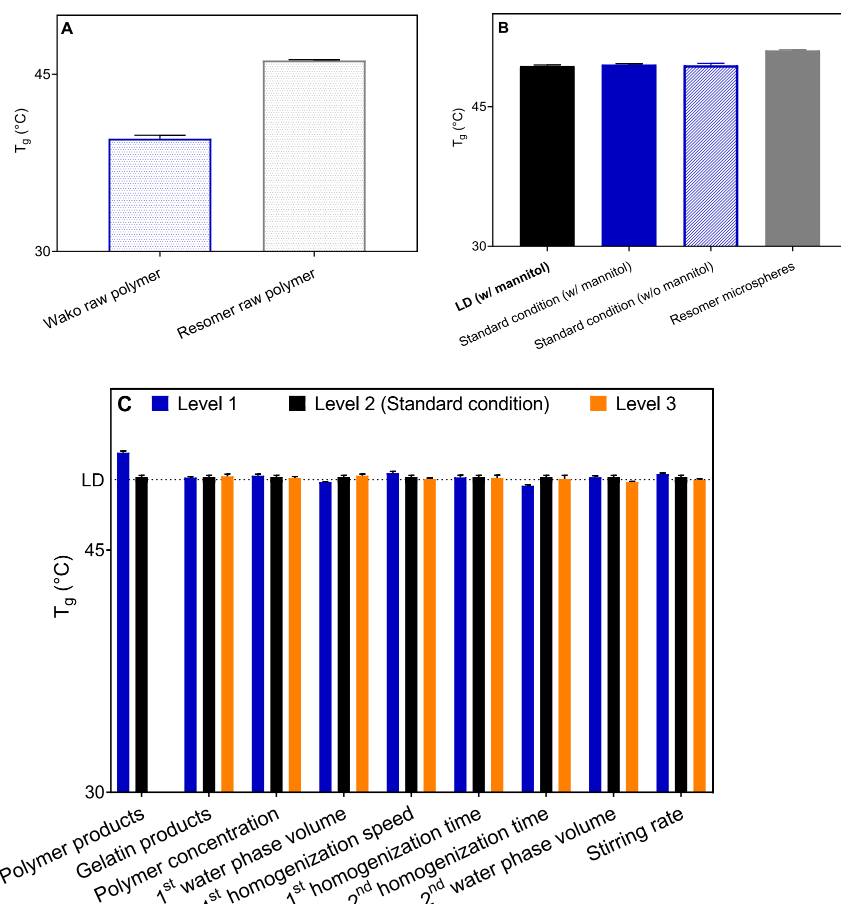

Figure 5. Glass transition temperatures (T_g) of (A) raw polymers, (B) LD, standard condition formulation with or without mannitol and Resomer microspheres, and (C) all other formulations. Data represent the mean ± SEM (n = 3).

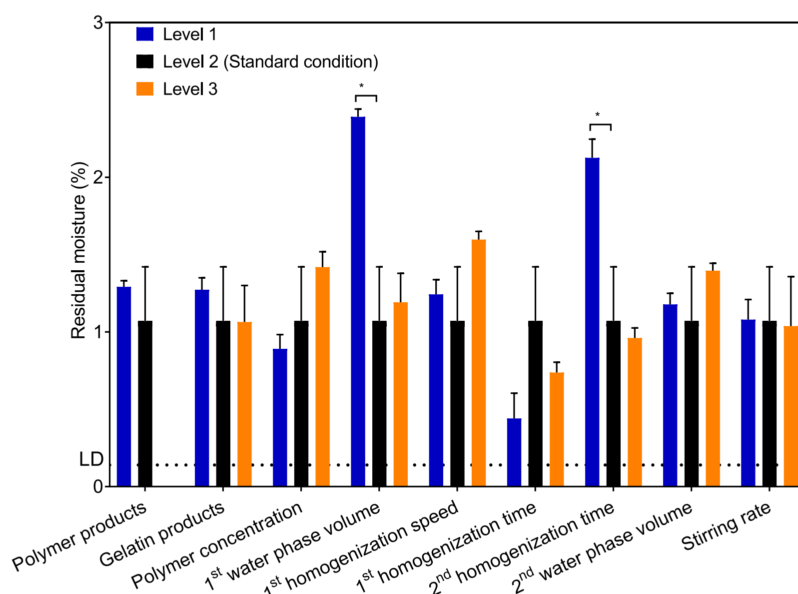

Figure 6. Residual moisture of formulations. Data represent the mean ± SEM (n = 3). The dash line indicates the value for LD: *$p \leq 0.05$.

3.4. Release Kinetics. The cumulative release of LD in PBST lasted for 7 weeks with a 22.8 ± 0.4% initial burst on day 1 followed by a zero-order release after day 3. The release of peptide from prepared formulations showed similar release pattern (Figure 9). In each parameter group, when the formulation had a higher EE, it usually also had higher initial burst. The loading and release of leuprolide in each formulation were normalized to the values of the standard condition formulation, and the comparison is shown in Figure 10. When the formulation was prepared with the Resomer polymer, it showed a higher EE and higher initial burst, but the release slowed down after 7 days and exhibited a slightly slower

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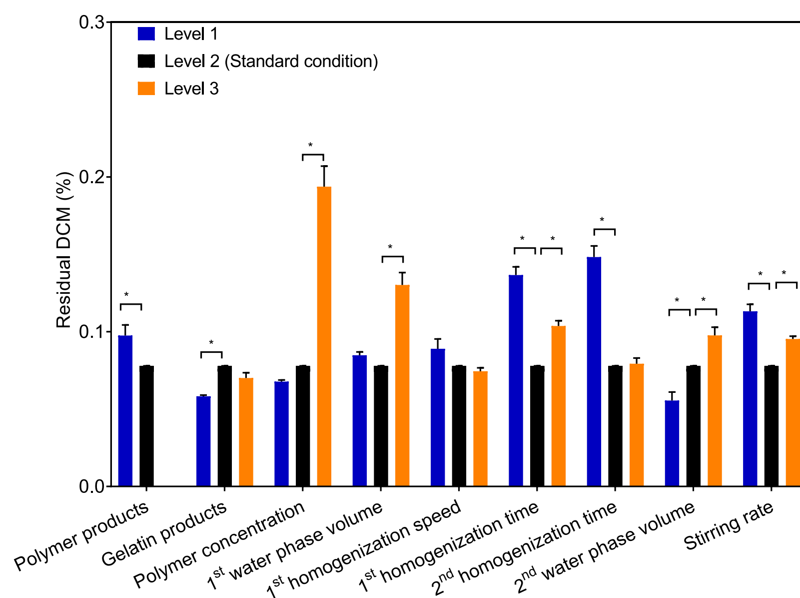

Figure 7. Residual DCM of formulations. Data represent the mean ± SEM (n = 3): *$p \leq 0.05$.

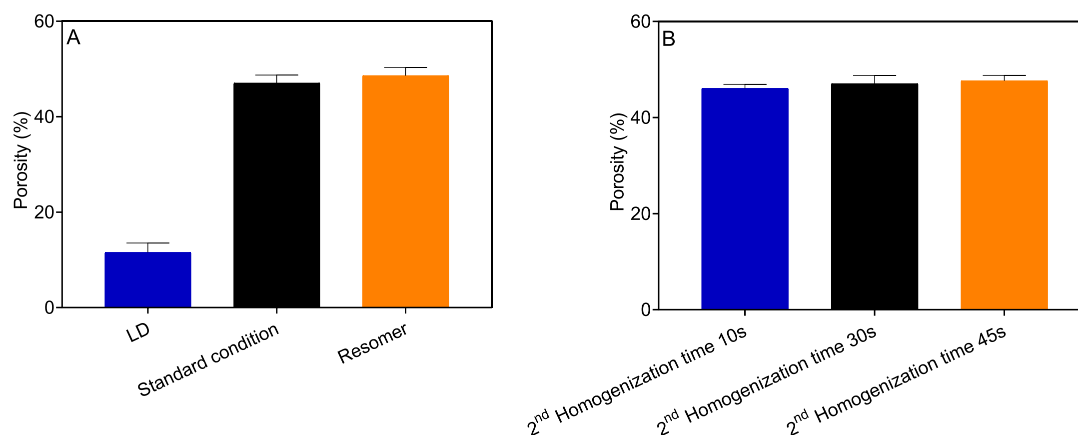

Figure 8. Porosity of formulations in raw material and second homogenization time groups. Data represent the mean ± range (n = 2).

release compared to the standard condition formulation (0.93%/day for Resomer formulation vs 1.12%/day for standard condition formulation; 1.05%/day for LD based on least squares slope for 7−63 days). The release mechanisms of standard condition formulation, Resomer formulation, and LD were compared by plotting kinetics of release after the initial burst versus the mass loss of the polymer, as shown in Figure 11. As seen in the figure, these curves superimposed, strongly suggesting an equivalent release mechanism for the three. In each case, the kinetics of peptide release was faster than mass loss. Slightly slower mass loss rate from Resomer formulation was consistent with its slower peptide release. The release mechanism was likely governed by a combination of factors (e.g., peptide desorption and mass loss), as discussed elsewhere.[21]

4. DISCUSSION

On the basis of the ionic interaction between basic residual of amino acids in the leuprolide and carboxylic group in the polymer chains, Okada et al. proposed the drug core structure in the microspheres loaded with leuprolide where polymer chain served as a hydrophobic diffusion barrier.[22] We observed ~60% EE of the leuprolide and ~100% EE of the gelatin (Figure 1 and Figure 2). The loss of peptide mainly occurred during the second homogenization step, and the loss content increased as homogenization intensity increased (Figure S1). In our study, small microspheres were preferably prepared. Thus, a balanced protocol was established to produce microspheres with desired EE, particle size, and release kinetics due to the efficiency of the homogenizer and small sample size. Both leuprolide and gelatin are considered water-soluble, but gelatin is only readily dissolved at relatively high temperature.[15] Though leuprolide and gelatin were fully dissolved in the primary water phase at 60 °C, the temperature of the primary emulsion formed after homogenization was close to room temperature (data not shown). It is surmised that the gelatin in the disperse phase became a rigid gel-like structure and cannot easily migrate into the outer water phase during the second homogenization.

The effect of raw materials/manufacturing variables on EE of leuprolide and D (0.5) were shown in (Figure 1 and Figure 4B) and also summarized in Table S1. When the Wako PLGA was replaced with the Resomer polymer with a marginal increase in Mw (up to 15.1 kDa from 13.9 kDa), increased EE and a slightly larger particle size were obtained in the formulation. The Resomer 752H has a similar average

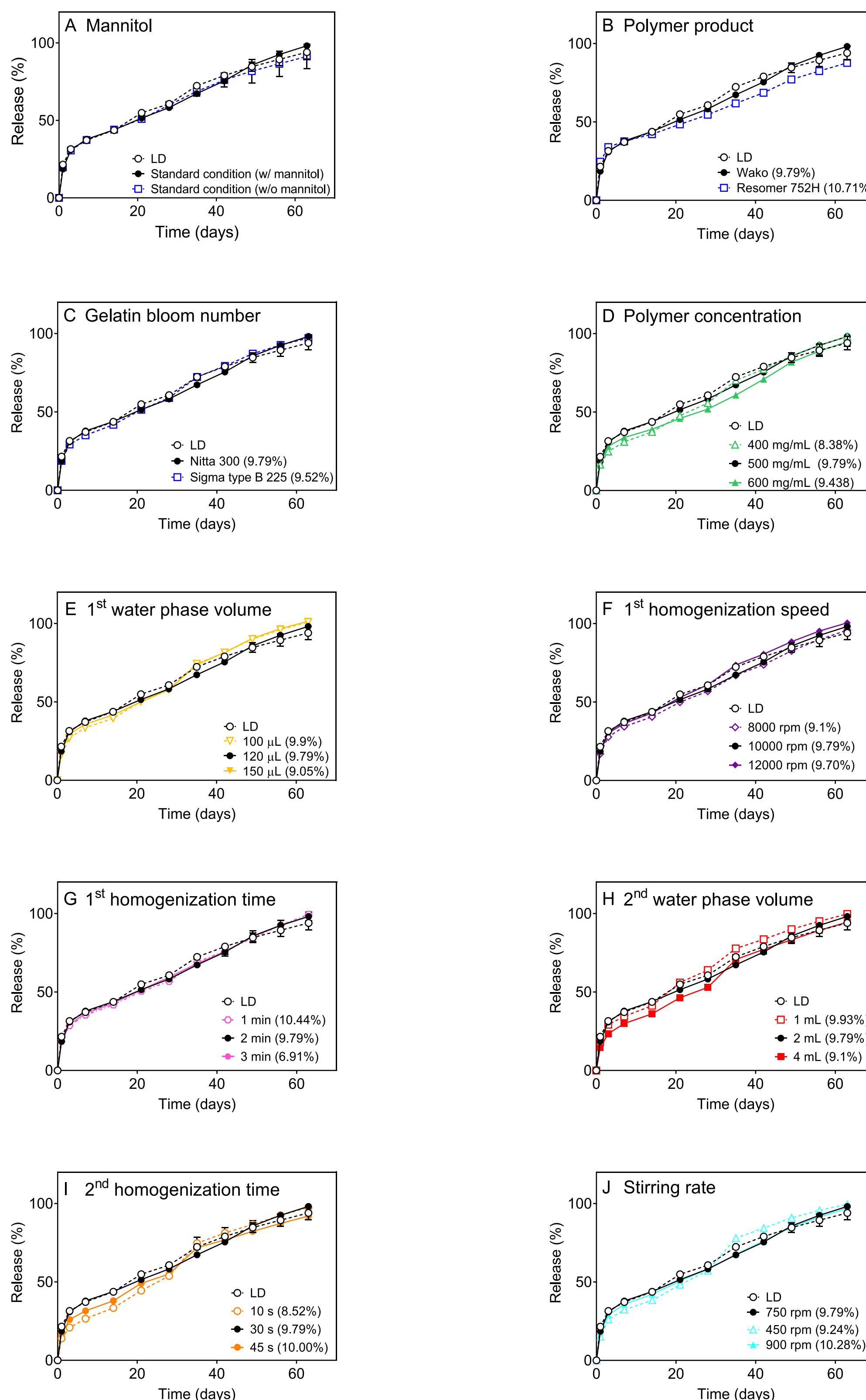

Figure 9. Release kinetics of formulations in different variable groups. Data represent the mean ± SEM (n = 3). The values in the parentheses are the loading of peptide in each formulation.

molecular weight to Wako polymer but the polymer solution of the formulation showed higher viscosity, which also raised the viscosity of the primary emulsion (data not shown) and finally decreased the drug loss during manufacturing. Gelatin products with bloom numbers 75, 225, and 250 were used to prepare three different microspheres. The EE of leuprolide and gelatin in microspheres loaded with gelatins with relatively high bloom numbers was comparable, while the EE for both ingredients was lower in the formulation with low bloom number gelatin (Figure 1 and Figure 2). The bloom number is

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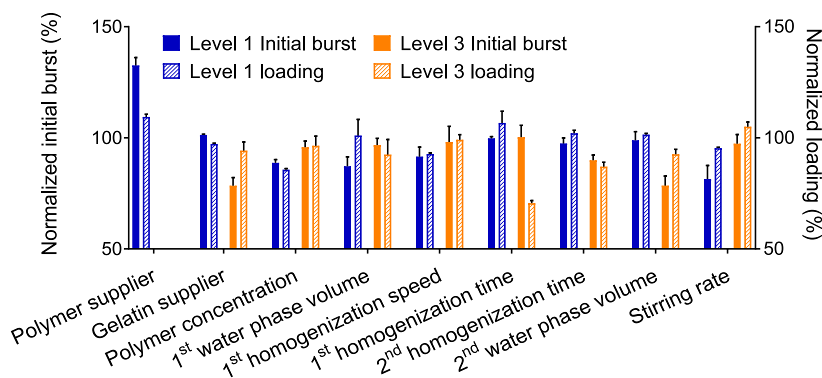

Figure 10. Relationships between initial burst and loading, which are normalized by the standard condition values (standard condition loading, 9.8 ± 0.2%; initial burst, 18.6 ± 0.6%). Data represent the mean ± SEM (n = 3).

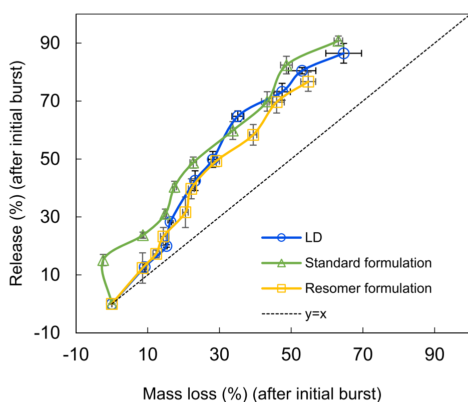

Figure 11. Kinetics of release after initial burst plotted against mass loss kinetics. Data on both axes represent the mean ± SEM (n = 3).

associated with the gelatin molecular weight and intrinsic viscosity.[15,23] The reduced viscosity of the gelatin 75 solution is a possible reason for decreased EE as the primary emulsion size would be expected to be smaller, increasing the mobility and escape of the disperse phase droplets. The slight reduction in gelatin loading for microspheres with 75 bloom gelatin is consistent with this explanation.

When the polymer concentration was decreased from 500 and 600 mg/mL to 400 mg/mL, a drop in the EE of peptide and median diameter of microspheres was observed (Figure 1 and Figure 4B). The concentrated polymer solution is expected to shorten the evaporation time of DCM[24] and thus more strongly inhibit drug leakage from the polymer matrix to the outer water phase.[25,26] The effect of W1/O ratio on the EE of drug could be complex. Lower W1/O ratio (i.e., lower primary water phase) usually results in an efficient emulsification to form the primary emulsion with good quality and minimize the drug loss during the secondary emulsification.[24,25,27] But lower primary water phase may also produce smaller microspheres[28,29] with relatively low EE of peptide. Ogawa et al. observed increased EE of peptide as the particle size of microspheres increased.[8] The water phase volume range (100, 120, and 150 μL) selected in this study did not significantly affect the EE of peptide (Figure 1), while it slightly decreased the particle size when the smaller primary aqueous phase volume was used. The first homogenization speed range (8000, 10 000, and 12 000 rpm) selected in this study did not significantly affect the EE of peptide, but low homogenization speed slightly decreased the EE, which was probably due to the larger emulsion droplets size that increased the chances of drug leakage. However, a significant reduction in the EE of peptide was obtained when the first homogenization time increased. Another dramatic decrease of peptide EE occurred when the second homogenization time or secondary water phase volume was increased. The intensity of second homogenization seems to be the critical factor that affects both EE of peptide and particle size. Particularly, when larger water phase was mixed with the primary emulsion, the emulsification efficiency was reduced and led to increased particle size.[25,29] Stirring rate is expected to affect the particle size by controlling breakup of emulsion into small droplets.[8,30,31] In our study, the dispersion of the oil phase into the water phase and separation of nascent emulsion droplets occurred during the second emulsification by the high shear force provided by the homogenizer. Since the soft microspheres droplets already formed before the in-liquid drying step, the stirring rate mainly affected the solvent removal rate. Higher stirring rate will lead to faster evaporation of DCM[32] and solidification of microspheres, and slightly increased EE of peptide was observed in this case.

The T_g of microspheres is mainly affected by the T_g of raw polymer and the interaction between leuprolide and polymer chains. When leuprolide acetate was loaded into microspheres, the basic amino acid residuals in peptide interacted with the carboxylic group in the polymer chains[22] and formed salts throughout the polymer matrix, increasing the T_g. Okada et al. found the T_g of the leuprolide loaded microspheres increased gradually from 42 to 47 °C as the loading increased in the range of 0−8%.[9,12] However, in our study, all microspheres prepared with Wako polymer displayed the same ΔT_g (~10 °C) independent of the peptide loading. As the loading of peptide was always ~10%, it is possible that the reported effect has a plateau at high peptide loading (>8%). Though the Resomer polymer showed a higher T_g than the Wako polymer, the ΔT_g during the encapsulation of peptide was only ~5 °C. Differences between T_g values between the manufacturers may be influenced by different blockiness and block length of the lactic and glycolic acid chains or changes in the polymer molecular weight distribution.[33] The small Mw difference between the two manufacturers of PLGA would be expected to affect these numbers to a negligible extent.

The residual moisture and residual DCM level for LD were lower than the formulations prepared. Particularly, the residual DCM in LD was lower than the lowest detection limitation of 1 ppm. These results indicated that the in-water drying protocol on a large scale is capable of achieving low levels of organic solvent in the manufactured microspheres. DCM is classified as solvent to be limited in USP General Chapter

<467> with the permitted daily exposure (PDE) at 6 mg/day.[34] The concentration limit could be calculated by[34]

$$\text{concentration (ppm)} = (1000\ \mu\text{g/mg} \times \text{PDE (mg/day)})/\text{dose (g)} \quad (6)$$

The total amount of 1-month LD product in a single administration is 88.2 mg, since most of the formulations in this study were considered as composition-equivalent and similar dose was proposed to calculate the concentration of DCM exposure. The maximum concentration of DCM in the formulations was 0.19%, and the corresponding amount of DCM in the formulation dosed was 0.17 mg, which was lower than the PDE. When the microspheres were prepared with the shortest second homogenization time, they displayed the high residual levels of both water and DCM. This is probably due to the relatively large particle size that increased the distance for the solvent to be transported through the polymer matrix and evaporate. When the second water phase volume was increased, the residual DCM was also increased. The DCM close to the surface of the nascent microspheres was removed quickly, which facilitated the formation of a thick skin layer impeding further removal of solvent.[35] Since solvents, like water and DCM, affect the polymer relaxation and work as plasticizer on PLGA,[36] the T_g of formed microspheres might be regulated by residual solvent content. However, in this study, we did not see significant difference of T_g in the formulations with different levels of residual solvents. This may indicate that the current difference of residual solvent is not large enough to induce significant changes on the microsphere T_g.

The release kinetics for all formulations were highly similar to LD. The biggest difference in the release curves was caused by the initial burst. The theoretical loading of leuprolide was kept constant in the formulation, but the actual loading varied based on the different input variables. The increased loading of water-soluble drug caused a slightly higher concentration gradient that induced more drug diffusion to release medium. The microspheres prepared with Resomer polymer had a slightly larger particle size, higher loading of peptide, and higher initial burst. Meanwhile, studies have shown that increased T_g might decrease the long-term release rate.[37,38] As described above, the T_g values of both the raw Resomer polymer and the final microspheres were higher than those based on the Wako polymer. After the initial burst, Resomer microspheres showed slightly slower release rate compared the Wako microspheres consistent with the slightly slower polymer erosion. The composition-equivalent microspheres showed much higher porosity than the LD, and they were expected to release the peptide more quickly. This is not what we observed. Instead, the in vitro release of all the formulations after the initial burst was strikingly similar. It is noteworthy that the long-term release of peptide from PLGAs has been shown to be very insensitive to the length-scale of the PLGA matrix at least when above a certain matrix size,[39] which is likely a result of a combination of factors such as the strongly likelihood of a molecular dispersion of the peptide-PLGA salt[19] and size-insensitivity of polymer erosion for the polymers used for peptide encapsulation. Moreover, after a short release period, microspheres of this lower Mw tend to aggregate and in vitro release may be more like that of a thin film.

In closing, we developed composition-equivalent PLGA microsphere formulations to the 1-month LD. The composition, T_g, and in vitro release kinetics of PLGA microspheres loaded with leuprolide can be largely replicated on the bench scale relative to the LD. The encapsulation efficiency of leuprolide was much lower than that of gelatin. The substitution of ring-opening polymerized PLGA, albeit with a marginal increase in Mw, in place of polycondensation PLGA increased T_g, EE, and initial burst and slightly reduced long-term release rate. Changing manufacturing variables centered at a standard formulation did not strongly affect release behavior. Changes in initial burst release mirrored changes in drug loading/encapsulation efficiency. The release mechanisms of formulation after initial burst appeared to be dependent on polymer erosion and not strongly affected by the microsphere characteristics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.9b01188.

Effect of increased variables on the EE of leuprolide and *D* (0.5) in formulations; kinetics of leuprolide loss during secondary homogenization process and in-liquid drying process (PDF)

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Notes

The authors declare no competing financial interest.

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