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Evaluating the Properties of Poly(lactic-*co*-glycolic acid) Nanoparticle Formulations Encapsulating a Hydrophobic Drug by Using the Quality by Design Approach[1)]

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We applied the Quality by Design (QbD) approach to the development of poly(lactic-*co*-glycolic acid) (PLGA) nanoparticle formulations encapsulating triamcinolone acetonide, and the critical process parameters (CPPs) were identified to clarify the correlations between critical quality attributes and CPPs. Quality risk management was performed by using an Ishikawa diagram and experiments with a fractional factorial design (ANOVA). The CPPs for particle size were PLGA concentration and rotation speed, and the CPP for relative drug loading efficiency was the poor solvent to good solvent volume ratio. By assessing the mutually related factors in the form of ratios, many factors could be efficiently considered in the risk assessment. We found a two-factor interaction between rotation speed and rate of addition of good solvent by using a fractional factorial design with resolution V. The system was then extended by using a central composite design, and the results obtained were visualized by using the response surface method to construct a design space. Our research represents a case study of the application of the QbD approach to pharmaceutical development, including formulation screening, by taking actual production factors into consideration. Our findings support the feasibility of using a similar approach to nanoparticle formulations under development. We could establish an efficient method of analyzing the CPPs of PLGA nanoparticles by using a QbD approach.

Key words Quality by Design (QbD); critical quality attribute; critical process parameter; poly(lactic-*co*-glycolic acid) (PLGA); nanoparticle formulation

The use of Quality by Design (QbD), an approach aimed at scientific and systematic pharmaceutical development to ensure quality throughout the life cycles of pharmaceutical products, has recently been recommended.[2–4)] Construction of such a control strategy in the future is also desirable for nanoparticle formulations as representative functional dosage forms. However, attempts to construct QbD-based quality-control strategies for nanoparticle formulations can be difficult, because the pharmaceutical properties and manufacturing processes required are more complex than are those for conventional pharmaceutical products. In addition, the approaches designed for establishing QbD-based quality management strategies may vary markedly depending on the product types of nanoparticle formulations. Accordingly, few examples of the application of the QbD approach to nanoparticle formulations are available currently.[5–7)]

Poly(lactic-*co*-glycolic acid) (PLGA) is prepared by random copolymerization of lactic acid and glycolic acid, with the two different monomers being connected by ester linkages. The ratio of these two monomers used for polymerization and the average molecular weight of the resulting polymer determine the kinetics of release of the drug from PLGA microparticles[8–11)] or from the drug–PLGA mixture in *in situ* implant form.[12)] An example of the utilization of these properties of PLGA microparticles is Leuplin (Takeda Pharmaceutical Co., Ltd., Osaka, Japan), a PLGA particle formulation composed of poly-lactic acid microcapsules with a diameter in the order of micrometers and encapsulating a derivative of luteinizing-hormone releasing hormone. The development of this product has made it possible to develop a long-term controlled-release system compatible with subcutaneous injection once monthly, once every 3 months, or once every 6 months. Furthermore, extensive research and development of PLGA nanoparticle formulations that retain controlled drug release and ensure greater drug concentrations at the target site are ongoing.[7,13)]

Our objectives here were to 1) establish methods of analyzing the critical quality attributes (CQAs) of PLGA nanoparticle formulations and manufacturing processes for these formulations; and 2) enhance our understanding of the correlations between product performance and quality attributes and the correlations between quality attributes and process parameters. Because many parameters interact with each other under actual manufacturing conditions, the quantities of mutually related factors were assessed in the form of ratios. Furthermore, we investigated two-factor correlations by using a fractional factorial design with resolution V, in which both main effects and two-factor interactions can be estimated without confounding each other. The system was then extended by using a central composite design, and the results obtained were visualized by using the response surface method to construct a design space. Through these results, we assessed the feasibility of applying QbD to more complex, higher-function formulations, namely nanotechnology-based formulations, and the usefulness of the QbD approach in the early stage of pharmaceutical development. Although a formulation optimization of PLGA nanoparticles by using the QbD approach was reported previously,[7)] that study was a basic one

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without the encapsulation of drugs in the nanoparticles tested; it therefore failed to evaluate drug leakage, which directly affects encapsulation efficiency and greatly influences product efficacy and safety. Therefore, we encapsulated a drug in PLGA nanoparticles. Furthermore, in constructing the design space, we incorporated, insofar as possible, actual manufacturing factors, such as operating conditions.

To set a quality target product profile (QTPP) of the PLGA nanoparticle formulations developed here, we selected a target disease and drug. We chose rheumatoid arthritis, because one of the established methods of therapy for this disease is local intraarticular administration of the appropriate drug. Furthermore, there is a report that PLGA nanoparticles could be an effective means of delivery to inflamed synovial tissue owing to their ability to penetrate the synovium, and PLGA particulate systems with biocompatibility in the joint can provide local-therapy action in joint disease.[14] A hydrophobic anti-inflammatory drug, triamcinolone acetonide, which is approved in Japan for administration *via* this route for the treatment of rheumatoid arthritis, was selected as the target drug and active pharmaceutical ingredient (API).[15] We performed a feasibility study for an encapsulation product containing the API in PLGA nanoparticles, designed to enhance penetration into the synovial cavity following intraarticular administration.[14] This formulation is a sterile injection for intraarticular administration and is to be used as a freeze-dried preparation; the PLGA nanoparticle suspension is prepared at the time of use, because the suspension is usually not stable. Considering these factors, the settings of the QTPP for this formulation are summarized in Table 1. The strength of the product is not less than 90% and not more than 110% of the label claim. This value was empirically determined.

Experimental

Proposed Formulation and Manufacturing Processes Screening of the composition of both constituents of PLGA and the molecular weight of the resulting polymer revealed that neither factor had any effect on relative drug loading efficiency in PLGA nanoparticles (data not shown). Accordingly, PLGA-7520 with a lactic acid to glycolic acid ratio of 3 : 1 and a molecular weight of 20000 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used for this formulation.

Partially saponified polyvinyl alcohol (abbreviated hereafter as PVA) was selected as an excipient, and acetone and water were selected as good and poor solvents, respectively, for PLGA. Gohsenol EG-05P (degree of saponification 86.5 to 89.0 mol%; viscosity 4.8 to 5.8 mPa·s, the Nippon Synthetic Chemical Industry Co., Ltd., Osaka, Japan), which is marketed as a pharmaceutical excipient, was used as a PVA preparation.

To prepare PLGA particles with a diameter in the order of nanometers we used the oil-in-water (O/W) emulsion solvent diffusion method.[16] Briefly, the procedures used were as follows:

(1) API and PLGA were dissolved in an appropriate quantity of acetone (good solvent preparation process).

(2) PVA was separately dissolved in water (poor solvent preparation process).

(3) To the poor solvent, stirred under temperature-controlled conditions, the good solvent was added to allow crystallization of PLGA nanoparticles encapsulating API (crystallization process). A 500-mL crystallization tank was used.

(4) After crystallization, the resulting suspension was transferred to a 500-mL recovery flask for acetone removal with a rotary evaporator (NVC-2200, EYELA, Tokyo, Japan) (solvent removal process).

(5) After solvent removal, the resulting aqueous suspension was filtered over Filter Paper No. 2 (size of retained particles, 5 μm) (Advantec, Tokyo, Japan) to remove API that had leaked from the PLGA particles and aggregated (crude filtration process).

(6) After crude filtration, the resulting suspension was transferred to a vial and lyophilized in a freeze dryer (FDU-1200, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) for approximately 60 h (lyophilization process).

Although a sterile filtration process may be needed in the overall manufacturing process for commercial products, we did not include this filtration step here, because our aim was to investigate only the early stage of pharmaceutical development; *i.e.*, this study did not involve administration of the product to animals.

Proposed CQAs Because the most desirable function of this formulation was penetration into the synovial cavity, it was possible that particle size and particle size distribution would be key factors. In addition, the drug loading efficiency of the PLGA particle formulation would obviously have a major influence on its efficacy and safety.

Table 1. Settings of the QTPP of a PLGA Nanoparticle Formulation

Dosage form and administration	Injection (suspension at the time of use, freeze-dried product), local administration
Description	Lyophilized dosage form of white powder or masses
Identification	Conformed (JP)
Assay (Relative drug loading efficiency)	90–110%
Impurity	Less than identification threshold
Particle size	Not more than 200 nm
Dissolution	Slow release
Uniformity of dosage	Conformed (JP)
Stability	Stable more than 2 years at 25°C/60%RH with packaging form
Bacterial endotoxins test	Conformed (JP)
Sterility test	Conformed (JP)
Insoluble particulate matter test for injections	Conformed (JP)
Foreign insoluble matter test for injections	Conformed (JP)
Residual solvent	Not more than limit value

JP, The Japanese Pharmacopoeia.

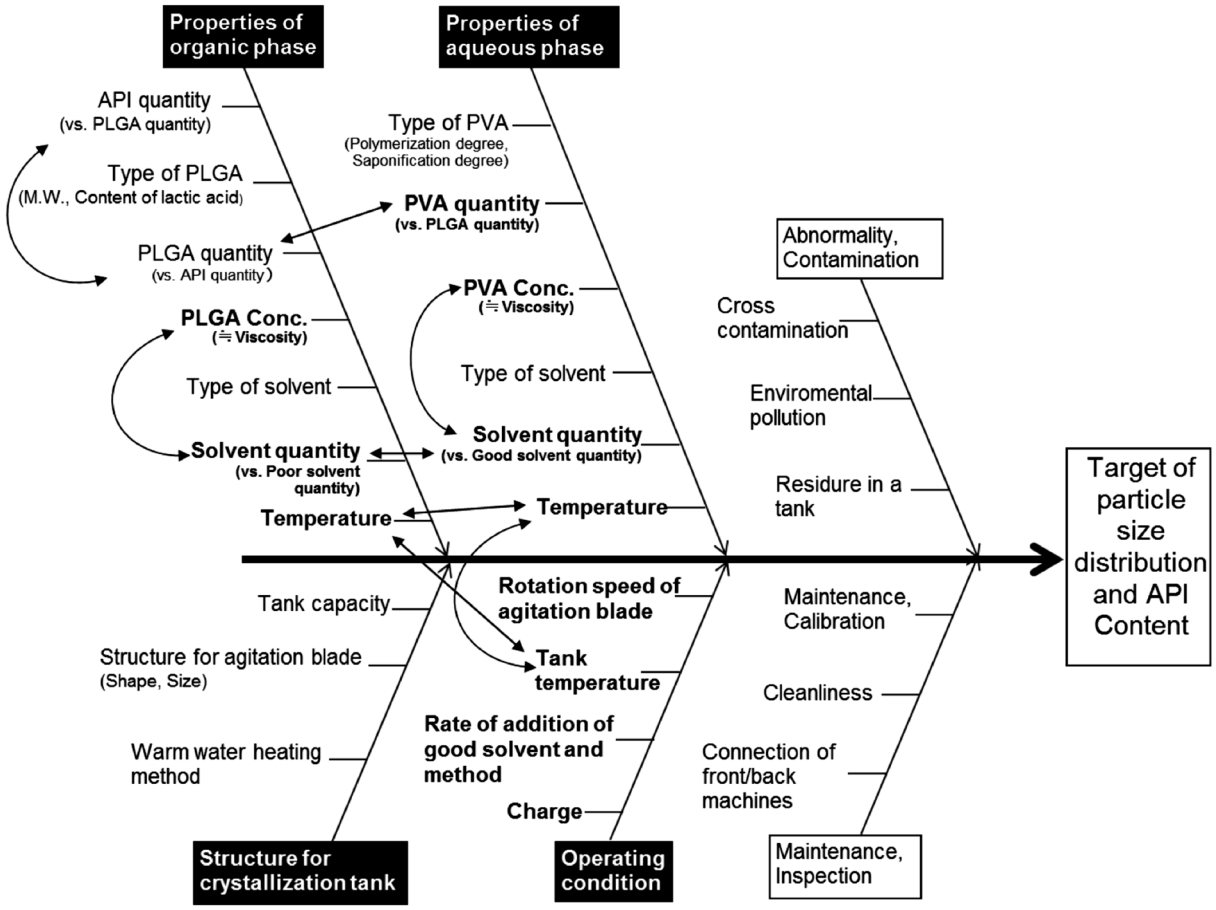

Fig. 1. Ishikawa Diagram

Double-headed arrows connect two related factors. M.W.: molecular weight.

In light of the above considerations, three parameters were selected as CQAs: particle size, particle size distribution, and drug loading efficiency. Ideally, the particle size used for a PLGA particle formulation should be controlled after redispersion of the lyophilized particles in an appropriate solvent for administration. However, this requires a QbD approach covering the multiple processes. Therefore, we focused on control of the primary particles, and redispersibility after lyophilization was evaluated for reference purposes only.

Development of an Ishikawa Diagram Analysis During the crystallization process using the O/W emulsion solvent diffusion method, microemulsion droplets are initially formed by self-emulsification upon mixing of the good and poor solvents; this is followed by precipitation of PLGA within the emulsion droplets, driven by counter-diffusion between water and the organic solvent, to generate nanoparticles.[16] Accordingly, the crystallization process is expected to determine the primary particle size and to influence the drug loading efficiency upon counter-diffusion between water and the organic solvent and through the mixing conditions thereafter. On the basis of these considerations, the crystallization process is likely to have the most marked effect on the CQAs. Therefore, we constructed an Ishikawa diagram for this process to organize all relevant factors, taking actual manufacturing conditions into consideration (Fig. 1). Major factors influencing the CQAs in the crystallization process included the following:

(1) Properties of the organic phase (good solvent)
(2) Properties of the aqueous phase (poor solvent)
(3) Structure (type and volume) of the crystallization tank
(4) Operating conditions
(5) Abnormalities of the manufacturing process and contamination
(6) Maintenance and inspection

Factors (1) to (4) were considered particularly important. Note that factor (1) Properties of the organic phase (good solvent) and factor (2) Properties of the aqueous phase (poor solvent) included several factors (*e.g.*, PLGA concentration in the case of factor (1) and PVA concentration in the case of factor (2)), as shown in the Ishikawa diagram (Fig. 1).

Risk Assessment by Using Fractional Factorial Designs Validation of all of the major factors and their related factors identified in the Ishikawa diagram, as illustrated in Fig. 1, requires an enormous number of experiments. Therefore, the crystallization tank capacity, the types of some of the solvents and the type of excipient were fixed (as indicated in non-bold text in Fig. 1). In addition, the quantities of mutually related factors (as indicated by the double-headed arrows in Fig. 1) were assessed in the form of ratios. Finally, the following seven factors were selected for validation:

(1) PLGA concentration in the good solvent
(2) PVA to PLGA quantity ratio
(3) Rotation speed of agitation blade
(4) Temperature of poor solvent (product temperature)
(5) Rate of addition of good solvent

(6) Charge added to crystallization tank (quantity of poor solvent)
(7) Poor solvent to good solvent volume ratio.

A two-level fractional factorial design for the experiments was developed by taking into account these seven factors (Table 2a). One advantage of using a fractional factorial design is that it has fewer runs than a full factorial design for the same number of factors, because all factors can cover a Latin hypercube with a smaller number of runs. This approach is therefore routinely used to reduce the number of experiments required.[17] The two-level values for individual factors represented the upper and lower limits of the range considered to yield nanoparticles, as determined by prior exploratory experiments.

Unscrambler X version 10.2 (CAMO Software AS, Oslo, Norway) was used to design experiments by assigning either of the two levels to each factor on an orthogonal array. Selection of Resolution IV (only main effects can be estimated and two-factor interactions are not confounded with each other) set the initial design for a total of 16 runs. Then, additional triplicate runs for one center point between the high and low levels (designated hereafter as center point runs) were designed to validate the reproducibility of the experiments. Nineteen runs in total were designed for validation (Supplementary Table 1).

The following four parameters were evaluated: particle size (*Z*-average) and particle size distribution (polydispersity index (PDI)) after crude filtration, redispersibility (rate of change in particle size before and after lyophilization), and drug loading efficiency. The particle size and particle size distribution were measured at 25°C with a Zeta Sizer Nano ZS analyzer (Malvern Instruments, Worcestershire, U.K.) after dispersion of the sample in an appropriate quantity of water. The drug loading efficiency was determined according to the assay method for triamcinolone acetonide described in The Japanese Pharmacopoeia, Sixteenth Edition.[18]

The experimental data obtained were then subjected to ANOVA using Unscrambler X version 10.2. A factor with a large effect and a p value not exceeding 0.05 was considered significant and was defined as a critical process parameter (CPP).

Identification of Two-Factor Interactions When the seven factors were assessed by using the fractional factorial design described in the above section, five of them ((1) PLGA concentration in good solvent, (2) PVA to PLGA quantity ratio, (3) Rotation speed of agitation blade, (5) Rate of addition of good solvent, and (7) Poor solvent to good solvent volume ratio) were found to influence CQAs. We next attempted to identify two-factor interactions by creating a new two-level design for the experiments (Table 2b).

Unscrambler X version 10.2 was used to design experi-

Table 2. (a) Factors Potentially Influencing CQA Candidates, and Their Levels; (b) Factors and Levels Used to Identify Two-Factor Interactions; (c) Four-Factor Central Composite Design Table for Optimization

a

Factor	Unit	Level	
		Low	High
PLGA conc.	mg/mL	5	15
Quantity ratio of PVA/PLGA	—	1	5
Rotation speed	rpm	100	1000
Temperature of poor solvent	°C	5	40
Rate of addition of good solvent	g/min	1	7
Charge (as poor solvent)	g	100	300
Volume ratio of poor solvent/good solvent	—	2	10

b

Factor	Unit	Level	
		Low	High
PLGA conc.	mg/mL	7	15
Quantity ratio of PVA/PLGA	—	2	5
Rotation speed	rpm	400	1000
Rate of addition of good solvent	g/min	3	7
Volume ratio of poor solvent/good solvent	—	4	10

c

Lot No.	PLGA Conc. (mg/mL)	Rotation speed (rpm)	Rate of addition of good solvent (g/min)	Poor solvent/Good solvent
322S-1	3	700	5	7
322S-2	19	700	5	7
322S-3	11	100	5	7
322S-4	11	1300	5	7
322S-5	11	700	1	7
322S-6	11	700	9	7
322S-7	11	700	5	1
322S-8	11	700	5	13

ments by assigning either of the two levels to each factor on an orthogonal array. Selection of Resolution V (both main effects and two-factor interactions can be estimated without confounding each other) set the initial design for a total of 16 runs. Then, additional triplicate center point runs for one center point between the high and low levels were designed to validate the reproducibility of experiments. The values for the two excluded factors—temperature of poor solvent (product temperature) and charge added to the crystallization tank (quantity of poor solvent)—were fixed at 40°C and 300 g, respectively. Thus, a total of 19 runs were designed for validation (Supplementary Table 2).

The following three parameters were evaluated: particle size and particle size distribution after crude filtration, and drug loading efficiency. Particle size and particle size distribution and drug loading efficiency were measured as described in the above section.

The experimental data obtained were then subjected to ANOVA by using Unscrambler X version 10.2. A factor with a large effect and a p value not exceeding 0.05 was considered significant and defined as a CPP.

Optimization Study by Using the Central Composite Design Additional experiments for optimization involving a central composite design were designed for the CPPs (PLGA concentration, rotation speed of agitation blade, rate of addition of good solvent, and poor solvent to good solvent volume ratio) identified by the experiments with a fractional factorial design and their newly defined levels (Table 2c). Herein, the values for factors that did not give significant effects on the CQAs in experiments with a fractional factorial design were fixed (charge of poor solvent added to crystallization tank, 300 g; temperature of poor solvent, 40°C; PVA to PLGA ratio, 3.5).

Construction of Design Space The relationships of the output factors obtained in the optimization study by using central composite design to the input factors were displayed by using the response surface method to construct a design space. We used the polynomial regression method.

Results and Discussion

Risk Assessment by Using Fractional Factorial Designs

Particle Size after Crude Filtration

A two-level seven-factor fractional factorial design for the experiments was developed to screen for risk factors (Supplementary Table 1). The mutually related factors were assessed in the form of ratios, so that many factors could be efficiently considered in the risk assessment (Fig. 1). Measurement of particle size demonstrated considerable variation, including values exceeding 200 nm (Supplementary Fig. 1), the upper limit defined in the QTPP. Because the three lots obtained in the triplicate center point experiments all yielded particles of similar size, the reproducibility of these experiments was considered acceptable, and in all experiments thereafter the triplicate center point experiments were reproducible. When the experimental data were subjected to ANOVA (Fig. 2a), the PLGA concentration in the good solvent and the rotation speed of the agitation blade had significant effects on particle size. Particle size increased as the PLGA concentration in the good solvent increased and decreased as the rotation speed of the agitation blade increased, as indicated by the positivity and negativity, respectively, of the individual effects. The positive effect of PLGA concentration on particle size was at-

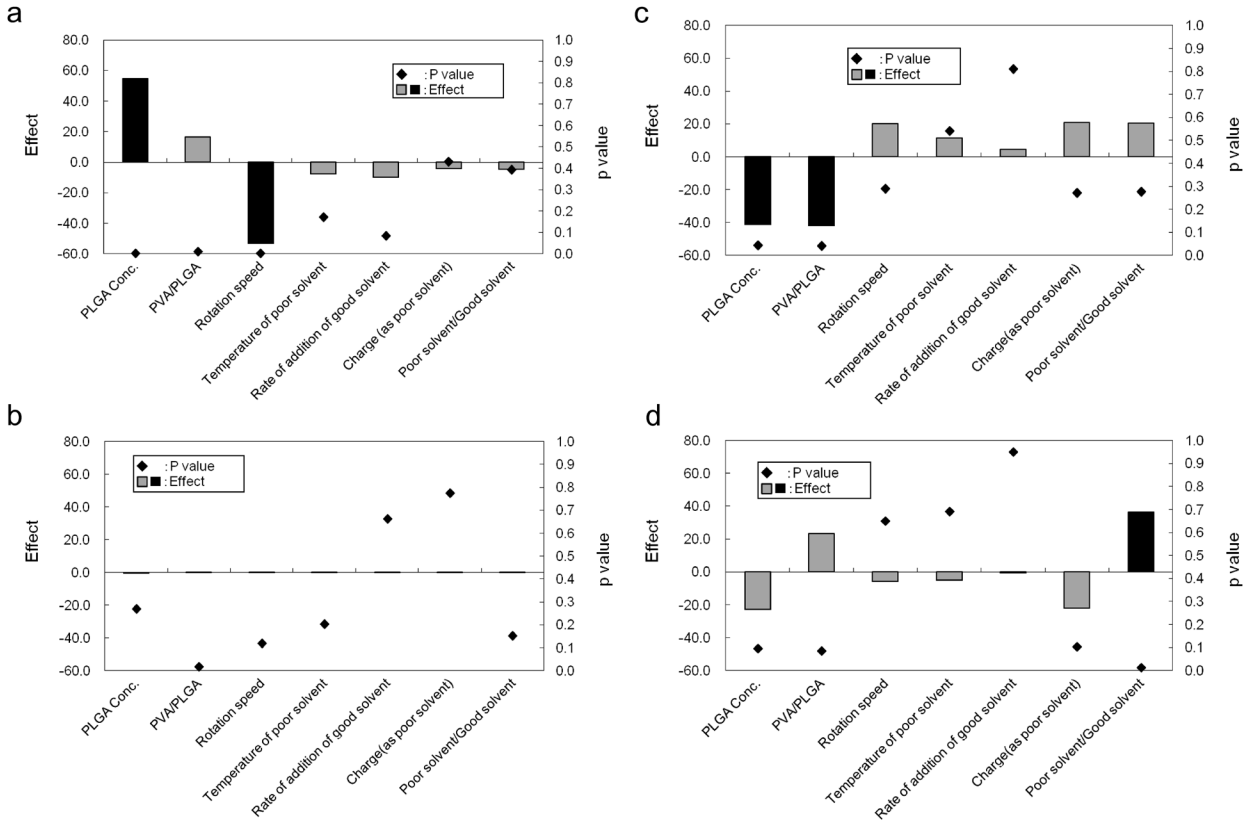

Fig. 2. ANOVA Results for (a) Particle Size, (b) Particle Size Distribution, (c) Rate of Change in Particle Size, and (d) Relative Drug Loading Efficiency Results Obtained in Experiments Using a Two-Level Seven-Factor Fractional Factorial Design

tributed to an increase in the PLGA concentration in the good solvent, leading to an increase in the viscosity of the good solvent and a subsequent increase in emulsion droplet size. We also inferred that solidification of PLGA due to counter-diffusion between acetone and water was accelerated with an increase in PLGA concentration in the good solvent, and that solidification of larger emulsion droplets yielded PLGA particles that were larger. The negative effect of the rotation speed of the agitation blade on particle size was attributed to an increase in shear force with the increase in rotation speed, yielding smaller emulsion droplets and smaller PLGA particles.

Although not meeting the criteria for a CPP, the PVA to PLGA ratio also had a significant effect on particle size; particle size tended to increase as the PVA to PLGA ratio increased. This effect was attributed to an increase in the viscosity of the poor solvent owing to an increase in PVA concentration in the poor solvent, which led to a decrease in the shear force applied to the PLGA particles. This finding suggested that the viscosity of the poor solvent might also be a factor that potentially influences particle size.

Particle Size Distribution after Crude Filtration

Measurement of particle size distribution demonstrated that the PDI was less than 0.1 in all lots (Supplementary Fig. 2).

When the experimental data were subjected to ANOVA (Fig. 2b), no factor was found to have a significant effect on particle size distribution. This result might have been attributable to the generation of uniform emulsion droplets under the experimental conditions used; it might also have been due to sufficiently expedited solidification of PLGA nanoparticles encapsulating API *via* counter-diffusion between the good and poor solvents, instead of mutual fusion of emulsion droplets.

Redispersibility after Lyophilization

The particle size of the lyophilized formulation was measured after redispersion. The rate of particle size change was then calculated as an index of redispersibility.

$$\text{Rate of particle size change (\%)} = \frac{\text{(post-lyophilization particle size} - \text{pre-lyophilization particle size)}}{\text{pre-lyophilization particle size}} \times 100$$

In some lots, the rates of particle size change thus determined demonstrated a marked increase after lyophilization (Supplementary Fig. 3).

When the data obtained were subjected to ANOVA (Fig. 2c), the PLGA concentration in the good solvent and the PVA to PLGA ratio had significant effects on redispersibility. Redispersibility tended to decrease as the PLGA concentration in the good solvent increased and as the PVA to PLGA ratio increased.

Relative Drug Loading Efficiency

Because we evaluated the effects of both the PVA to PLGA ratio and the PLGA concentration, the quantities of PVA and PLGA used in the formulation varied from lot to lot. As a consequence, the theoretical drug loading efficiency varied from lot to lot as well. Accordingly, the relative drug loading efficiency was calculated by dividing the drug loading efficiency (determined by assay) by the theoretical drug loading efficiency of each lot for comparative evaluation.

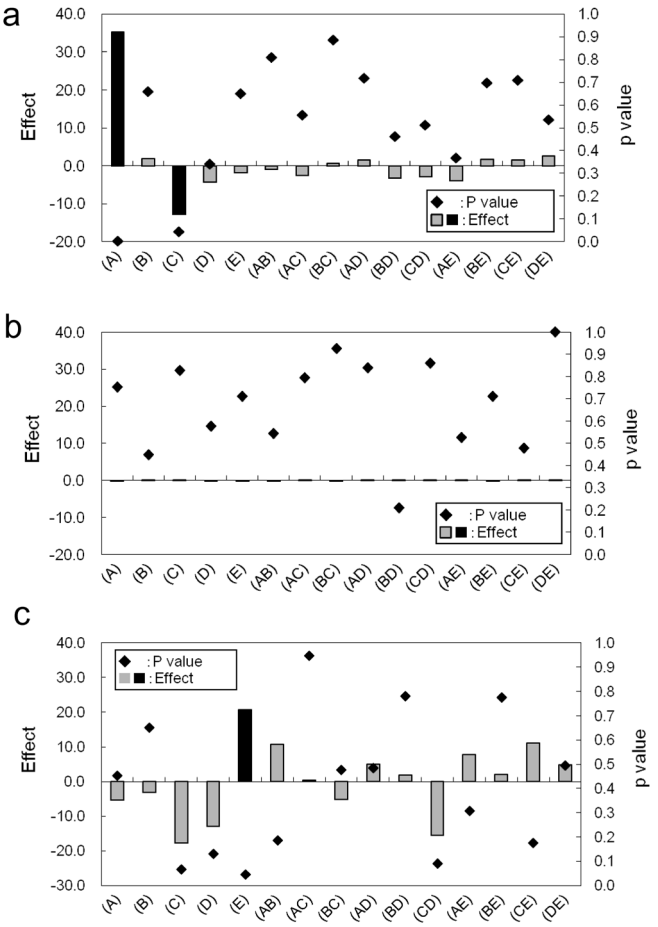

Fig. 3. ANOVA Results for (a) Particle Size, (b) Particle Size Distribution, and (c) Relative Drug Loading Efficiency for Identification of Two-Factor Interactions between CPP Candidates

A: PLGA concentration; B: PVA to PLGA quantity ratio; C: rotation speed; D: rate of addition of good solvent; E: poor solvent to good solvent volume ratio.

$$\text{Relative drug loading efficiency (\%)} = \frac{\text{drug loading efficiency}}{\text{the theoretical drug loading efficiency in each lot}} \times 100$$

The relative drug loading efficiency calculations identified numerous lots with efficiencies exceeding the limit defined in the QTPP (Supplementary Fig. 4). This was attributed to excipient loss.

When the data obtained were subjected to ANOVA (Fig. 2d), the poor solvent to good solvent volume ratio was found to have a significant effect on the relative drug loading efficiency. Relative drug loading efficiency increased as the poor solvent to good solvent volume ratio increased. This effect was attributed to increased problems with leakage of the API encapsulated in the resulting PLGA particles into the external solution as the quantity of good solvent (acetone) relative to the quantity of poor solvent (water) increased.

In conclusion, PLGA concentration and rotation speed were found to have effects on particle size (Fig. 2a); these two factors were thus regarded as CPP candidates. Evaluation of particle size distribution demonstrated monodispersity under all combinations of experimental conditions tested here. Evaluation of redispersibility as the rate of change in particle size for reference purposes revealed that the PLGA concentration and

the PVA to PLGA quantity ratio affected this parameter (Fig. 2c). This means that the formulation composition affected redispersibility. Although we did not investigate lyophilization conditions here, the PVA to PLGA quantity ratio was taken as a potential CPP candidate. The poor solvent to good solvent volume ratio was also found to be a CPP, influencing relative drug loading efficiency (Fig. 2d).

Table 3a summarizes the results described above. We identified four factors (PLGA concentration, PVA to PLGA quantity ratio, rotation speed, and poor solvent to good solvent volume ratio) as potential CPP candidates. Because the rate of addition of good solvent also had a minor effect on particle size ($0.05<p\leq0.10$), identification of two-factor interactions was performed for a total of five factors.

Identification of Two-Factor Interactions

Particle Size after Crude Filtration

For the five factors regarded as CPP candidates on the basis of the screening study involving experiments with a fractional factorial design, identification of two-factor interactions was performed. We used a fractional factorial design with resolution V, in which both main effects and two-factor interactions can be estimated without confounding each other (Supplementary Table 2). Measurement of particle size identified no lots with a particle size exceeding 200 nm (Supplementary Fig. 5)—the upper limit defined in the QTPP.

When the experimental data were subjected to ANOVA (Fig. 3a), the PLGA concentration in the good solvent and the rotation speed of the agitation blade had substantial effects on particle size. PLGA concentration in the good solvent showed a positive correlation with particle size, whereas the rotation speed of the agitation blade was negatively correlated with particle size. These results were consistent with those of the screening study (Fig. 2a).

The effects of two-factor interactions were minor for all combinations of factors investigated. Therefore, two-factor interactions were considered to have virtually no influence on particle size.

Particle Size Distribution after Crude Filtration

Measurement of particle size distribution demonstrated that the PDI was less than 0.15 in all lots (Supplementary Fig. 6).

When the experimental data were subjected to ANOVA (Fig. 3b), no factor had a significant effect on particle size

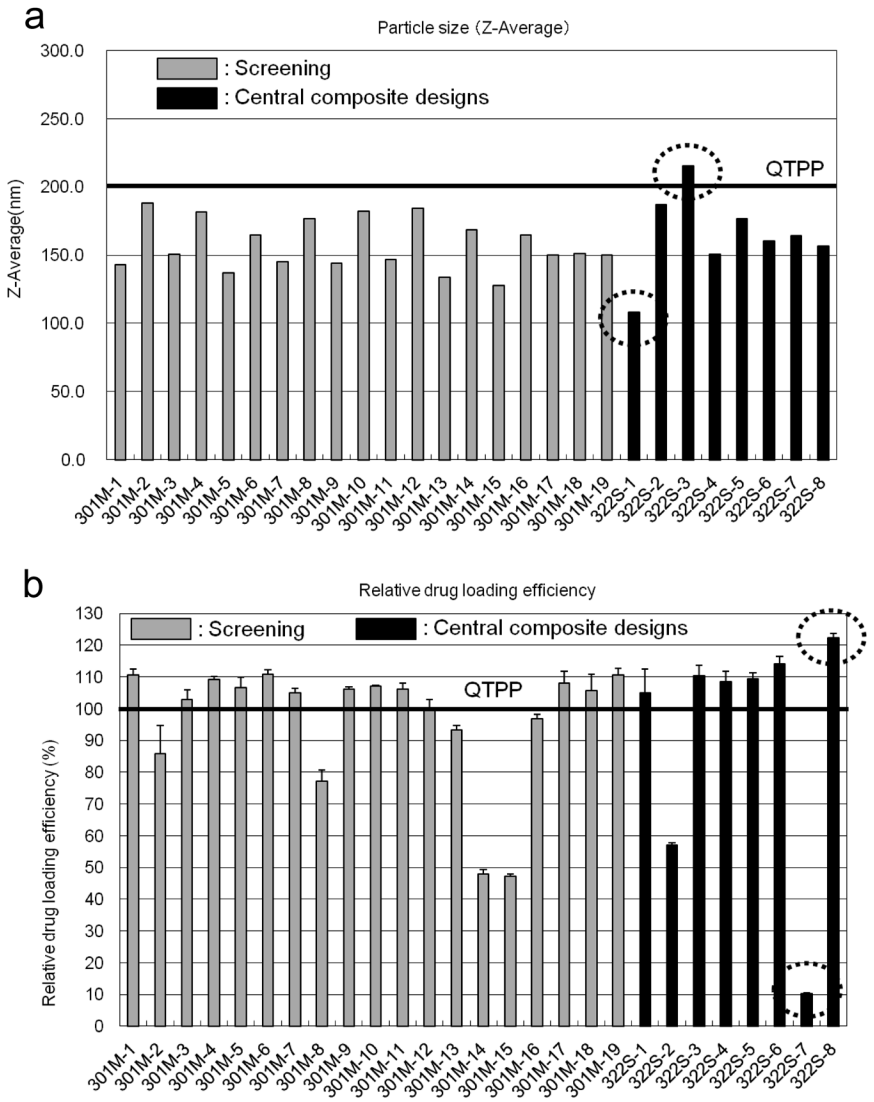

Fig. 4. Measurements of (a) Particle Size and (b) Relative Drug Loading Efficiency, Obtained by Using the Central Composite Method

(a) Minimum and maximum particle sizes shown by dotted circles correspond to the lowest PLGA concentration and the lowest rotation speed, respectively. (b) Minimum and maximum relative drug loading efficiencies shown by dotted circles correspond to the lowest and highest poor solvent to good solvent volume ratio, respectively.

Table 3. Summary of (a) ANOVA Results of Screening; and (b) ANOVA Results of Identification of Two-Factor Interactions

a

Factor	Particle size	Particle size distribution	Re-dispersibility (Rate of change of particle size)	Relative drug loading efficiency
PLGA conc.	+++*	NS	—*	±
PVA/PLGA	++	+	—*	±
Rotation speed	—*	NS	NS	NS
Temperature of poor solvent	NS	NS	NS	NS
Rate of addition of good solvent	±	NS	NS	NS
Charge (as poor solvent)	NS	NS	NS	NS
Poor solvent/Good solvent	NS	NS	NS	+*

b

Factor		Particle size	Particle size distribution	Relative drug loading efficiency
A	PLGA Conc.	+++*	NS	NS
B	PVA/PLGA	NS	NS	NS
C	Rotation speed	—*	NS	±*
D	Rate of addition of good solvent	NS	NS	NS
E	Poor solvent/Good solvent	NS	NS	+*
Interaction	AB	NS	NS	NS
	AC	NS	NS	NS
	BC	NS	NS	NS
	AD	NS	NS	NS
	BD	NS	NS	NS
	CD	NS	NS	±*
	AE	NS	NS	NS
	BE	NS	NS	NS
	CE	NS	NS	NS
	DE	NS	NS	NS

Cells corresponding to potential CPP candidates are marked with an asterisk. p values are represented as follows: NS, $p>0.10$; ±, $0.05<p\leq0.10$; +, −, $0.01<p\leq0.05$; ++, −, $0.005<p\leq0.01$; +++, −, $p\leq0.005$.

distribution. In addition, there was virtually no discernible two-factor interaction influencing particle size distribution. This effect might have been attributable to the generation of uniform emulsion droplets under the experimental conditions employed; it might also have been due to sufficiently expedited solidification of the PLGA nanoparticles encapsulating API *via* counter-diffusion between the good and poor solvents, instead of mutual fusion of emulsion droplets.

Relative Drug Loading Efficiency

The relative drug loading efficiency calculations identified numerous lots for which the relative drug loading efficiency exceeded the limit of 100% defined in the QTPP (Supplementary Fig. 7, Table 1). This was attributed to excipient loss during the preparation process.

When the data obtained were subjected to ANOVA (Fig. 3c), the poor solvent to good solvent volume ratio was found to have a significant effect on the relative drug loading efficiency. The poor solvent to good solvent volume ratio was positively correlated with relative drug loading efficiency. These results were consistent with those of the screening study (Fig. 2d).

Although not meeting the criteria for CPPs, rotation speed and rate of addition of good solvent, and the interaction between these factors, had modest negative effects on relative drug loading efficiency (Table 3b, Fig. 3c). The effect of rotation speed was attributed to decreased particle size as rotation speed increased, leading to an increase in the area in contact with the solvent, which in turn accelerated leakage of the API. Furthermore, the effect of the rate of addition of good solvent was attributed to insufficient shear force from the agitation paddle at increased rotation speed; the resulting particles, which were enlarged, were easily eliminated during the crude filtration process, resulting in a reduction in relative drug loading efficiency.

Table 3b summarizes the above-described results of the experiments with a fractional factorial design involving five factors. PLGA concentration and rotation speed had effects on particle size, whereas the poor solvent to good solvent volume ratio had an effect on relative drug loading efficiency. These results were consistent with those of the screening study using a fractional factorial design. Accordingly, these three factors were considered to be potential CPP candidates. In addition, because an identifiable two-factor interaction was observed between rotation speed and rate of addition of good solvent, and because this interaction exerted a negative effect on relative drug loading efficiency, the rate of addition of good solvent was included in an optimization study conducted by using the central composite method. The Box–Behnken method is often used for the experimental design of optimization studies.[7] However, we used the central composite method because it contains extreme factor combinations, whereas the Box–Behnken method does not examine borderline regions.[19] Moreover, a smaller number of experiments are required by the Box–Behnken method, leading to the possibility that the

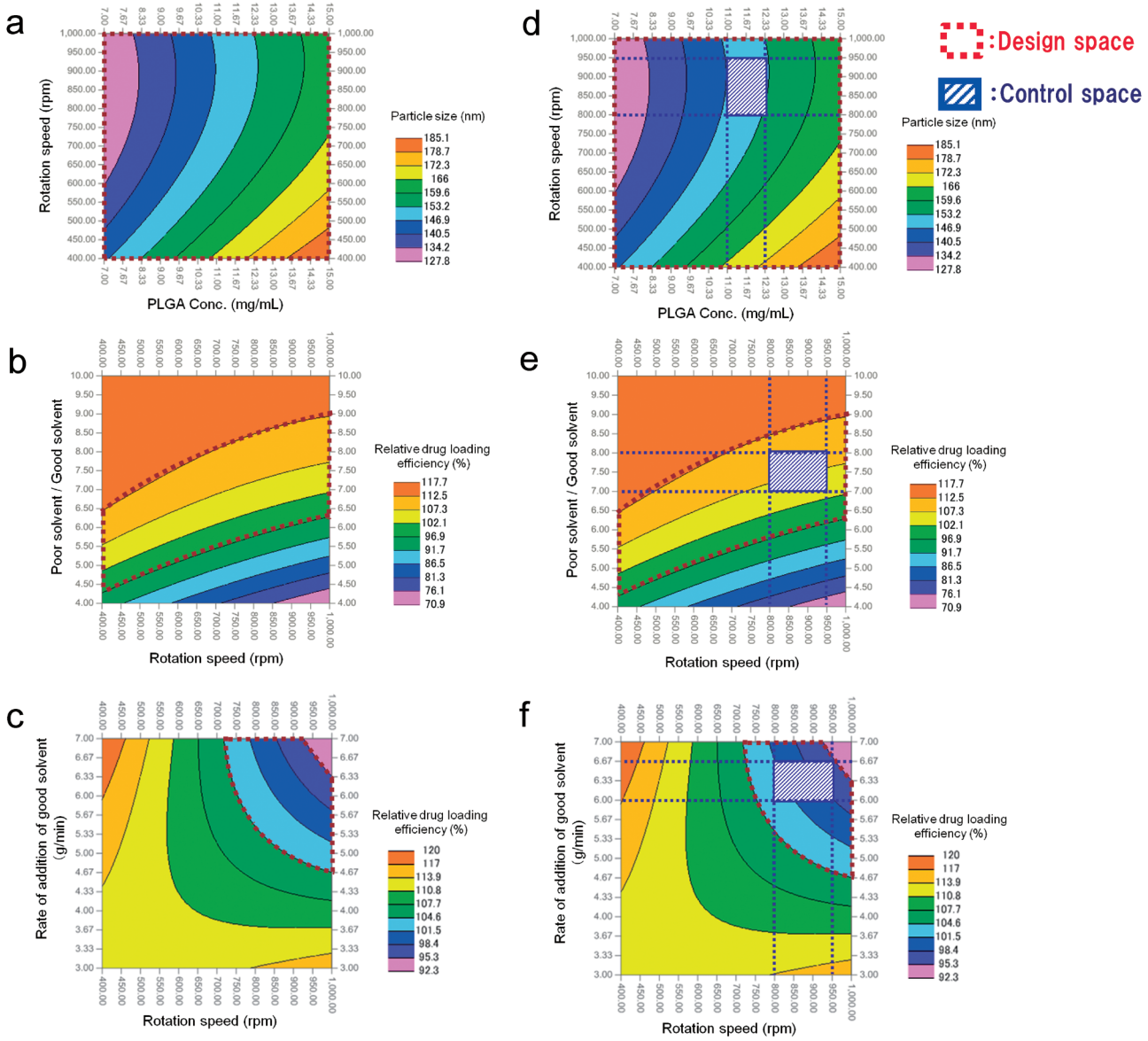

Fig. 5. Counter Plots Representing (a) Particle Size Distribution with Regard to PLGA Concentration and Rotation Speed; (b) Relative Drug Loading Efficiency Distribution with Regard to Poor Solvent to Good Solvent Volume Ratio and Rotation Speed; and (c) Relative Drug Loading Efficiency Distribution with Regard to Rate of Addition of Good Solvent and Rotation Speed; The Area Surrounded by the Dotted Line Represents the QTPP; (d) Response Surface Plot and Design Space for Particle Size; (e) Response Surface Plots and Design Space for Relative Drug Loading Efficiency with Regard to Poor Solvent to Good Solvent Ratio and Rotation Speed; (f) Response Surface Plots and Design Space for Relative Drug Loading Efficiency with Regard to Rate of Addition of Good Solvent and Rotation Speed

CPPs could be overlooked. The Box–Behnken method also requires other types of factorial designs. Therefore, in the drug development process, the central composite method is generally used.

Optimization Study by Using the Central Composite Method We conducted an optimization study employing the central composite method for a total of four factors, namely the three aforementioned CPP candidates plus the rate of addition of good solvent. The minimum and maximum particle sizes obtained by using the central composite method were beyond the range of values obtained in the experiments with a five-factor fractional factorial design, with the maximum value exceeding the limit defined in the QTPP (Fig. 4a). The minimum and maximum particle sizes corresponded to the lowest PLGA concentration and the lowest rotation speed, respectively, yielding results consistent with those of ANOVA (Fig. 3a).

The minimum and maximum relative drug loading efficiencies obtained by using the central composite method were also beyond the range of values obtained in the experiments with a five-factor fractional factorial design (Fig. 4b). The minimum and maximum efficiencies corresponded to the lowest and highest poor solvent to good solvent volume ratio, respectively, yielding results consistent with those of ANOVA (Fig. 3c).

Visualization by Using the Response Surface Method, and Construction of a Design Space The results obtained by using the central composite method and the experiments with fractional factorial design are combined and summarized in Supplementary Table 3. We used the polynomial regression method. We also added the terms and confounding patterns and the resulting F and p values calculated from the regression equation (Supplementary Table 4). On the basis of the

results of these 27 runs, individual CQAs were separately plotted against different pairs of CPPs by using the response surface method (Figs. 5a–c). From the P and F values shown in Supplementary Table 4, we found that PLGA concentration and rotation speed had significant effects on particle size ($p \leq 0.10$). Because no significant two-factor interactions were observed, the third CPP effect was not considered in Fig. 5a. Similarly, rotation speed and poor solvent to good solvent ratio had significant effects on relative loading efficiency ($p \leq 0.10$) (Fig. 5b). Because the two-factor interaction of rotation speed and rate of addition of good solvent had a significant effect on relative loading efficiency ($p \leq 0.10$), relative loading efficiency was also plotted against rotation speed and rate of addition of good solvent (Fig. 5c).

Figure 5a is a contour plot representing particle size distribution (the first CQA) in regard to a pair of CPPs, namely PLGA concentration and rotation speed. Particle size increased as PLGA concentration increased and rotation speed decreased; these results were consistent with those of ANOVA (Fig. 3a). The relationships between the two CPPs (PLGA concentration and rotation speed) and particle size were thus successfully visualized. The figure also demonstrates that all points on the response surface for particle size met the QTPP, provided that each CPP fell within a range between the high and low levels that we investigated. The response surface method is thus likely to be an effective approach when a narrower range of QTPPs is defined in future.

Figure 5b is a contour plot representing relative drug loading efficiency distribution (the second CQA) with regard to another pair of CPPs, namely the poor solvent to good solvent volume ratio and rotation speed. Relative drug loading efficiency increased as the poor solvent to good solvent volume ratio increased; these results were consistent with those of ANOVA (Fig. 3c). In contrast, rotation speed had a smaller effect than the poor solvent to good solvent volume ratio on relative drug loading efficiency; these results were also consistent with those of ANOVA. Figure 5c is a contour plot representing the relative drug loading efficiency distribution (the second CQA) in regard to another pair of CPPs, namely the rate of addition of good solvent and rotation speed. Relative drug loading efficiency decreased as these two factors increased; these results were consistent with those of ANOVA (Fig. 3c).

In addition, the QTPP range for relative drug loading efficiency (defined in Table 1 as 100±10%) is represented by the area surrounded by the dotted line on the response surface (Fig. 5b); this demonstrates the existence of an optimal region.

The region meeting the QTPPs for both particle size and relative drug loading efficiency on the response surface—*i.e.*, the design space—is surrounded by the red dotted lines in Figs. 5d (for particle size) and e and f (for relative drug loading efficiency). The relationships between the CPPs (rotation speed, PLGA concentration, rate of addition of good solvent, and poor solvent to good solvent volume ratio) and individual CQAs were thus successfully visually represented. With the control space defined as indicated in Fig. 5d, the target particle size (not more than 200 nm) was expected to be achieved at a PLGA concentration of 11.0 to 12.3 mg/mL and a rotation speed of 800 to 950 rpm. Furthermore, when the rotation speed was controlled to within the above range (800 to 950 rpm), the target relative drug loading efficiency (100±10%) was expected to be achieved at a poor solvent to good solvent volume ratio of 7.0 to 8.0 (Fig. 5e). In addition, when the rotation speed was controlled to within the above range (800 to 950 rpm), the target relative drug loading efficiency (100±10%) was also expected to be achieved at a rate of addition of good solvent of 6.00 to 6.67 g/min (Fig. 5f).

We selected particle size and drug loading efficiency of the PLGA particle formulation as CQAs on the basis of the QTPP; however, other factors such as the drug release rate, which could also affect the performance of formulations, can be evaluated by using the QbD approach.

With conventional methods, the quality of drug products is assured by the use of end-product testing (*i.e.*, quality by testing). However, for complex formulations such as PLGA nanoparticles, end-product testing alone is often insufficient to define quality. As shown here, use of the QbD approach enables scientific verification of how the formulation and manufacturing method affects product quality, and this in turn leads to continuous and real-time quality assurance. Although we have not covered scale in this study, it could be another factor to be considered in a commercial manufacturing setting.

Conclusion

We established an efficient method of analyzing the CPPs of PLGA nanoparticles by using the QbD approach. To clarify the correlations between CQAs and CPPs in relation to the product performance of a PLGA nanoparticle formulation, we performed quality risk management by using an Ishikawa diagram and ANOVA, in which factors were set with actual manufacturing in mind and the quantities of mutually related factors were assessed in the form of ratios. The results demonstrated that the CPPs for particle size were PLGA concentration and rotation speed and that the CPP for relative drug loading efficiency was the poor solvent to good solvent volume ratio. A two-factor interaction was analyzed by using a fractional factorial design with resolution V. We found a two-factor interaction between rotation speed and the rate of addition of good solvent by considering operating conditions in actual manufacturing; this interaction exerted a negative effect on relative drug loading efficiency. This information is very important when the hydrophobic API is encapsulated in PLGA nanoparticles. Our research provides a case study of application of the QbD approach to pharmaceutical development, including formulation screening in the early stages of development, and it supports the feasibility of using a similar approach to nanoparticle formulations under development. Extensive investigation at the stage of basic research, as we performed here, to enhance our understanding of quality attributes and process parameters should allow risk factors to be refined before implementation of the QbD approach at the production scale. Ultimately this would reduce production costs and expedite the process of marketing authorization application.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

References and Notes

1) The contents of this publication are the personal views of the authors and may not be understood or quoted as being made on behalf of, or reflecting, the position of the Japan Ministry of Health, Labour and Welfare or its affiliated institutes.
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