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Understanding the quality of protein loaded PLGA nanoparticles variability by Plackett–Burman design

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Abstract

The aim of this investigation was to screen and understand the product variability due to important factors affecting the characteristics CyA-PLGA nanoparticles prepared by O/W emulsification-solvent evaporation method. Independent variables studied were cyclosporine A (CyA) (X_1), PLGA (X_2), and emulsifier concentration namely SLS (X_3), stirring rate (X_4), type of organic solvent employed (chloroform or dichloromethane, X_5) and organic to aqueous phase ratio (X_6). The nanoparticles properties considered were encapsulation efficiency (Y_1), mean particle size (Y_2), zeta potential (Y_3), burst effect (Y_4) and dissolution efficiency (Y_5). The statistical analysis of the results allowed determining the most influent factors. The nanoparticles were characterized by scanning electron microscopy (SEM), differential scanning calorimetry (DSC), X-ray powder diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy. The factors combination showed variability of entrapment efficiency (Y_1), mean particle size (Y_2) and zeta potential (Y_3) from 10.17% to 93.01%, 41.60 to 372.80nm and 29.60 to 34.90 mV, respectively. Initially, nanoparticles showed burst effect followed by sustained release during the 7-day in vitro release study period. The dissolution efficiency (Y_5) varied from 52.67% to 84.11%. The nanoparticles revealed Higuchi release pattern and release occurred by coupling of diffusion and erosion. In conclusion, this study revealed the potential of QbD in understanding the effect of formulation and process variables on the characteristics on CyA-PLGA nanoparticles.

Keywords

CyA; PLGA; QbD; Plackett–Burman; Nanoparticles and dissolution efficiency

1. Introduction

Cyclosporine (CyA) is a cyclic neutral undecapeptide produced by fungus *Tolypocladium inflatum* which contains mainly D-amino acid, with a potent immunosuppressive activity that has been used to prevent allograft rejection in various organ transplantation such as kidney, liver, heart, lung and pancreas (Matzke and Luke, 1988; Lemley and Katz, 1988), in psoriasis (Costanzo et al., 2009) and atopic dermatitis (Akhavan and Rudikoff, 2008). It has been explored in the treatment of autoimmune disorders such as rheumatoid arthritis (Richardson and Emery, 1995) and Behçet's uveitis disease (Akman-Demir et al., 2008).

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New evidences are emerging its role in controlling ulcerative colitis (Yadav and Liu, 2009), and as a neuroprotective agent (Hatton et al., 2008).

Despite its promising pharmacological profile and great therapeutic value, the bioavailability after oral administration is low with high inter-patient variability (20–50%) (Lindholm et al., 1988; Fahr, 1993). The low oral bioavailability is due to its poor aqueous solubility (0.02 mg/ml) (Miyake et al., 2000) and furthermore, it is a substrate of p-glycoprotein (Charuk et al., 1995).

Many formulation strategies were investigated to improve solubility and bioavailability of CyA such as complexation with cyclodextrin (Matilainen et al., 2006), and particulate delivery system including microspheres (Yeung and Chaw, 2009) and liposome (Czogalla, 2009). The formulation of CyA in nanoparticles dosage has received much attention in the last few years mainly due to its ability to improve bioavailability and could be a better alternative to current delivery system. Biodegradable materials investigated for nanoparticles of CyA are chitosan (El-Shabouri, 2002) polycaprolactone (Varela et al., 2001), PLGA (Italia et al., 2007) and hydroxypropylmethyl cellulose phthalate (Wang et al., 2004). Investigators claimed 1.8-fold increase in bioavailability of CyA by chitosan based nanoparticles when compared with neoral microemulsion in Beagle dogs (El-Shabouri, 2002). Similarly, PLGA nanoparticles of CyA showed 119.2% relative bioavailability, low toxicity and prolonged release when compared with Sandimmune neoral dosage (Italia et al., 2007). PLGA based nanoparticles have distinct advantage of being FDA approved excipient, and will not encounter regulatory hurdle in approval.

Quality by design (QbD) is a FDA initiative to pharmaceutical development (FDA guidance of industry, 2006). It is a deliberate design effort from product conceptualization to commercialization. The objective of QbD approach is to design a process in such a way that manufactures pharmaceuticals that consistently meet critical quality attributes. Another objective is to identify and control critical source of variability in the process, and understand the impact of formulation components and process parameters on the critical quality attributes. Thus, one of the components of QbD strategy is to understand variables and their interactions, and their impact on the critical quality attributes. A process and formulation can be understood by developing them based on multivariate analysis of designed experiments and/or historical data that identify and characterize the critical-to-quality process parameters, and also identify the root causes of variability. To understand process and formulation, many statistical designs of experiment (DOE) are used. The most commonly used (DOE) is Plackett–Burman, which is a very efficient screening design used when only main effects are of interest to be investigated (Plackett and Burman, 1946).

The focus of this study was to design nanoparticles by QbD approach and evaluate the effects of different formulation and processing parameters on the characteristics CyA-PLGA nanoparticles. A Plackett–Burman screening experimental design was used to identify critical parameters that influence nanoparticles characteristics including entrapment efficiency, particle size, zeta potential, burst release and dissolution efficiency.

2. Materials and methods

Cyclosporine (Purity 99%) was purchased from Poli Industria Chemica S.P.A. (Rozzano, Milano, Italy). Poly(lactide-co-glycolide) (PLGA, lactide:glycolide = 50:50, inherent viscosity: 0.58 dL/g in hexafluoroisopropanol, Mw≈31,000 Da) was purchased from Lactel International Absorbable Polymers (Pelham, AL, USA). Dichloromethane and chloroform (HPLC grade) was obtained from Fisher Scientific Co. (Norcross, GA, USA). Sodium lauryl

sulphate and sodium azide was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used were analytical or HPLC grade.

2.1. Design of experiment

Traditional development of pharmaceutical formulation is based on time and energy consuming approach of changing one variable at a time while keeping other variables constant. Use of experimental design (DOE) technique allows testing of large number of variables simultaneously in a few experimental run. Screening design are the most powerful DOE techniques that determine the most critical factors in the pharmaceutical development. Most common screening design is Plackett–Burman (PB) design that screens large number of factors and identify critical one in a minimal number of run with good degree of accuracy. Generally, number of run needed to investigate the main effects are equal to 2^n or multiple of 4 in PB designs instead of 2 as in the case of full factorial design (Plackett and Burman, 1946). PB screening design with 12 experiments was constructed using software JMP version 7.0.1 (SAS, NC, USA). The linear equation of the model is as follows:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_5X_5+\ldots+b_nX_n$$

where Y is the response, b_0 is the constant and b_1, $b_2 \ldots b_n$ are the coefficient of factor X_1, $X_2 \ldots X_n$ (representing the effect of each factor ordered within −1, +1).

Independent process and formulation variables selected were drug (X_1), polymer (X_2), and surfactant concentration (X_3), stirring rate (X_4), type of solvent (X_5) and organic to aqueous phase ratio (X_6). The parameter level selection was based on preliminary study and on literature. Parameter studied in preliminary investigation was homogenization time and mechanical stirrer speed. Homogenization time did not have a significant impact on particle size and was kept constant for all the experiments. Mechanical stirrer speeds have impact on nanoparticle size and entrapment and included in the design. Solvents are selected based on the report of Italia et al. who reported effect of solvent on particle size (Italia et al., 2007). Similarly, level of drug, polymer, surfactant level and external volume are selected based on the literature (Shi et al., 2009).We could not conduct study all the variables. That is why we selected the ones that we thought are critical. The two levels of independent factors for the screening design and experiment domain of each variable were summarized in Tables 1 and 2. The dependent variables were encapsulation efficiency (Y_1), particle size (Y_2), zeta potential (Y_3), burst release (Y_4) and dissolution efficiency (DE) (Y_5).

2.2. Preparation of CyA-PLGA nanoparticles

CyA-PLGA nanoparticles were prepared according to emulsification-solvent evaporation technique (Kawashima et al., 1999). Briefly, CyA and PLGA were codissolved in 10 ml of organic solvent (dichloromethane or chloroform). Sodium lauryl sulphate solution (0.05%, w/v or 0.10%, w/v) was prepared in deionized water. Drug and polymer solution was added drop-wise to surfactant solution to make organic to aqueous phase ratio of 1:10 or 1:20 while stirring at 300 rpm and homogenizing by probe type homogenizer PowerGen 125 (Fisher Scientific, PA, USA) and continued homogenization for 10 min at 6000 rpm after complete addition of organic phase into aqueous phase. The nanoparticles formation and subsequent hardening was effected as a result of solvent evaporation by mechanical stirring at 600 rpm or 900 rpm at room temperature. The nanoparticles were retrieved from the aqueous solution by centrifugation at 49,500 × *g* (RC-5C, Sorwall Instruments/Thermo Scientific, MA, USA) for 30 min. The obtained nanoparticles were washed twice with 20 ml of deionized water, frozen at −80 °C and freeze dried in Freeze Dry/Shell Freeze System

(Labconco Corp., MI, USA) at −10 °C for 48 h. The dried particles were stored in fridge until further study.

2.3. Drug entrapment efficiency

Five milligrams of freeze-dried nanoparticles were dissolved in 5ml of chloroform, sonicated for 5min and vortexed. Hundred microliters of solution was diluted with 900 µl of the mobile phase for CyA quantization using a Hewlett–Packard (HP) HPLC instrument (Agilent technologies, CA, USA) that consist of a quaternary HP 1050 pump, HP 1050 autosampler, and 1050 HP UV detector set at a wavelength of 203 nm and column compartment thermostated at 70 °C. The HPLC stationary phase was composed of a C8, 4.6mm × 250mm (3.5 µm packing) reverse phase chromatography Zorbax SB-C8 column and a C8, 4.6mm × 12.5mm (5 µmpacking) Zorbax SB-C8 reliance guard column (Agilent technologies, CA, USA). The mobile phase consisted of acetonitrile:methanol:water:phosphoric acid (8:4:3:0.05) and was pumped isocratically at a flow rate of 1.25 ml/min. Each experiment was performed in triplicate and entrapment efficiency (EE) was calculated according to the formula:

$$\mathrm{EE}\,(\%)=\frac{\text{mass of drug in nanoparticles}}{\text{mass of drug used in formulation}}\times 100$$

2.4. Particle size and zeta potential measurements

Dried nanoparticles were suspended in CyA saturated MiliQ water and sonicated for 2–3 min to obtain a uniform suspension before measurements. The particle size distribution was expressed as mean number and determined by photon correlation spectroscopy at 23 °C (Particle Size/Zeta Potential PSS NICOMP 380 ZLS, Particle sizing Systems, Santa Barbra CA, USA). For zeta potential measurements, nanoparticles were suspended in MiliQ and measurements were made at 23 °C, at a diffraction angle of 14°, under an electrical field of 15 V/cm, by Zetasizer (NICOMP 380 ZLS). The measurements were conducted in triplicate.

2.5. In vitro drug release studies

The dried nanoparticles were also evaluated for in vitro drug release studies by horizontal shaker method. The CyA-PLGA nanoparticles equivalent to 5mg of drug were suspended in 200 ml of phosphate buffer (0.20M) pH 7.4 containing 0.1% (w/v) sodium lauryl sulphate and 0.02% (w/v) sodium azide. Sodium lauryl sulphate was used to maintain sink condition and sodium azide was used to prevent the microbial growth in the release medium. Various replicates were placed on biological shaker at 37 ± 0.5 °C and 120 rpm. 0.5 ml samples were withdrawn at specified time intervals (0.08, 0.16, 0.33, 1, 2, 3, 4, 5 and 7 days) and centrifuged at 14000 rpm for 15 min and supernatant were analyzed for percentage of drug released by RP-HPLC. The experiment was performed in duplicate.

2.6. SEM measurements

Surface morphology and shape of freeze-dried nanoparticles were investigated by SEM (JSM-6390 LV, JEOL, Tokyo, Japan) measurements at the working distance of 15 mm and an accelerated voltage of 20 kV. Nanoparticles were gold coated with sputter coater (Desk V, Denton Vacuum, NJ, USA) before SEM observation under high vacuum and high voltage 10 mV to achieve film thickness of 30 nm.

2.7. Differential scanning calorimetric studies

DSC of CyA, PLGA, physical mixture of drug and polymer and nanoparticles were performed with SDT 2960 Simultaneous DSC/TGA (TA Instruments Co., New Castle, DE,

USA). The physical mixture prepared with blank nanoparticles of PLGA and CyA by blending in mortar and pastle. Accurately weigh sample (2–4 mg) were sealed in an aluminum pan and empty pan was used as a reference. The samples were scanned from 50 to 300 °C at a scanning rate of 10 °C/min. Nitrogen was used for purging the sample holders at a flow rate of 20 ml/min.

2.8. Powder X-ray diffraction studies

X-ray diffraction (XRD) experiments were performed on X-ray diffractometer (MD-10 mini-diffractometer, MTI Corporation, Richmond, CA, USA) using Cu K2α rays (λ = 1.54056 Å) with a voltage of 25 kV and a current of 30 mA, in flat plate θ/2θ geometry, over the 2θ ranges 25–70°, with a step width 0.05° and a scan time of 2.0 s per step. Diffraction patterns for CyA, PLGA, physical mixture of drug and PLGA and drug loaded nanoparticles were obtained.

2.9. FTIR studies

FTIR spectra of drug, polymer, their physical mixture and drug loaded nanoparticles were performed by ATR–FTIR (Thermo Nicolet Nexus 670 FTIR, GMIInc., Ramsey, Minnesota, USA), and OMNIC ESP software (version 5.1) was used to capture and analyze the spectra.

3. Results and discussion

3.1. Influence of investigated parameters on entrapment efficiency (Y_1)

Entrapment efficiency varied from 10.17% (formulation 12) to 93.01% (formulation 7) for the various factors combination (Table 3). The most significant factors were drug (X_1) and emulsifier concentration (X_3) ($p < 0.05$) used in the formulation relative to other factors influencing entrapment efficiency (Tables 4 and 5 and Fig. 1). The linear model explaining the effects of various factors on entrapment efficiency (Y_1) is

$$Y_1 = 48.35 + 7.30X_1 - 5.16X_2 - 29.95X_3 + 2.63X_4 + 4.86X_5 - 2.94X_6$$

The confidence that model can predict the observed value better than the mean was 87.4% and good correlation was obtained between observed and predicted value as indicated by R^2 value of 0.942 (Table 3). Further analysis by ANOVA indicated significant effect of independent factors (Prob > *F*, 0.0018) on response Y_1 (Table 5). Positive value in the model for a response represents an effect that favors and negative value indicates an inverse relationship between response and a factor (Chopra et al., 2007). To increase the entrapment efficiency (Y_1), drug concentration (X_1) has to be high in the formulation as more of the drug would be available for entrapment. Increased encapsulation efficiency would be expected with increased polymer concentration (X_2); this would increase the viscosity of the medium and result in faster solidification. This would further prevent drug diffusion to external phase, and also increased viscosity would limit the diffusion of drug from inner phase to outer phase (Yang et al., 2000). But on the contrary, we observed the negative effect of polymer (X_2) on entrapment efficiency (Y_1). It is reported that polylactic acid oligomers have surface tension reducing activity which results from the carboxylate group that get introduced at the oil/water interphase and stabilize the emulsion by electrostatic repulsion (Carrio et al., 1995). Similarly, oligomers of PLGA could have surfactant like effects which helps in the diffusion of drug from internal to external phase of emulsion that account low entrapment of CyA with increase in the polymer.

Another factor that significantly impacted the entrapment efficiency (Y_1) was emulsifier concentration (X_3). Higher concentration resulted in lower entrapment, as this would

increase the partitioning of drug from internal phase to external phase. This increased partitioning might result from increased solubility of drug in the external phase (Yang and Owusu-Ababio, 2000). Volume of external phase (X_6) has negative effect on the entrapment. This could be explained by the fact that more volume will be available for the drug to diffuse from internal to external phase and it also decrease the viscosity of the system and further, increases the diffusion of CyA.

Type of organic solvent (X_5) employed in the manufacturing of nanoparticles had positive impact on the entrapment but effect of this factor was not significant and chloroform increased the entrapment efficiency. This could be explained by low miscibility of chloroform (0.8 g/100 ml at 20 °C, http://www. inchem.org/documents/icsc/icsc/eics0027.htm) in water in comparison to dichloromethane (1.3 g/100 ml at 20 °C, http://www. inchem.org/documents/icsc/icsc/eics0058.htm) that reduce the diffusion of drug into external medium and accounted for high entrapment of CyA. Stirring rate (X_4) had non-significant effect on entrapment efficiency (Y_1).

3.2. Influence of investigated parameters on particle size (Y_2)

The mean particle (Y_2) size ranged from 41.60nm (formulation 7) to 372.80nm (formulation 2) depending on the variable level selected during production (Table 3). The fitted model describing the influence of variables on the mean particle size is

$$Y_2=162.59+95.89X_1 - 15.32X_2 - 1.82X_3 - 40.04X_4 - 27.71X_5 - 21.78X_6$$

Statistical analysis (Tables 4 and 5) revealed that the most significant factors effecting mean particle size (Y_2) were drug (X_1) ($p < 0.05$) used in the formulation and stirring rate (X_4) ($p < 0.05$) during emulsification and solvent evaporation step (Fig. 2). There was good correlation between actual and predicted value as shown by R^2 value of 0.93 (Table 3). Other investigated levels of factors did not have significant impact on the mean particle size.

Increase in particle size was observed with increase in drug (X_1) and polymer (X_2) as supported by other investigator (Couvreur et al., 1997). This could be explained by increased viscosity of organic phase that ultimately affect the shearing efficiency of stirrer. This also resulted in decreased collision during emulsification and droplet solidification resulting in the aggregation of semi-solid particles and increased particle size. Similar effect was observed with increase in the ratio of organic to aqueous phase ratio (X_6). Increasing the ratio would result in increased volume of external phase that reduced the agitation efficiency (Jeffery et al., 1991). Smaller particles were obtained with increasing stirrer speed (X_4) and this could be explained by the fact that increasing the stirring rate, provided necessary shearing energy to break the large droplet into smaller one (Yang et al., 2001). Emulsifier concentration (X_3) also played a role in decreasing the particle size. This could be attributed to increased diffusion of drug from droplet to external phase and resulted in smaller particle size. It also stabilized the smaller droplet and prevented coalescence into bigger droplet (Yang et al., 2001). Type of solvent (X_5) has negative impact on the particle size (Y_2) and chloroform produces smaller nanoparticle when used as solvent. This phenomenon could be explained probably by viscosity difference between chloroform and dichloromethane (0.38 and 0.9 centistokes at 20 °C, respectively, http://www.engineersedge.com/fluid flow/fluid data.htm) that cause easy homogenization during initial phase of emulsification and produces smaller particle.

3.3. Influence of investigated parameters on zeta potential (Y_3)

Zeta potential is the overall charge acquired by particles in a particular medium and its value gives the indication of potential physical stability of nanoparticles dispersion. If all the particles have large positive or negative of zeta potential they will repel each other and system is considered to be stable. Higher the value, more stable the system. It is reported that the value of ±30 mV assure the stability of dispersed system (Motwani et al., 2008). The zeta potential of nanoparticles formulations ranged from 29.60 mV (formulation 9) to 34.90 mV (formulation 6) (Table 3, Fig. 3). Model describing the effects of factors on the zeta potential is

$$Y_3=33.12-0.87X_1-0.38X_2+0.19X_3-0.57X_4+0.51X_5-0.09X_6$$

Statistical analysis results are summarized in Tables 4 and 5 revealed that none of the factor was significant ($p < 0.05$) in predicting the value of zeta potential.

3.4. Influence of investigated parameters on burst effect (Y_4)

The in vitro release of CyA from nanoparticles in phosphate buffer of pH 7.4 showed biphasic release pattern typical of sustained/controlled release formulation, an initial burst release followed by sustained release. Burst release was due to the presence of drug at the nanoparticles surface and possibly, could be ascribed to the solvent flux out of the organic phase during the solvent evaporation step that cause drug transportation to the particle surface. Subsequently, solvent partition into water phase leading to reduction of its solubilizing power, and the drug precipitates at the particle surface or in the suspension medium. Additionally, drug appears steadily at the nanoparticles surface by diffusion through the polymer-rich phase may also be expected to provide a source of particle surface drug crystallization (Jalil and Nixon, 1989).

Equation describing the effect of factors on the burst release of CYA from nanoparticles in 2 h is

$$Y_4=25.97+3.02X_1-2.44X_2-6.89X_3+0.44X_4-1.96X_5-0.51X_6$$

Statistical analysis (Tables 4 and 5) suggested that most significant factor influencing the burst effect is emulsifier concentration (X_3) ($p < 0.05$, Fig. 4) used in the manufacturing of nanoparticles. Other factors too had effect but were not very significant.

Burst release varies from 9.39% (formulation 6) to 36.33% (formulation 7) (Table 3). There is direct relationship between burst release (Y_4) and amount (X_1) of drug used in the formulation which cause higher entrapment of drug. This could also be explained by phenomenon of drug nanoparticle. Not all drugs will be entrapped by polymer and some of it might appear as drug nanoparticles which has higher solubility due to higher surface area that contributes to burst effect. Similar effect of stirring rate (X_4) was also observed. Increasing the stirring rate led to an increase in the shearing of the primary emulsion nanodroplet, exposing more area for solvent diffusion to external medium and generation of smaller nanoparticle and hence more burst effect would be observed. High polymer (X_2) was associated with low burst effect. High polymer (X_2) is connected with increase viscosity of organic phase that hamper the drug migration from inner core to outer surface. Similar results were obtained with increasing emulsifier concentration (X_3). Increased emulsifier concentration (X_3), lead to increase solubilization of the precipitated drug at the nanoparticles surface. Additionally, burst release was lessening with increase in the organic to aqueous ratio (X_6). This was possibly due to large volume of medium available for the

drug to diffuse from particle surface to external medium and hence, resulted in less drug at the surface of particle. Organic to aqueous phase ratio (X_6) had negative impact on burst release due to the low entrapment and the formation of bigger particles at high organic to aqueous ratio, therefore, less surface area available for drug diffusion. Type of organic solvent (X_5) had negative effect on burst effect that was insignificant. The burst effect is less in the formulation that used dichloromethane than chloroform. This is probably due to low entrapment and bigger size nanoparticle produced in that formulation.

3.5. Influence of investigated parameters on dissolution efficiency (Y_5)

In vitro drug release form PLGA polymer occurs by diffusion and erosion mechanism. The initial drug release from PLGA nanoparticles occur by diffusion of CyA from polymer matrix. On the other hand, drug release during later phase is mediated by both diffusion of drug and erosion of PLGA itself. PLGA degrades through a process of autocatalytic hydrolysis of ester bonds. The end products of degradation are acidic monomers and oligomers that further catalyze the hydrolysis. Any factor that influences formation or retention of monomers could affect the release of drug from polymer matrix (Wischkel and Schwendeman, 2008).

Dissolution efficiency (DE) was calculated from the in vitro release data as per Khan (Khan, 1975):

$$\mathrm{DE}\,(\%)=\frac{\int_0^t Y \times dt}{Y_{100} \times t} \times 100$$

Y is the drug release at time t.

The DE of prepared formulation lies between 52.67% (formulation 6) and 84.11% (formulation 5) (Table 3). The following model can describe the effect of various factors on DE:

$$Y_5=68.87+3.17X_1 - 0.09X_2 - 10.36X_3+1.53X_4+3.01X_5 - 1.06X_6$$

Statistical analysis results (Tables 4 and 5) revealed that the most significant factor affecting the DE (Y_5) was emulsifier concentration (X_3) ($p < 0.05$, Fig. 5) used during the manufacturing of nanoparticles. An increase in emulsifier concentration (X_3) used caused decrease in the entrapment efficiency and hence, DE decreased. The drug (X_1) and polymer (X_2) concentration had positive and negative effect on DE, respectively that is probably due to corresponding effect on entrapment efficiency (Y_5). High stirring rate (X_4) increased the value of DE due to the formation of smaller particles which produced large surface area, and hence increased diffusion of drug from PLGA matrix. Organic to aqueous phase ratio (X_6) had negative effect on DE due to decrease in the entrapment efficiency (Y_1).

To understand the mechanism of drug release form PLGA matrix the release data was fitted into zero, first order Higuchi model (Merchant et al., 2006). It was found that in vitro CyA release from PLGA matrix was best fitted in Higuchi diffusion release model as indicated by highest value of determination coefficient 'R^2' (0.84–0.98) followed by first order (0.81–0.95), and then zero order (0.43–0.75). This is further confirmed by fitting the release data in Korsmeyer–Peppas equation:

$$\frac{M_t}{M_\alpha} = Kt^n$$

(M_t/M_α) is the fraction release up to time *t*, '*k*' is a constant incorporating structural and geometrical characteristics of dosage forms and 'n' is an exponent that characterize release mechanism. For spherical matrix system, if the exponent $n = 0.43$, then the drug release mechanism is Fickian diffusion, and if $0.43 < n < 0.85$, then it is non-Fickian or anomalous diffusion. An exponent value of 0.89 is indicative of Case-II Transport or typical zero order release (Siepmann and Peppas, 2001). The plot between log of cumulative percentage release vs. log of time for Korsemeyer–Peppas model showed good linearity (R^2, 0.85–0.98) and the value of exponent coefficient was between 0.561 and 0.701 which appear to indicate coupling of diffusion and erosion mechanism, so-called anomalous diffusion that indicate drug release is controlled by more than one process.

3.6. Visualization and compatibility studies

To further prove the Higuchi release model, and verify some of the assumption of this model that dosage forms are spherical, drug are homogenously distributed in the matrix and no interaction occurring between the drug and matrix (Higuchi, 1963). Sphericity analysis of nanoparticles were performed by SEM. SEM photograph revealed that nanoparticles are spherical, non-porous and with smooth surface morphology (Fig. 6). DSC studies were performed to determine drug status inside the formulated nanoparticles and drug–polymer interaction during the manufacturing of nanoparticles. It is reported that CyA shows characteristic melt peak at 190 °C for an orthorhombic crystal form and around 110 °C for a tetragonal form (Lechuga-Ballestros et al., 2003). DSC thermogram showed no peak for pure drug and nanoparticles formulations (Fig. 6). It proved that starting drug is amorphous in nature, and also there was no change in the physical characteristics of CyA in the nanoparticles formulation. Also, thermogram did not reveal any extra endo/exothermic peaks which are indicative of no interaction between PLGA and CyA. XRD studies further verified the amorphous nature of drug as it showed no peaks in diffractogram (Fig. 6). Moreover, FTIR confirmed these results (Fig. 6). Characteristics amorphous form CyA showed peaks at 2873 and 2836 cm^{-1}. The crystalline form of CyA shows characteristic peak at 2855 cm^{-1} and another peak at 2928 cm^{-1} that shifted to 2836 cm^{-1} in amorphous form (Bertacche et al., 2005).

4. Conclusion

In this study, several process and formulation variables were screen by a DOE/QbD approach to understand the most significant impact on the characteristics of CyA-PLGA nanoparticles. It was found that emulsifier level was most significant factor effecting entrapment efficiency, burst effect and dissolution efficiency. For particle size, drug level and stirring were most important factors. Other formulation and processing factors did not have significant impact on nanoparticles properties. The in vitro release profile of CyA-PLGA nanoparticles showed biphasic release pattern and followed Higuchi kinetic and release occurred by combination of diffusion and erosion mechanism as shown by Korsemeyer–Peppas model.

Following this study, parameters which have the greatest influence on the nanoparticles properties were determined. Only the main effects have been evaluated. Further studies to understand their interactions and quadratic response surfaces are in progress.

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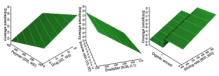

Fig. 1.
Surface profilers showing effect of drug (X_1), polymer (X_2), emulsifier (X_3), stirring rate (X_4), solvent (X_5) and organic to aqueous phase ratio on entrapment efficiency (Y_1).

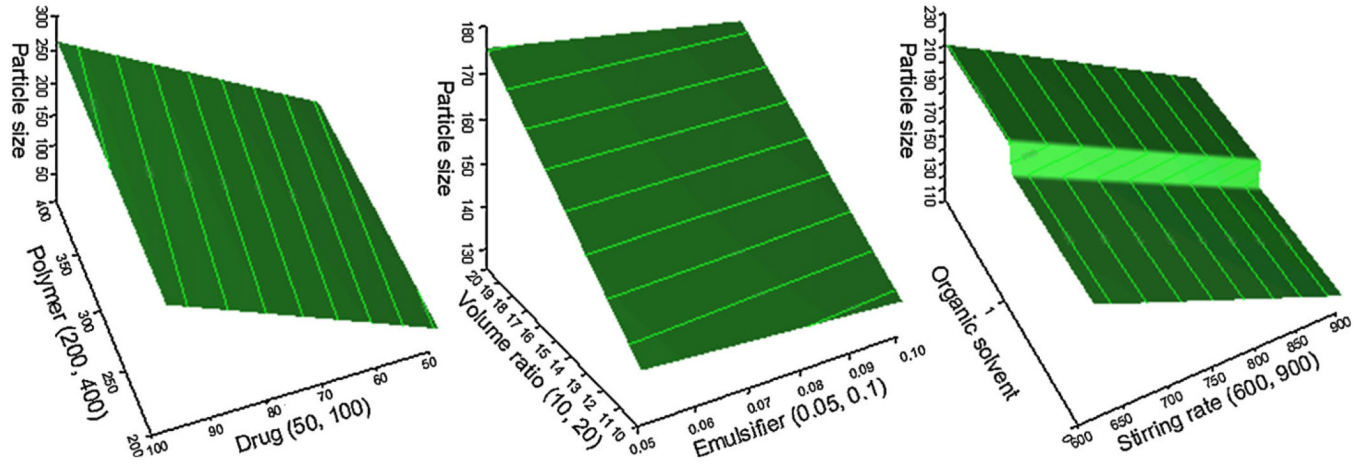

Fig. 2.
Surface profilers showing effect of drug (X_1), polymer (X_2), emulsifier (X_3), stirring rate (X_4), solvent (X_5) and organic to aqueous phase ratio on mean particle size (Y_2).

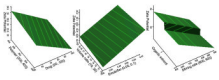

Fig. 3.
Surface profilers showing effect of drug (X_1), polymer (X_2), emulsifier (X_3), stirring rate (X_4), solvent (X_5) and organic to aqueous phase ratio on zeta potential (Y_3).

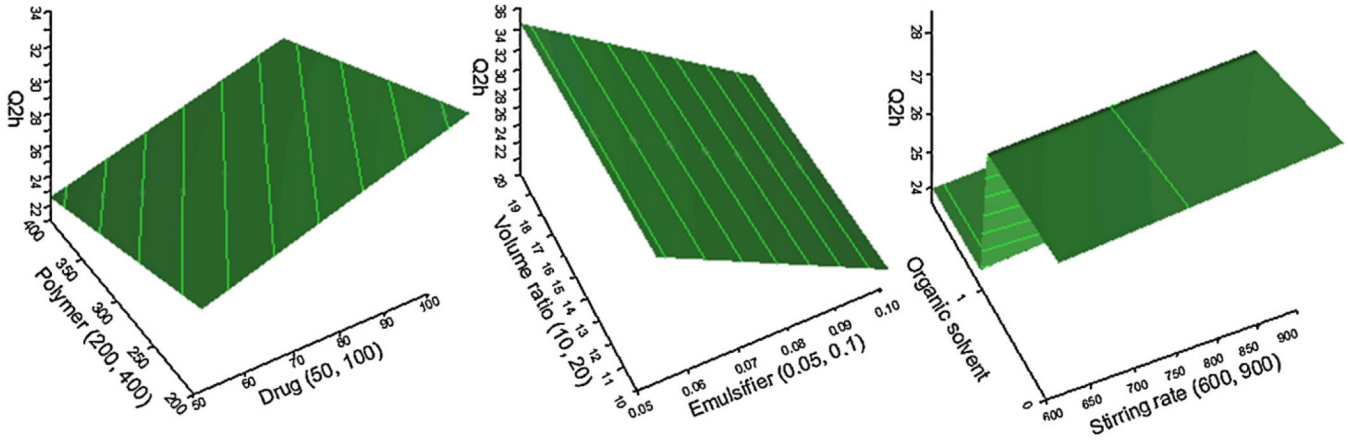

Fig. 4.

Surface profilers showing effect of drug (X_1), polymer (X_2), emulsifier (X_3), stirring rate (X_4), solvent (X_5) and organic to aqueous phase ratio on burst effect (Y_4).

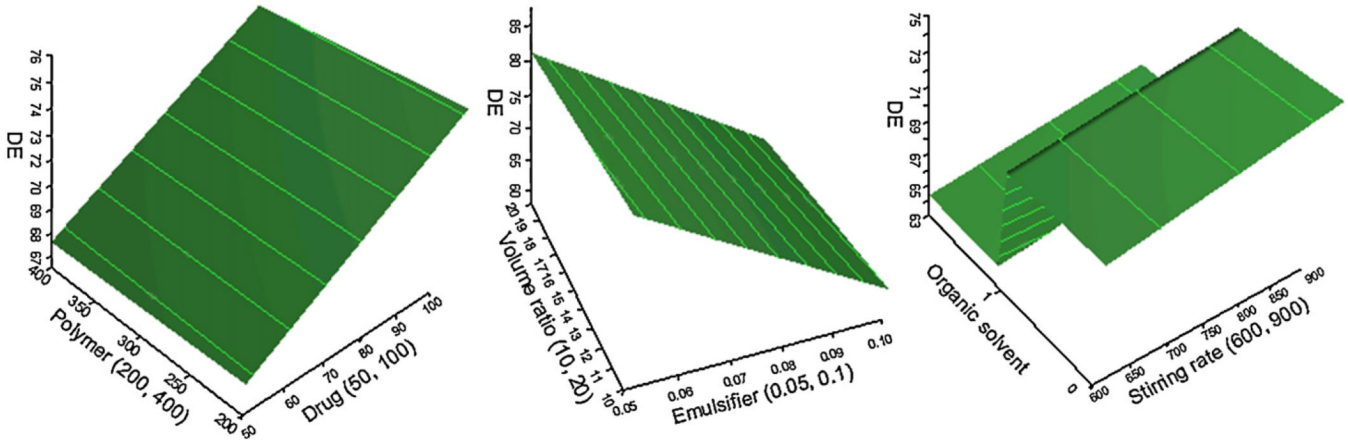

Fig. 5.

Surface profilers showing effect of drug (X_1), polymer (X_2), emulsifier (X_3), stirring rate (X_4), solvent (X_5) and organic to aqueous phase ratio on dissolution efficiency (Y_5).

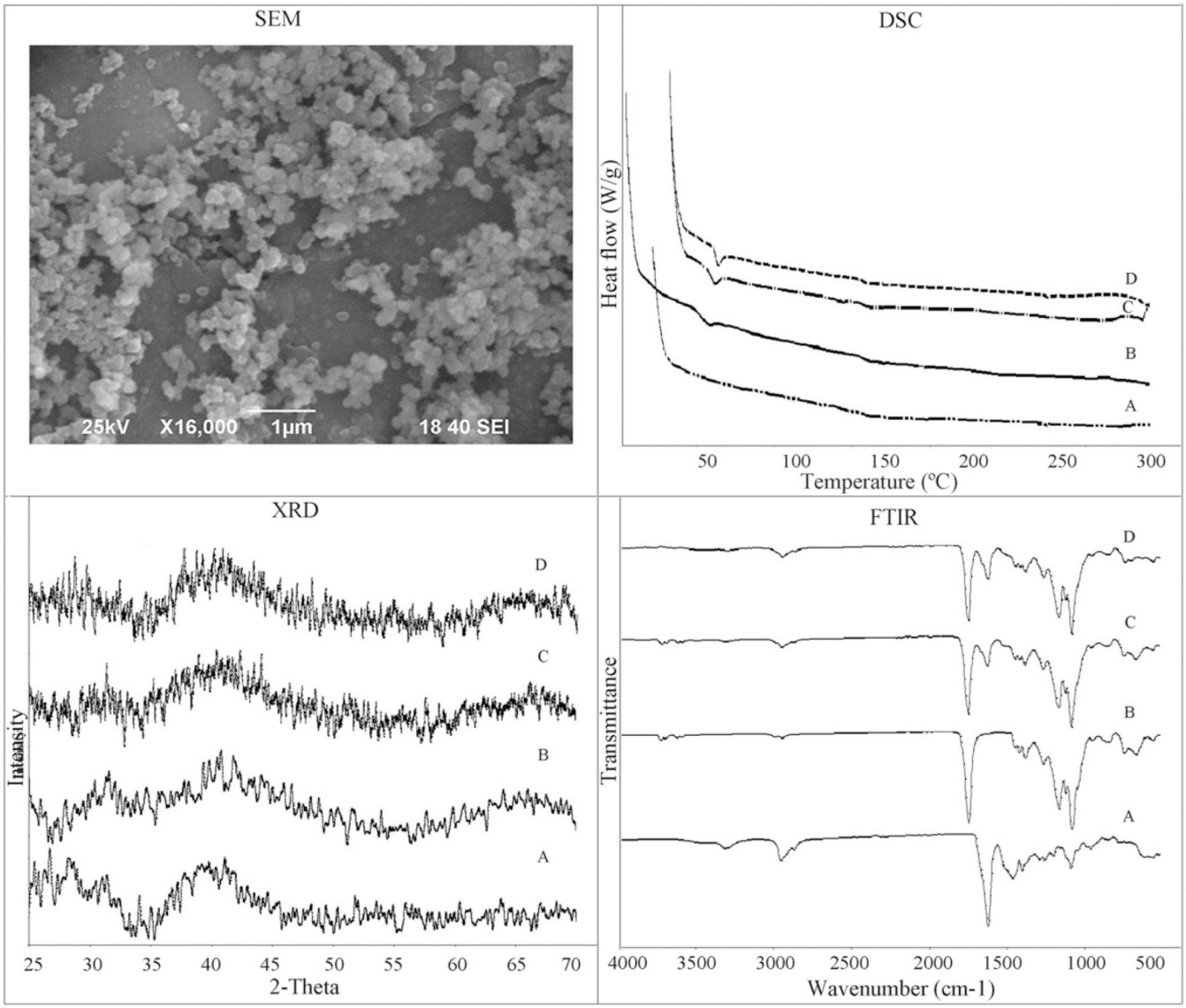

Fig. 6.

SEM of nanoparticles and FTIR, XRD and DSC of (A) CyA, (B) PLGA, (C) physical mixture of CyA and PLGA and (D) CyA loaded nanoparticles.

Table 1

Experimental factors and their level.

Factor	Factor significance	Level (−1)	Level (+)
X_1	Drug (mg)	50	100
X_2	Polymer (mg)	200	400
X_3	Emulsifier concentration (%)	0.05	0.10
X_4	Stirring rate (rpm)	600	900
X_5	Type of organic solvent	Dichloromethane	Chloroform
X_6	Organic to aqueous phase ratio	1:10	1:20

Table 2

Experiment design.

Formulation	Drug loading (mg) (X_1)	Polymer loading (mg) (X_2)	Emulsifier concentration (%) (X_3)	Stirring rate (rpm) (X_4)	Type of organic solvent (X_5)	Organic to aqueous ratio (X_6)
1	50	200	0.05	900	Dichloromethane	1:10
2	50	200	0.10	600	Chloroform	1:20
3	50	200	0.10	600	Dichloromethane	1:20
4	50	400	0.05	600	Chloroform	1:10
5	50	400	0.05	900	Chloroform	1:20
6	50	400	0.10	900	Dichloromethane	1:10
7	100	200	0.05	600	Chloroform	1:10
8	100	200	0.05	900	Dichloromethane	1:20
9	100	200	0.10	900	Chloroform	1:10
10	100	400	0.05	600	Dichloromethane	1:20
11	100	400	0.10	600	Dichloromethane	1:10
12	100	400	0.10	900	Chloroform	1:20

Table 3

Observed and predicted value of entrapment efficiency (Y_1), particle size (Y_2), zeta potential (Y_3), burst effect (Y_4) and DE (Y_5).

Formulation	Observed Y_1	Predicted Y_1	Observed Y_2	Predicted Y_2	Observed Y_3	Predicted Y_3	Observed Y_4	Predicted Y_4	Observed Y_5	Predicted Y_5
1	86.63	82.07	136.30	180.46	34.01	32.69	30.48	33.72	75.39	81.74
2	10.30	9.43	330.80	311.26	34.80	34.63	18.23	15.96	52.80	49.83
3	11.08	11.93	275.50	295.04	34.11	34.28	16.88	19.14	52.89	55.85
4	49.19	64.86	372.80	302.01	33.27	34.83	29.84	25.87	76.34	72.47
5	65.75	67.54	275.70	243.85	32.82	33.39	32.70	29.95	84.11	79.45
6	23.21	10.24	159.80	218.28	34.90	34.08	9.39	12.87	52.67	54.84
7	93.01	91.47	41.60	68.76	32.53	32.09	36.33	38.98	82.20	85.02
8	87.64	89.15	42.60	43.04	31.38	31.37	35.13	36.72	78.13	79.96
9	32.35	36.85	56.80	60.06	29.60	31.35	33.48	25.98	78.40	67.39
10	87.40	74.43	117.50	148.37	33.52	33.14	32.81	32.03	79.19	76.71
11	23.25	22.13	77.60	90.36	33.87	33.12	19.20	21.29	60.62	64.14
12	10.17	19.82	64.10	64.64	32.59	32.40	17.10	19.03	53.78	59.08
R^2	0.942	0.930	0.627	0.857	0.850					

Table 4

Statistical analysis of entrapment efficiency (Y_1), particle size (Y_2), zeta potential (Y_3), burst effect (Y_4) and DE (Y_5) results.

	Entrapment efficiency (Y_1)		Particle size (Y_2)		Zeta Potential (Y_3)		Burst Effect (Y_4)		DE (Y_5)	
	Coefficient	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**	**Coefficient**	***p*-value**
Constant, b_0	48.349	<0.0001*	162.592	<0.0001*	33.117	<0.0001*	25.975	<0.0001*	68.8767	<0.0001*
Drug loading, X_1	7.301	0.0456*	95.892	0.0008*	−0.868	0.0772	3.025	0.0722	3.177	0.2003
Polymer loading, X_2	−5.166	0.1199	15.325	0.3003	0.378	0.2955	−2.442	0.1260	−0.088	0.9709
Emulsifier concentration, X_3	−29.951	0.0001*	−1.825	0.8960	0.195	0.5735	−6.892	0.0035*	−10.35	0.0049*
Stirring rate, X_4	2.629	0.3842	−40.042	0.0295*	−0.567	0.1407	0.442	0.7534	1.537	0.5076
Type of organic solvent, X_5	4.861	0.1383	−27.708	0.091	0.515	0.1728	−1.958	0.2011	3.01	0.2726
Organic to aqueous ratio, X_6	−2.944	0.3345	21.775	0.162	−0.087	0.7997	−0.508	0.7182	−1.057	0.6656

*Most significant value.

Table 5

ANOVA results of entrapment efficiency (Y_1), mean particle size (Y_2), zeta potential (Y_3), burst effect (Y_4) and DE (Y_5).

Response	Source	DF	Sum of square	Mean of square	*F* ratio	Prob > *F**
	Model	6	12194.985	2032.50		
Y_1	Error	5	456.344	91.27	22.2693	0.0018
	Cumulative total	11	12651.329			
	Model	6	138744.590	23124.1		
Y_2	Error	5	19164.140	3832.8	6.0332	0.0337
	Cumulative total	11	157908.730			
	Model	6	15.461	2.577		
Y_3	Error	5	9.184	1.837	1.403	0.3637
	Cumulative total	11	24.645			
	Model	6	786.303	131.051		
Y_4	Error	5	130.733	26.147	5.0122	0.0488
	Cumulative total	11	917.036			
	Model	6	1584.685	264.114		
Y_5	Error	5	278.458	55.692	4.7424	0.0543
	Cumulative total	11	1863.144			

* Prob > *F* is the significance level and a value less than 0.05 considered significant.