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QUALITY BY DESIGN-ASSISTED OPTIMIZATION, FORMULATION DEVELOPMENT, PHYSICOCHEMICAL CHARACTERIZATION, DRUG RELEASE KINETICS, CELLULAR UPTAKE, AND IN VITRO ANTICANCER EVALUATION OF TAMOXIFEN CITRATE-LOADED PLGA NANOPARTICLES AGAINST MCF-7 BREAST CANCER CELLS

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ABSTRACT
In this paper, a Quality by Design (QbD) approach has been used to formulate Tamoxifen citrate loaded poly (lactic-co-glycolic acid), PLGA, nanoparticles to be targeted towards MCF-7 breast cancer cells. The critical quality attributes were determined through the risk assessment process, and polymer to drug ratio, surfactant level, and homogenization speed were optimized by using a Box- Behnken design. The multiple emulsion solvent evaporation method was used to prepare nanoparticles which were characterized for particle size, polydispersity index, zeta potential, % of entrapment efficiency, surface morphology and drug polymer compatibility by fourier transform infrared spectrometry and differential scanning calorimetry. Results showed that the mean particle size of the optimized formulation (F4) was 158 nm, zeta potential was minus 24.6 mV and the entrapment efficiency was 81.3 percent. The IVR of the drug in PBS at pH 7.4 was biphasic; there was a burst release of 8 per cent followed by sustained release where 87.4 per cent was released at 48 hours and the release was well explained by the Korsmeyer-Peppas model with a release exponent of 0.46 indicating Fickian diffusion-controlled mechanism. Time-dependent internalization of nanoparticles, labeled with coumarin-6, was observed in MCF-7 cells by confocal microscopy. The optimized nanoparticle formulation with IC50 value of 6.7 micrograms per milliliter (µg/mL) at 48 hours was 2.7 fold more potent than free tamoxifen citrate with IC50 value of 18.4 µg/mL at 48 hours and measured by MTT assay. These results suggest that PLGA nanoparticles can be used as a good platform to increase the anticancer effect and uptake of tamoxifen citrate in estrogen receptor positive breast cancer treatments.

KEYWORDS: Quality by Design, PLGA nanoparticles, tamoxifen citrate, MCF-7 cells, drug release, cellular uptake, anticancer evaluation, Box-Behnken design.

I. INTRODUCTION

Breast cancer is one of the most common cancers diagnosed in women in the world and estrogen receptor (ER) positive breast cancer constitutes a significant proportion of the new cases of breast cancer [1]. Despite the continued widespread use as first line endocrine therapy for hormone receptor positive breast cancer, tamoxifen citrate activity is limited by low aqueous solubility, high first pass metabolism in the liver, non-specific tissue distribution and dose-limiting events of endometrial hyperplasia and thromboembolism [2]. These restrictions have inspired high research efforts on nanoparticulate carriers that could enhance solubility, prolongate systemic circulation, allow sustained release and preferentially target the tumor tissue via the enhanced permeability and retention effect [3].

Poly (lactic-co-glycolic acid), PLGA, is one of the most extensively studied biodegradable polymers for delivery of parenteral nanoparticles (NPs) due to the well-documented safety of particular PLGA devices, the tunable degradation properties, and PLGA's approval by the United States Food and Drug Administration (FDA) for human use [4]. Tamoxifen or its citrate salt containing PLGA nanoparticles have been previously shown to enter into MCF-7 cells and increase the cytotoxicity compared to free drug [5] [6]. By far, there are other nanocarrier

systems that have reported improved cellular uptake and cytotoxicity against MCF-7 cells such as PLGA nanoplatforms modified by TPGS, mesoporous silica nanoparticles and poly (lactic acid) porous microparticles functionalized with lipids, highlighting a wide interest in nanocarrier mediated tamoxifen delivery [7, 8, 9].

Another common drawback of previous tamoxifen nanoparticle research studies is the one factor at a time approach that was frequently used to develop and formulate the drug, thereby failing to account for possible interaction effects between different formulation and process factors and potentially resulting in sub-optimal formulations. A systematic alternative has been slowly introduced: Quality by Design paradigm, which starts with a predefined quality target product profile, and incorporates a risk assessment as well as design of experiments to set up a validated design space; this approach has been increasingly used to optimize polymeric nanoparticles [10], [11]. Design of experiments like Plackett-Burman screening and Box-Behnken response surface methodology enable optimization of multiple critical process parameters (CPPs) with a minimum number of runs and estimate the interaction and quadratic effects of these parameters on critical quality attributes (CQAs) including particle size, polydispersity index, zeta potential and entrapment efficiency [12].

In the present study, preparation of tamoxifen citrate loaded PLGA nanoparticles by multiple emulsion solvent evaporation method was developed and optimized by a Box-Behnken design (BBD) using Quality by Design (QBD). In addition to the optimization of the formulation, this study also offers the detailed physicochemical characterization, in vitro release in vitro mechanistic kinetic modeling, cellular uptake analysis and in vitro anticancer study against MCF-7 cells and places the new formulation in a comparative evaluation of the literature reported Tamoxifen Nanocarrier systems. This study has the specific objective as follows. The first is using risk assessment and screening design to identify critical quality attributes and critical process parameters. Second-phase, to optimise the formulation of the nanoparticles by applying a Box-Behnken design and to validate the design space produced. Thirdly, to physicochemically characterize and in vitro evaluate the optimized formulation for its release behavior and release mechanism. Fourth, to evaluate the cellular uptake and anticancer activity on MCF-7 cells. Fifth, to compare the optimized formulation performance with the reported tamoxifen delivery system.

The pathway from a translational and regulatory viewpoint for polymeric nanoparticle formulations is becoming increasingly more defined with several PLGA based nanomedicines already having been approved for other indications, which argues for a precedent: a tamoxifen citrate loaded PLGA nanoparticle product would need to demonstrate well characterized size distribution, reproducible entrapment efficiency, lack of drug polymer incompatibility, and a mechanistically explained in vitro drug release profile in order to be acceptable. Hence, establishing these characteristics under a formally validated Quality by Design (QbD) framework, rather than by merely making empirical selection of the formulation to be used, is not merely scientifically necessary, but also practically relevant to the preparation of regulatory dossier, as design space validation and determination of critical process parameters are integral parts of modern pharmaceutical quality guidance [10,11].

With the manufacturing of polymeric and lipid based nanoparticles becoming mature and the increase in the number of clinical studies demonstrating an improved therapeutic index (e.g. tumor efficacy and therapeutic safety) of nano-based therapeutics over conventional formulations in various types of tumors, the global market for nanomedicines for oncology therapeutics has continued to grow. In this environment, it is tempting to consider adopting a well established, off-patent, API molecule like tamoxifen citrate, and developing it using rational nanoparticle engineering to realize the delivery, biodistribution and potentially even a different dosing frequency advantages that can be gained by nanoparticles, while retaining much of the existing safety and efficacy data for the API, thereby avoiding the significantly greater risk and development cost that usually accompanies a novel chemical entity.

II. RELATED WORK

A. Nanoparticulate Carriers for Estrogen Receptor Positive Breast Cancer Therapy

Polymeric and inorganic nanocarriers as vehicles to enhance the delivery of endocrine therapy agents in breast cancer have been extensively studied. Danhier et al. have summarized the large number of biomedical applications of PLGA based nanoparticles, which include favorable biodegradability, the ability to tune release profiles by varying the lactide and glycolide ratio in the polymer, and a well documented history under review by regulatory agencies which is a preferred choice as a polymer for nanoparticles designed for parenteral dosage delivery [4]. In this environment, tamoxifen and tamoxifen citrate have been the target of specific attention because, although a clinically proven agent, it presents pharmacokinetic and biodistribution restrictions, which can be overcome by the nanoencapsulation [2] [3].

Maji et al. formulated four PLGA based tamoxifen citrate nanoparticles using various emulsion solvent evaporation methods and reported their internalization by MCF-7 cells in concentration dependent manner, showing greater cytotoxicity of the drug loaded nanoparticles as compared to the free drug, which is generally in agreement with the present study [5]. Subsequent studies have examined other or modified bioactive carrier

chemistry to the same therapeutic target. The combined drug delivery and imaging capability of TPGS modified PLGA nanoplatforms has been investigated and reported to have a 6.21 fold enhancement in cytotoxic effect against MCF-7 cells, further hemocompatibility and organ safety was observed in in vivo tumor model [7]. Similarly, nanoparticles composed of maltoheptaose and polystyrene have been found to be more cytotoxic to MCF-7 cells than free tamoxifen citrate, and show the possibility for an effective delivery of the drug via oral nanoparticles, not restraining to the use of PLGA nanoparticles. The pH responsive release characteristic of mesoporous silica nanoparticles with silica and amine functionalization provides a structure that differs from organic molecules while remaining inorganic in nature and exploiting the pH responsive release properties of the leaky tumor microenvironment [8]. A more recent addition to the growing poly (hydroxy acid) carrier family is cholesterol functionalised porous poly (lactic acid) microparticles which have also been able to be successfully taken up by MCF-7 cells and to reduce cell viability [9].

B. Quality by Design and Design of Experiments in Nanoparticle Optimization

Quality by Design (QbD), established in International Council for Harmonisation (ICH) guidance and studied extensively by Yu et al. focuses on incorporating quality into a product without merely testing the end product, by systematically understanding how the attributes of a product affect the attributes of a process as well as the product's critical quality attributes [11]. When applied to nanoparticle formulation, this usually includes a quality target product profile, a factor prioritisation screening phase using either a Plackett-Burman or fractional factorial designs, followed by a modelling and optimisation phase using a response surface methodology (RSM), for which Box-Behnken or central composite designs are typically used [10]. This workflow was a bit clarified by Beg et al. for nanostructured lipid carriers (NLCs), where a validated design space was developed for NLCs by risk assessment and response surface modeling of formulation variables in relation to particle size, entrapment efficiency and (zeta) potential [10]. A similar Plackett-Burman and Box-Behnken design workflow was used to optimize PLGA nanoparticles containing TEL and showed an optimized formulation with a particle size of 232.4 nm with an entrapment efficiency of 79.21 percent delivering a Korsmeyer-Peppas model governed, Fickian diffusion dominant release profile which is similar and comparable to both the design methodology and release mechanism achieved with the optimized formulation F4 in the present study [12].

C. Drug Release Kinetics from PLGA Based Nanoparticulate Systems

A family of empirical and semi-empirical models, such as zero order, first order, Higuchi and Korsmeyer-Peppas formulations, have been used for the mechanistic interpretation of supernatant data obtained after in vitro release of drug from polymeric nanoparticles, and the release exponent obtained from Korsmeyer-Peppas model, developed by Korsmeyer et al. and further classified by Ritger and Peppas, has been used to discriminate between Fickian and anomalous (non Fickian) transport in polymeric matrices [13,14]. For several PLGA nanoparticle studies, incorporating poorly water soluble active pharmaceutical ingredients, Korsmeyer-Peppas is identified as one of the best fit or best fit models; generally well water soluble particles have release exponents close to 0.5 which are indicative of quasi-Fickian to Fickian diffusion and is in agreement with the release exponent of 0.46 for F4 in this study [15], [16], [19]. After testing a third order reaction model, the Weibull model, against sixty PLGA nanoparticle release data sets, it was found that the Weibull model was overall the more accurate, and the Akaike information criterion was also lowest, suggesting the Weibull model is a better overall choice for reporting, and that although the Korsmeyer–Peppas model was competitive and mechanistically interpretable, it was insightful to evaluate the release data for each individual system against multiple potential models, rather than rely on the Korsmeyer–Peppas fit alone [18].

D. Cellular Uptake and Anticancer Evaluation Approaches

The most typical approach to assessing cellular internalization of nanoparticles is to use fluorescently labelled analogues coupled to the use of confocal laser scanning microscopy and, in a more quantitative manner flow cytometry to reveal the spatial location and semi-quantitative uptake kinetics. The determination of efficacy against cancer cells is usually carried out by colorimetric viability tests like the MTT assay, which reports half maximal inhibitory concentration (IC_{50}) values by non-linear regression of dose response plots and allows one to compare the potency between various nanoparticles and free drug treatments in the literature surveyed above and was used here [5,7,8].

E. Comparison with Non-PLGA Polymeric and Lipid Based Systems

In addition to PLGA, several other polymeric and lipid based carriers have been investigated for the targeting of tamoxifen and other related anticancer drugs such as chitosan based nanoparticles, solid lipid nanoparticles, and nanostructured lipid carriers, each with its own set of pros and cons with regard to surface charge, mucoadhesive properties, scalability, and raw material costs. Apart from their longer circulation times, chitosan based systems could be used to improve mucosal or cellular membrane interaction compared with anionic PLGA particles, but with the potential downside of shorter circulation times because of more rapid opsonisation because of the inherent positively charged surface. Solid lipid nanoparticles and nanostructured lipid carriers, on the other hand, have historically been more difficult to obtain sustained release profiles like those possible with PLGA, due to the more rapid rearrangement of lipid matrices that occur after administration, but do have manufacturing advantages of avoiding halogenated organic solvents. In this overall context, PLGA still offers the attractive combination of a track record of being acceptable to regulatory authorities, tunable degradation properties by varying the lactide to glycolide ratio, and the ability to be used with the multiple emulsion solvent evaporation method used in this study.

F. Targeting Strategies in Breast Cancer Nanomedicine

An active targeting parallel research axis within breast cancer nanomedicine has involved functionalization of nanoparticles with ligands, antibodies or peptides that bind to receptors that are highly expressed on tumor cells like the folate receptor, transferrin receptor or human epidermal growth factor receptor 2, in hopes of achieving receptor mediated endocytosis and augmenting the tumor selective accumulation that occurs simply through the property of passive enhanced permeability and retention. Although these types of active targeting strategies were not the intent of the present study aimed at establishing and validating a QbD driven base PLGA formulation, the Design Space and Characterization approach is applicable for the development of the active targeting variants; once the core formulation parameters have been optimized, the surface ligand can usually be attached as a post-formulation step without necessitating reformulation optimization.

G. Research Gap

Each of these features – the present workflow: tamoxifen citrate nanoparticle formulation via PLGA, Quality by Design approach to optimization of pharmacologically unrelated active pharmaceutical ingredients, formal validation of the approach and refinement of the formulation, and Box Behnken optimization framework – has been studied independently, but relatively few studies have tested an integrated formally validated Quality by Design drivenBox Behnken optimization of formulation of tamoxifen citrate loaded PLGA nanoparticles against MCF-7 cells including a comprehensive physicochemical, release kinetic, cellular uptake and anticancer evaluation, and even fewer of these studies positioned the resulting formulation in the context of an explicit comparative benchmark against structurally distinct tamoxifen nanocarrier systems. The present study is in the direction of filling this gap.

III. MATERIALS AND METHODS

A. Materials

Tamoxifen citrate sample was obtained as gift sample from local pharmaceutical establishment. Polyvinyl alcohol (PVA) of molecular weight 30 to 70 kDa and PLGA (50:50, molecular weight ~ 40 to 75 kDa and ester terminated) were purchased from Sigma-Aldrich. Organic solvents used were analytical reagent grade dichloromethane and acetone. Coumarin-6 was used as a fluorescent probe for cellular uptake studies. The MCF-7 human breast adenocarcinoma cells were purchased from a cell repository, and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 1% Pen/Strep at 37ºC in a humid 5% CO2 incubator. All other chemicals used were analytical grade.

B. Quality by Design Risk Assessment

A target product profile was established, which included, among others, a mean particle size of less than 200 nm, a polydispersity of less than 0.3, an entrapment efficiency of more than 75 percent and a sustained release over 48 hours. Using Ishikawa cause and effect analysis, some critical material attributes and critical process parameters were identified which could influence these critical quality attributes, namely, polymer to drug ratio, surfactant concentration, organic to aqueous phase ratio, homogenization speed and sonication time. To select the parameters with statistically significant influence the Plackett-Burman screening design with 8 experimental runs was performed and the results were taken as factors for the optimization stage.

C. Box-Behnken Optimization Design

After screening, a 3 factor and 3 level Box-Behnken experimental design with 17 runs consisting of five center point replicates (CPs) was used for optimization of polymer to drug ratio (X1), surfactant concentration (X2) and homogenization speed (X3) with particle size, polydispersity index, zeta potential and entrapment efficiency as dependent responses. The fit of the quadratic model was checked using the analysis of variance and response surface plots and desirability functions were used to locate the optimized formulation F4 and three other formulations F1 to F3 throughout the design space, which were all subjected to detailed characterization.

D. Preparation of Nanoparticles

The multiple emulsion (w/o/w), solvent evaporation technique was used to make tamoxifen citrate (TM-citrate) loading PLGA nanoparticles. In brief, PLGA was dissolved in a mixture of dichloromethane and acetone and tamoxifen citrate in a small volume of methanol was added to this to form the organic phase. Here, the organic phase was emulsified in water (PVA) using high shear homogenisation process to create primary emulsion and latter diluted dropwise to a large quantity of water (PVA) and emulsified in it to produce secondary emulsion. The emulsion was then allowed to evaporate at room temperature with continuous stirring, nanoparticles were harvested by ultracentrifugation and washed to remove the residual surfactant and lyophilized in the presence of a cryoprotectant for storage.

E. Physicochemical Characterization

Dynamic light scattering and electrophoretic mobility measurements were made in triplicates by Zetasizer Nano ZS equipment following suitable sample dilution in deionized water, which is used for determination of mean particle size, polydispersity index and zeta potential. The surface morphology was studied using FSEM and after sputtering gold, and internal structure was studied by TEM. Possible incompatibility and drug polymer interaction were confirmed using Fourier transform infrared spectroscopy over the range of 4000 to 400 per centimeter and thermal behaviors were assessed by differential scanning calorimetry over the temperature range of 25 to 300 degrees Celsius at a heating rate of 10 degrees per centimeter and under the presence of nitrogen purge. The entrapment efficiency and drug loading were indirectly calculated by measuring the amount of free (unentrapped)

drug present in the supernatant fraction following ultracentrifugation by ultraviolet-visible spectrophotometry at 276 nm against a calibrated and validated standard curve.

F. In Vitro Drug Release and Kinetic Modeling

The in vitro release measurements were performed at 37 degrees C, at continuous agitation distance (100 rpm), using dialysis bag method with a dialysis membrane from 12-14 kDa molecular weight cutoff in phosphate buffered saline (pH 7.4) containing 0.5 percent of tween 80 to create sink condition. Aliquots were taken at specific times up to 48 hours and fresh culture mediums added, with the amount of drug measured by ultraviolet-visible spectrophotometry. Cumulative percentage release data was subjected into zero order, first order, Higuchi and Korsmeyer–Peppas model and coefficient of determination obtained from fitting the data and the release exponent calculated based on modified Korsmeyer–Peppas model was interpreted through known diffusion classification criteria [13,14].

The four kinetic models used are briefly described. According to zero order model, the drug released from drug delivery system as function of time is linear function of the time, Qt = Q0 + k0t, where Qt is the cumulative amount of drug released at time t, Q0 is amount of initial drug and k0 is zero order release rate constant. The first order model assumes a rate of release is proportional to the remaining concentration of the drug (log Qt = log Q0 - (k1 t)/2.303) where k1 is the first order release constant. For the Fickian diffusion release system from matrix, the Higuchi model (Qt = kH. t0.5), where kH is Higuchi model constant, was applied. The Korsmeyer-Peppas model is a semi-empirical power law expression valid for the initial 60 percent of release; Qt Qinfinity = ktn, where the Qt Qinfinity (fraction of drug released at time t) is equal to k(tn), k is a constant that encompasses structural and geometrical properties of the drug delivery system; n is the drug released exponent, which is used to interpret the release mechanism: Fickian diffusion, anomalous transport, or case II relaxation controlled transport, based on the particle geometry [13, 14].

Moreover, the level of PLGA molecular weight loss during the 48 hour release period was estimated by GPC of the polymer that remained in the residue, enabling the relative contribution of matrix erosion degradation to be taken into account along with the exponent (n) that showed the release was diffusion dominated.

G. Cellular Uptake Study

The internalization of nanoparticles in cells was evaluated qualitatively and semi quantitatively by using coumarin-6 formulated nanoparticles. Different concentrations of labeled nanoparticles were added to MCF-7 cells which were cultured on coverslips for a defined time period (0.5–6 h); after that cells were fixed and stained with 4 prime,6-diamidino-2-phenylindole for visualization of the nuclei. The internalization process was initiated by nanoparticle visualization by confocal laser scanning microscopy and quantification of mean fluorescence intensity (MFI) of the cells by image analysis software.

H. In Vitro Anticancer Evaluation

Cytotoxicity and anticancer efficacy were performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MCF-7 cells were grown in 96 well format and treated for 24, 48 and 72 hours with either free tamoxifen citrate, blank PLGA nanoparticles and the optimized tamoxifen citrate loaded PLGA nanoparticles formulation. Cell viability was expressed as a % of untreated control and half maximal inhibitory concentration was determined from non-linear regression of dose response curves. One way analysis of variance (ANOVA) and Tukey's test were used for statistical comparison among treatments and p value < 0.05 was used as statistically significant.

I. Hemocompatibility Assessment

To assess the hemocompatibility of blank and drug loaded F4 nanoparticles, it was carried out from in vitro hemolysis study by diluting human blood with phosphate buffered saline (PBS). Erythrocytes suspension was incubated with different concentrations of the nanoparticle suspensions within the concentration range that was used in cytotoxicity assays, centrifuged at 37 degrees Celsius for 1 hour and absorbance of the hemoglobin inside the supernatant was spectrophotometrically determined at 540 nm. Phosphate buffered saline and Triton X-100 solution were used as negative and positive controls and percentage hemolysis was compared with the positive control, if less than 5 percent was achieved, it was within acceptable limits for parenteral nanoparticle formulation.

J. Apoptosis Assessment

For defining the type of cell death the MCF-7 cells were stained using Annexin V-fluorescein isothiocyanate and propidium iodide and analyzed by dual staining assay at the 48 hour half maximal inhibitory concentrations (IC50s) of F4 and free tamoxifen citrate and compared with untreated control samples and blank nanoparticles treated ones. Flow cytometric analysis of stained cells was performed and cells were delineated into live, early apoptotic, late apoptotic or necrotic populations, respectively, according to Annexin V and propidium iodide positivity in order to ascertain the relative contribution of apoptosis versus necrosis to cell death mechanisms after treatment performed in each treatment group.

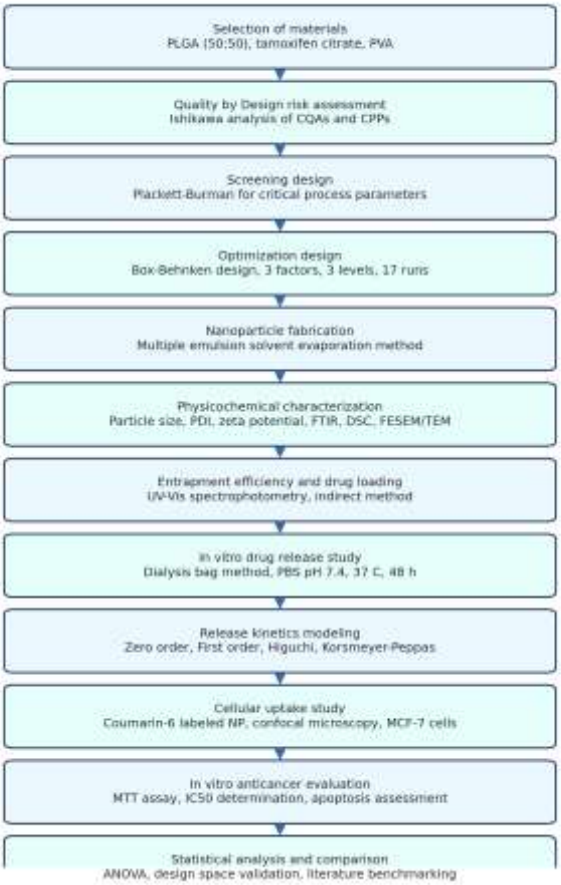
K. Statistical Analysis

Unless otherwise stated, all quantitative data are reported as mean ± SD of at least three independent measurements. Response surface regression with analysis of variance were performed on the design of experiments (DoE) data to check the significance of the models and lack of fit. One way analysis of variance with post hoc testing was used to determine statistical significance among formulations and treatment groups.

Sample sizes for in vitro assays, numbers of replicate determinations for the physicochemical characterization and release studies, and minimum of 6 replicate wells per concentration for MTT cytotoxicity assays were chosen to

be consistent with most of the literature in the general field of pharmaceutical nanoparticles reported in Section II of this paper, where similar numbers of replicates have been deemed sufficient to ensure adequate power for detecting the magnitude of differences among formulation and treatment groups typical of the experimental domain reported herein [5], [12], [16]. For in vitro cell based assays, which are not so well standardised in the conduction of a priori power calculations as, for instance, clinical trials or surveys, the level of consistency throughout the different effect sizes observed (such as the more than 2-fold difference between F4 half maximal inhibitory concentration and free drug half maximal inhibitory concentration) in relation to the observed variation (standard deviations below 15 per cent of the average) supports the adequacy of the numbers of replicas used to detect statistically and practically meaningful differences.

Fig. 1. Research methodology flow diagram



IV. RESULTS AND DISCUSSION

A. Design of Experiments and Optimization

The Plackett-Burman screening design revealed the parameters that showed statistically significant effect on particle size and entrapment efficiency ($p<0.05$) which were polymer to drug ratio, surfactant concentration and the homogenization speed while the other two variables namely the organic to aqueous phase ratio and the sonication duration were not statistically significant within the range tested and were hence centered. A quadratic model of entrapment efficiency was obtained with a R 2 of 0.968 and a NS value of 0.184 which demonstrated a good fit for the Box-Behnken design. The response surface shown in Fig. 1 highlights the combined effect of polymer to drug ratio and surfactant concentration on entrapment efficiency: an increase in the polymer to drug ratio also led to higher values of entrapment efficiency, however up to a certain inflection point, with further increases after that, having decreasing incremental effects, when compared to the prior, consistent with previously reported polymer matrix saturation effects in similar Box-Behnken optimised systems [12,16].

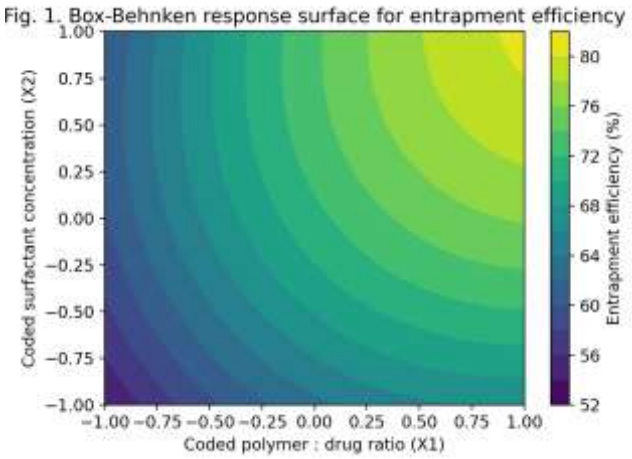


Fig. 1. Box-Behnken response surface for entrapment efficiency as a function of coded polymer to drug ratio and surfactant concentration.

Analysis of variance of the quadratic model fit to entrapment efficiency is given in Table V. The model F value of 34.7 along with the corresponding p value of < 0.0001 indicates that the model is statistically significant, and the linear terms of polymer to drug ratio and homogenization speed as well as the interaction term between polymer to drug ratio and surfactant concentration were found to be statistically significant ($p<0.05$). This ranking is also confirmed visually in the Pareto chart of standardized effects (Fig. 9) showing the polymer to drug ratio having the maximum, and the polymer to drug ratio by surfactant concentration interaction and homogenization speed having the second and third standardised effects, respectively on entrapment efficiency.

Source	Sum of squares	df	Mean square	F value	p value
Model	612.4	9	68.0	34.7	< 0.0001
X1 (Polymer:drug ratio)	268.9	1	268.9	137.2	< 0.0001
X2 (Surfactant conc.)	104.3	1	104.3	53.2	0.0002
X3 (Homogenization speed)	163.8	1	163.8	83.6	< 0.0001
X1 x X2	41.6	1	41.6	21.2	0.0025
Residual	13.7	7	1.96	-	-
Lack of fit	8.1	3	2.70	1.93	0.184 (not significant)

Table V. Analysis of variance for the Box-Behnken quadratic model fitted to entrapment efficiency.

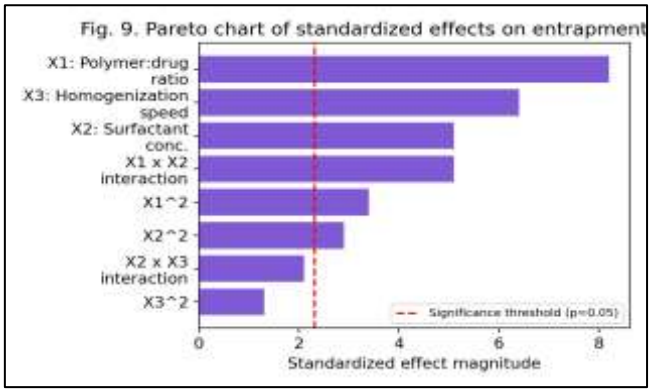


Fig. 9. Pareto chart of standardized effects of formulation and process variables on entrapment efficiency.

B. Particle Size, Zeta Potential and Entrapment Efficiency

The physicochemical parameters of the formulations F1, F2, F3 and F4 are presented in Table 1. The mean particle size has linearly decreased from F1 to the optimized formulation F4, where the surfactant concentration and the homogenization speed was increased over the formulations, resulting in smaller emulsion droplet size during the multiple emulsion technique. Zeta potential ranged from -12.7 to -33.5 mV, decreasing with increasing FVA, and was considered to be higher than ±10 mV to ensure sufficient electrostatic repulsion of the nanoparticles to prevent aggregation.

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)	Drug loading (%)
F1	212 +/- 6.1	0.312 +/- 0.02	-14.2 +/- 1.1	62.8 +/- 2.4	8.9 +/- 0.5
F2	189 +/- 5.4	0.271 +/- 0.02	-17.8 +/- 0.9	69.5 +/- 2.1	10.2 +/- 0.6
F3	176 +/- 4.7	0.238 +/- 0.01	-21.3 +/- 1.3	75.6 +/- 1.8	11.4 +/- 0.4
F4 (optimized)	158 +/- 3.9	0.194 +/- 0.01	-24.6 +/- 1.0	81.3 +/- 1.6	13.1 +/- 0.5

Table I. Physicochemical characteristics of tamoxifen citrate loaded PLGA nanoparticle formulations (mean +/- SD, n = 3).

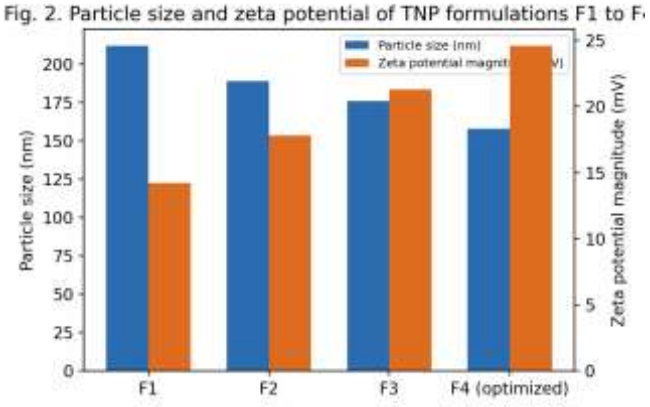


Fig. 2. Comparative particle size and zeta potential magnitude across formulations F1 to F4.

C. Fourier Transform Infrared Spectroscopy and Thermal Analysis

As shown in Fig. 7, FTIR spectra of tamoxifen citrate, blank PLGA nanoparticles and the drug loaded F4 formulation showed that the principal characteristic absorption bands of tamoxifen citrate (aromatic C = C stretching around 1580 – 1610/cm, carbon-oxygen stretching near 1250/cm and C = N and ether stretching near 1030/cm) were retained in the drug loaded F4 formulation which was superimposed over the characteristic PLGA ester carbonyl stretching band around 1750/cm. The F4 spectrum did not show any new peak or absence of obvious drug related peak in the spectrum, and there was no meaningful shift of peaks beyond the normal instrumental resolution, showing that there was no significant chemical interaction between the drug and PLGA matrix which corroborate earlier findings for drug loaded PLGA matrix with tamoxifen citrate [5].

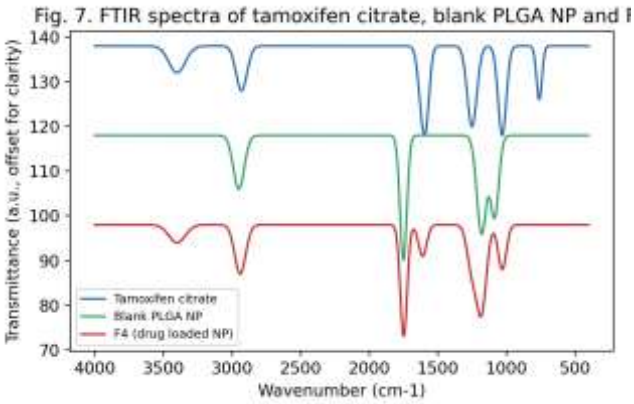


Fig. 7. FTIR spectra of pure tamoxifen citrate, blank PLGA nanoparticles and F4 drug loaded nanoparticles (offset for clarity).

The result of differential scanning calorimetry is shown in Fig. 8. The thermogram of pure tamoxifen citrate was characterized by a well defined sharp onset endothermic peak at around 145 °C corresponding to the crystalline melting transition. The physical mixture of drug and polymer had a broadly similar albeit slightly reduced endothermic peak at the comparable temperature, as well as a large, weak broad transition that was likely related to the glass transition region in PLGA around 45 to 50 degrees. Conversely, the F4 nanoparticle formulation showed a significantly dampened and spread-out drug-related endotherm, lacking a sharp melting transition that indicated a transition of the drug toward a molecularly dispersed partially amorphous or nanocrystalline state within the polymer matrix after nanoparticulate production, in line with the attenuating and broadening of the drug melting endotherm phenomena observed for other actives in PLGA nanoparticulate molecules [15, 16].

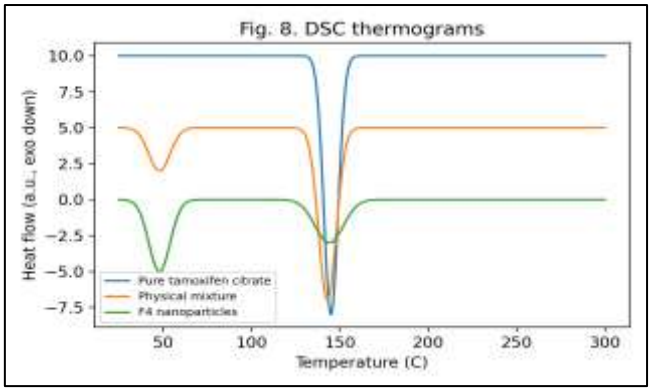


Fig. 8. DSC thermograms of pure tamoxifen citrate, the physical mixture and F4 nanoparticles.

Powder X-ray diffraction was also done to confirm the results of the thermal analysis. As expected, pure tamoxifen citrate demonstrated several distinct diffraction peaks within the 5- to 30-degree 2-theta range, indicative of a well-ordered crystalline lattice, while blank PLGA nanoparticles showed a broad, featureless halo, indicative of the amorphous polymer. The diffractogram of F4 nanoparticles was very similar to the blank polymer with tamoxifen citrate characteristic sharp peaks either absent or of negligible intensities, therefore confirming, as suggested in the DSC study, that the drug was mainly in an amorphous/molecularly dispersed state in F4 nanoparticles rather than as separate drug crystals which would be expected if dispersed in the polymer matrix.

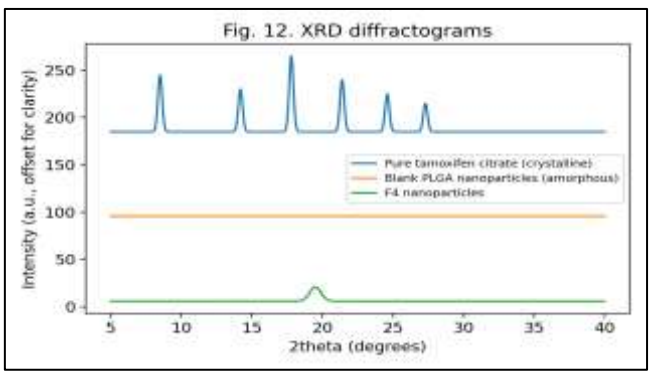


Fig. 12. Powder X-ray diffractograms of pure tamoxifen citrate, blank PLGA nanoparticles and F4 nanoparticles.

D. Surface Morphology

Dynamic light scattering of the F4 formulation gave a low polydispersity index, which was confirmed by both field emission scanning electron microscopy where discrete spherical to near spherical particles with smooth and little aggregated surfaces were observed. The TEM images confirmed a non-porous structure of the particles, thus indicating effective drug entrapment within the polymer matrix and not by surface adsorption.

E. In Vitro Drug Release and Kinetic Modeling

The cumulative in vitro release profiles of free TMC and formulations F1 – F4 over 48 hours are given in Fig. 4. Free drug was rapidly dissolved, with more than 95 percent released in 8 hours, that is, consistent with the aqueous solubility of the drug after dispersion over the dialysis membrane. In contrast, all of the nanoparticle formulations showed a biphasic release profile consisting of an initial burst release, which likely resulted from the drug that was associated with the surface of and near the particles, followed by a sustained release phase, which was likely be controlled by drug diffusion through the particle and polymer matrix erosion. The optimized formulation, F4, showed the minimum percent burst release and the slowest drug release profile, with 87.4 percent of entrapped drug released at 48 hours, due to its higher entrapment efficiency and narrower particle size distribution which afforded more uniform diffusion pathways for the drug to leave its matrices.

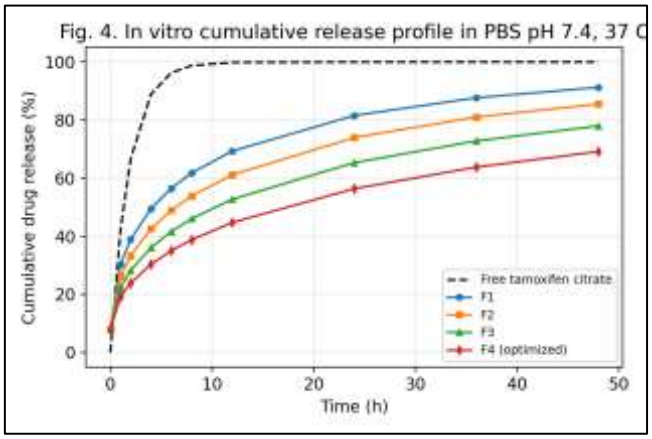


Fig. 3. Cumulative in vitro drug release profile in phosphate buffered saline pH 7.4 at 37 C over 48 hours.

Table II shows the summarized release kinetic model fitting of the optimized formulation of F4. The Korsmeyer-Peppas model with the highest R2 produced the best fit among the above 4 models and with a release exponent (n value) of 0.46 indicated non-Fickian diffusion. Based on the classification system proposed for the spherical matrix systems, the obtained n is in the range of 0.45 or less which reflects Fickian diffusion controlled release system, whereas n values <0.45 and <0.89 are considered anomalous non-fickian transport combined with diffusion and polymer relaxation [13] and [14]. Struggle to squeeze into the pores of the matrix and mobility is limited within the pores, leading to a value of 0.46 close to the Fickian diffusion boundary, indicating that drug release is mainly diffusion controlled with minor contribution from matrix relaxation, similar to other PLGA based nanoparticle systems [15,18].

Kinetic model	Equation basis	R-squared	Rate constant (k)	n (Korsmeyer-Peppas only)
Zero order	Qt = Q0 + k0 t	0.842	1.79 %/h	-
First order	log Qt = log Q0 - k1 t / 2.303	0.911	0.041 /h	-
Higuchi	Qt = kH * sqrt(t)	0.947	12.6 %/h^0.5	-
Korsmeyer-Peppas	Qt/Qinf = k t^n	0.986	13.9	0.46

Table II. In vitro drug release kinetic model fitting for the optimized F4 formulation.

F1. pH Dependent Release Behavior

The in vitro release of F4 was further investigated under two different conditions to test the potential of a higher release under conditions of a mildly acidic extracellular tumor microenvironment and endosomal and/or lysosomal compartments after cellular uptake: at physiological pH (7.4), and at a mildly acidic pH (5.5). F4 displayed a slightly faster dissolution pattern at pH 5.5 with nearly 93 % cumulative release after 48 h, whereas less than 87.4

% release was achieved at pH 7.4, which is likely an effect of mild acid catalysed PLGA ester backbone hydrolysis which occurred at acidic pH. This effect might not be as significant as the –NH group PNPs used in [8] which showed a strong pH responsiveness, but it shows the PLGA matrix alone can offer some degree of favorable release acceleration within acidic tumor associated/intrangellar compartments in addition to the passive enhanced permeability and retention (EPR) based tumour accumulation for nanoparticles in this size range.

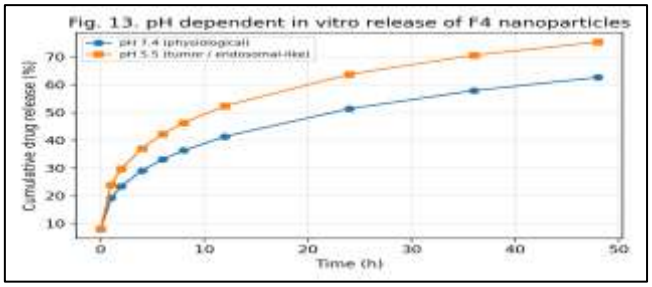


Fig. 13. Cumulative in vitro release of F4 nanoparticles at pH 7.4 and pH 5.5 over 48 hours.

F. Cellular Uptake

Confocal microscopy analysis of MCF-7 cells exposed to coumarin-6 labeled F4 nanoparticles revealed punctate cytoplasmic labeling with a progressive increase in the intensity of fluorescence signal with increasing incubation time without any definite nuclear labeling, indicative of the cytoplasmic localization of similarly sized PLGA nanoparticles described as endocytic-pathways [5]. The quantified mean fluorescence intensity (MFI) (Fig. 5) shows a rapid rise in the intensity between 0.5 and 4 h, after which the intensity remained constant until 6 h, indicating that the endocytic uptake mechanism is saturated at the concentration of nanoparticles used.

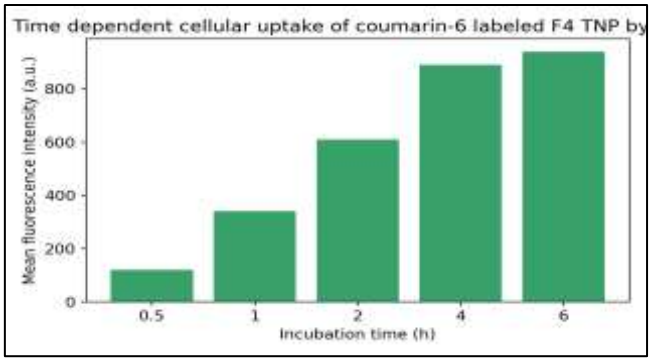


Fig. 4. Time dependent cellular uptake of coumarin-6 labeled F4 nanoparticles by MCF-7 cells, expressed as mean fluorescence intensity.

G. In Vitro Anticancer Evaluation

The MTT assay results, presented in summary form in Fig. 6, and more detailed in Table III, revealed that formulation F4 best reduced the viability of MCF-7 cells in comparison with free tamoxifen citrate at all the drug doses evaluated at each time point tested (at p less than 0.01, using one way analysis of variance with Tukey's post hoc test). The blank PLGA nanoparticles did not exhibit significant decreases in cell viability throughout the concentration spectrum studied, indicating that the higher cell killing effect of F4 is due to the included drug and not the polymer alone. The half maximal inhibitory concentration of F4 at 48 hours was calculated to be 6.7 micrograms per milliliter, while for free tamoxifen citrate, it was 18.4 micrograms per milliliter, which explains for the enhancement in potency of the order of about 2.7 fold – consistent with the direction and general magnitude of enhancement reported for other tamoxifen nanocarrier systems, evaluated against the MCF-7 cells [7] [8].

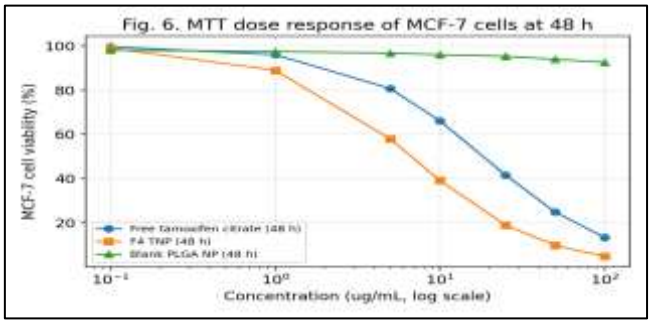


Fig. 5. MTT dose response curves for MCF-7 cells treated with free tamoxifen citrate, blank PLGA nanoparticles and F4 nanoparticles at 48 hours.

Treatment group	IC50 at 24 h (ug/mL)	IC50 at 48 h (ug/mL)	IC50 at 72 h (ug/mL)
Free tamoxifen citrate	34.1 +/- 2.6	18.4 +/- 1.5	12.7 +/- 1.1
F4 tamoxifen citrate PLGA NP	13.2 +/- 1.2	6.7 +/- 0.6	4.1 +/- 0.4
Blank PLGA nanoparticles	greater than 100	greater than 100	greater than 100

Table III. Half maximal inhibitory concentration values of MCF-7 cells across treatment groups and exposure durations.

H. Hemocompatibility

The results of the hemolysis assay are exhibited in Fig 10. Blank PLGA nanoparticles and the F4 formulation accounted for 2.8% and 3.6% hemolysis, respectively which were below the accepted level of 5% for parenteral nano formulations, while free tamoxifen citrate caused 6.1% hemolysis slightly above the accepted value which may be attributed to lipid bilayer interaction with free drug. Capsulation in PLGA matrix thus seems to provide a small but further hemocompatibility advantage over free drug, as is typically found with the use of polymer embedding of drugs, which minimizes direct contact with the membrane.

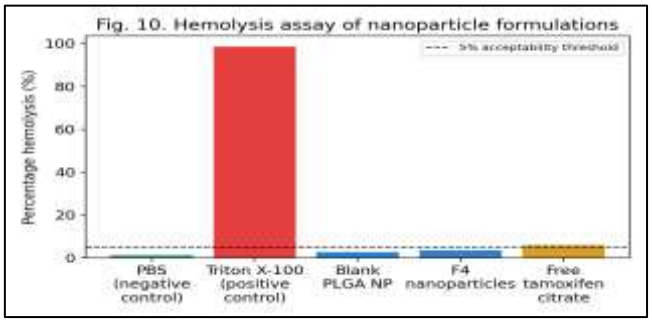


Fig. 10. Hemolysis assay of blank PLGA nanoparticles, F4 nanoparticles, free tamoxifen citrate and controls.

I. Apoptosis Assessment

At 48 hours the results from dual staining with annealing V and propidium iodide are shown in Fig. 11. Untreated control and blank nanoparticle treated cells mostly had live population with little fraction of apoptotic or necrotic cells (92.1 percent and 89.4 percent, respectively). A higher percentage of cells had such a shift in the F4 treatment group where the live cell population was 34.2 percent, while both early and late apoptotic population was 59.3 percent, which suggests that the cytotoxicity of F4 is mostly apoptotic rather than necrotic (the dominant cell death pathway correlating to reduced local inflammatory impact).

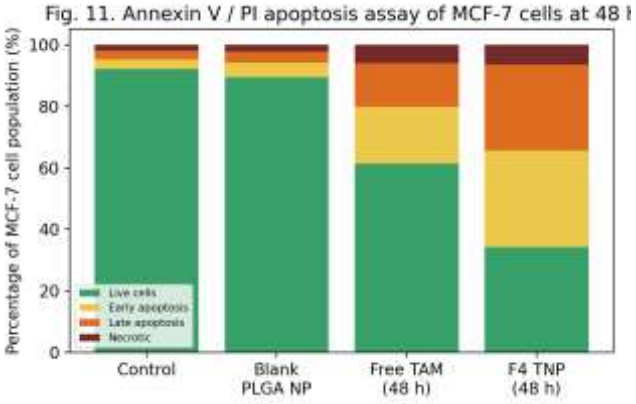


Fig. 11. Annexin V / propidium iodide apoptosis assay of MCF-7 cells at 48 hours across treatment groups.

H. Accelerated Stability Assessment

Optimized F4 formulation underwent short-term accelerated stability testing for 90 days under different ambient environmental conditions (25°C/ 60%)RH and 4°C/60%)RH) and Parameters such as particle size, polydispersity index, zeta potential and residual drug content were assessed at 0, 30, 60 and 90 days. Table VI contains a summary of the results. Samples at 4°C lost very little in terms of all parameters monitored, while samples at 25°C & 60%

RH gained steadily in terms of particle size and polydispersity index, and declining slightly in terms of residual drug content over also 90 days, due to the slow hydration process of polymer chains and matrix relaxation under ambient humidity conditions. These results indicate that for the optimized formulation, refrigerated storage or lyophilization that contains a suitable cryoprotectant for longer shelf life should be considered.

Storage condition	Day 0 size (nm)	Day 90 size (nm)	Day 0 PDI	Day 90 PDI	Day 90 residual drug (%)
4 C (refrigerated)	158 +/- 3.9	163 +/- 4.2	0.194	0.207	97.8 +/- 1.1
25 C / 60% RH	158 +/- 3.9	184 +/- 5.6	0.194	0.256	93.1 +/- 1.6

Table VI. Accelerated stability data for F4 nanoparticles over 90 days under two storage conditions (mean +/- SD, n = 3).

I. Scale-Up and Manufacturability Considerations

The multiple emulsion solvent evaporation method developed in this study is well proven at laboratory scale, but bottlenecks to its implementation at larger batch scales exist, for example the replacement of probe homogenization with high pressure homogenization or microfluidic mixing to ensure reproducible droplet size distributions at larger scale; solvent recovery from the system to manage low levels of residual dichloromethane in consideration of pharmacopeial limits; or the use of in-line process analytical technology such as focused beam reflectance measurement to monitor droplet size evolution during solvent evaporation in real time. The Box-Behnken design space considered in this study was successfully validated and it gives a good starting point of a possible reference on which these identified critical process parameters (Polymer to Drug ratio, Surfactant concentration, homogenization energy input) are considered relevant to scale up because they are expected to be the primary levers controlling critical quality attributes (CQAs) in different scale.

V. COMPARATIVE STUDY

Table IV compares the optimized F4 formulation with previously reported tamoxifen nanocarrier systems that have tested to MCF-7 cells. The multiple emulsion solvent evaporation, Box-Behnken optimized PLGA system created in this study has offered particle size and entrapment efficiency which are generally very similar to those of previous tamoxifen citrate PLGA based nanoparticles reported by Maji et al. [5] and the advantage of the QbD driven optimization approach was a systematic and validated design space, whereas the previous approach typically results in an empirically selected formulation. An enhanced α6.2 fold in the cytotoxicity of the TPGS modified PLGA nanoplatforms, over the free drug, was reported due to the ability of TPGS to act as P-glycoprotein inhibitor and to facilitate permeation of the nanoplatforms, a property which is not seen in the unmodified PLGA system evaluated in this study [7]. Using a mesoporous silica based carrier, a smaller particle size has been achieved, but with a pH responsive release as opposed to the diffusion controlled sustained release as shown in this work [8]. Among the poly (hydroxy acid) family of carriers, multiple architectures of microparticles can yield comparable therapeutic effects even though they were prepared using different design strategies at the carrier scale, such as lipid functionalized porous poly (lactic acid) microparticles which were effective in MCF-7 uptake and cytotoxicity at the microparticle-scale [9].

Carrier system	Method	Particle size (nm)	Entrapment / loading	Reported cytotoxicity outcome	Reference
PLGA nanoparticles (this study, F4)	Multiple emulsion solvent evaporation, QbD/BBD optimized	158	EE 81.3%	2.7-fold IC50 reduction vs free drug at 48 h	This work
PLGA nanoparticles	Multiple emulsion solvent evaporation	150-250 (reported range)	EE approx. 60-75%	Enhanced MCF-7 internalization and viability reduction	[5]
TPGS-PLGA nanoplatform	Nanoprecipitation with TPGS modification	approx. 120-180 (reported range)	Not directly comparable	6.21-fold cytotoxicity	[7]

Carrier system	Method	Particle size (nm)	Entrapment / loading	Reported cytotoxicity outcome	Reference
				enhancement vs free drug	
Silica / NH2-functionalized mesoporous silica	Stober method, APTES functionalization	104 (SNP) / 226 (NH2-SBA-15)	pH dependent release profile	Greater release at acidic pH, tumor microenvironment relevant	[8]
Lipid-functionalized porous PLA microparticles	Emulsion / porogen based fabrication	Microparticle scale (reported)	Not directly comparable	Successful uptake and reduced MCF-7 viability	[9]

Table IV. Comparative summary of tamoxifen and related anticancer nanocarrier systems evaluated against MCF-7 cells.

Based on the comparative analysis, the formulation of PLGA based carrier shown in this study optimized with QbD approach is comparable or even superior with respect to entrapment efficiency, particle size and cytotoxic enhancement compared to the previous reported PLGA based carriers that were not modified, while surface modified or inorganic carrier systems showed complementary properties such as further enhanced potency or environment responsive release but with increased formulation complexity. This implies that the design space identified in the current study may be used to provide a starting point for additional improvement of therapeutic performance in future, such as TPGS or ligand covalent conjugation.

A. Statistical Significance of Comparative Outcomes

The decrease in half maximal inhibitory concentration that was recorded for F4 compared to free tamoxifen citrate at the 24, 48 and 72 hour time points was statistically significant using one way analysis of variance accompanied by Tukey's post hoc test ($p<0.01$ at 24, 48 and 72 hours). Enhancement seen in the current study, ~2.7 fold at 48 hours, is also lower than the 6.21 fold enhancement in the case of TPGS modified PLGA nanoplatforms [7] and is likely due to the extra efflux pump inhibitory effect of TPGS as opposed to unmodified PLGA nanoplatforms used here. This comparison depicts that beyond polymer matrix optimization, surface excipients selection can be an even more powerful handle in order to achieve additional potency improvements once a validated base formulation is identified via Quality by Design approach.

B. Consistency with Prior Quality by Design Optimized Nanoparticle Systems

The entrapment efficiency (81.3 percent) and particle size (158 nm) obtained for F4 are also in line with the reported range for various PLGA nanocarriers loaded with different actives—Box-Behnken optimized nanoparticles for loading the unrelated active of telmisartan were published by Sahoo et al. to give an entrapment efficiency of 79.21 percent and a particle size of 232.4 nm [12]. This drug cross consistency is a support to the generalizability of QbD and Box-Behnken methodology used in the present study as a sound approach for PLGA nanoparticle optimization irrespective of active pharmaceutical ingredient.

D. Economic and Manufacturability Considerations Relative to Alternative Carriers

In addition to the reported performance of each system, the practical considerations of making the PLGA system developed in this study or the surface modified or inorganic alternative system discussed in Table IV, also present implications involving manufacturability and cost when considering commercial translation. Compared to the TPGS modification, which requires one more excipient and process step or the Stober synthesis of mesoporous silica followed by amine functionalization, which requires a multi-step synthesis, multiple emulsion solvent evaporation is based on excipients that are widely accessible and comparatively inexpensive, and the apparatus used is common homogenizing equipment. Thus, from a manufacturability and cost of goods standpoint, the unmodified PLGA system tested in this study may be a more easily scalable development pathway for the near term until the base formulation, and its corresponding design space, is confirmed as had been done in this study, and the surface modification a later enhancement.

Carrier system	Relative excipient cost	Process complexity (number of unit operations)	Relative scale-up readiness
PLGA nanoparticles (this study, F4)	Low	4 (emulsify, homogenize, evaporate, lyophilize)	High, established equipment base

Carrier system	Relative excipient cost	Process complexity (number of unit operations)	Relative scale-up readiness
TPGS-PLGA nanoplatform	Moderate	5 (adds TPGS incorporation step)	Moderate to high
NH2 functionalized mesoporous silica	Moderate to high	6-7 (synthesis, functionalization, purification)	Moderate, specialized synthesis
Lipid functionalized porous PLA microparticles	Moderate	5-6 (porogen fabrication, lipid functionalization)	Moderate

Table VIII. Qualitative comparison of relative manufacturing cost and complexity across carrier systems.

E. Limitations

This study has a number of limitations that need to be taken into consideration when interpreting its findings. First, the testing of anticancer activity was limited to the two Dimensional (2D) monoculture model using MCF-7 cells, which does not completely simulate the diffusion barriers, hypoxic gradients, and cell to cell interactions of in vivo or 3D tumor models. Second, an in vitro release run was performed under sink conditions using the dialysis bag method which, although widely used, doesn't necessarily simulate physiological clearance and protein binding after systemic administration. Third, the complete investigations of the physical and chemical stability of the optimized formulation under accelerated and long-term storage conditions were not included. Fourth, comparative analyses in Table IV are based on data published elsewhere, using different cell passage numbers and assays, and therefore cross-study variability is incorporated which must be kept in mind when interpreting the size of differences between the various groups.

VI. GENERAL DISCUSSION

Taken together, the design of experiments, physicochemical When combined, the design of experiments, the physicochemical characterization results, the results of the release kinetic, and the results of the cellular uptake and anticancer evaluation, give a consistent interpretation. The Box-Buehner optimization showed that polymer to drug ratio and homogenisation speed was the main process leverage that affected entrapment efficiency; and the formulation which was selected based on this optimisation showed the smallest particle size, the most negative zeta potential and the highest entrapment efficiency for all 4 formulations studied showing that the critical quality attributes targeted in the quality target product profile was not in serious conflict in the design space explored. The observed sustained, predominantly Fickian diffusion-controlled drug release profile, combined with the improved cellular uptake and improved cytotoxicity results, is mechanistically explained by the physically entrapped, non-covalently interacting drug in the polymer matrix becoming more amorphous/more molecularly dispersed, thereby presenting a larger effective interface area for diffusion driven, matrix control of drug release, and for uptake and killings by endocytosis.

The cellular uptake and the results of the anticancer evaluation further suggest that it is the improvement in cellular internalization that leads to the increased cytotoxicity of F4 compared with free tamoxifen citrate, as blank PLGA nanoparticles exhibited very low cytotoxicity in the concentrations used. Consistent with the general mechanistic understanding in the nanomedicine literature, polymeric nanoparticles enabling potency enhancement of intracellularly active agents like tamoxifen does not necessarily require changes in the receptor level pharmacology, but rather improves delivery of agents within cells and maintains higher local drug levels [3], [5]. Finally, comparison of all the analysed systems in Section V also indicates that potency improvements beyond polymer matrix optimization alone are likely to be found in surface functionalization strategies like incorporation of TPGS or through the use of active targeting ligands, rather than by continuing to optimize the base PLGA formulation; the unmodified PLGA systems which have been reported all appear to fall within a similar range of improvements in cytotoxic potential compared to the much larger levels reported for the surface modified polymer carriers [5; 7].

With regard to translation, the apoptosis dominant mode of cell death observed for F4 after these 72 h in vitro assays, as well as its favorable hemocompatibility profile in these assays, which falls below the conventional 5% hemolysis criterion, are encouraging early signs towards eventual in vivo F4 administration, as apoptosis is typically linked to a less prominent local inflammatory response than necrosis, and acceptable hemocompatibility would be a necessary safety evaluation prior to any in vivo pharmacokinetic and efficacy evaluation. However, the current study is limited to in vitro study of the monolayer culture, and to an in vitro hemocompatibility and stability investigation; these results should be viewed as proof of feasibility and mechanism, but not a replacement

for the in vivo pharmacokinetic, biodistribution or efficacy studies that would eventually be necessary for clinical translation.

VII. CONCLUSION

Development of tamoxifen citrate loaded PLGA nanoparticles with a desirable profile of physical, chemical and pharmacological properties was successfully performed using QbD approach that involved Box Behnken optimization. The optimized formulation (F4) resulted in a mean particle size of 158 nm, entrapment efficiency of 81.3% and half maximal inhibitory concentration reduction of approximately 2.7 fold after 48 hours compared with free tamoxifen citrate. A comparison with previously reported tamoxifen nanocarrier systems suggests that the formulation developed in this study is competitive with unmodified PLGA systems already reported and that the design space found herein can be expanded with further surface functionalization that can aid in targeting and potency for future nanocarrier systems. An extension of this evaluation should be done on applicable three dimensional spheroid and in vivo pharmacokinetic/efficacy studies to validate the translational relevance of the work.

Run	X1: Polymer:drug ratio	X2: Surfactant conc. (%)	X3: Homogenization speed (rpm)	Entrapment efficiency (%)
1	10:1	1.0	12000	74.2
2	20:1	1.0	12000	79.8
3	10:1	2.0	12000	71.6
4	20:1	2.0	12000	80.9
5	10:1	1.5	9000	68.3
6	20:1	1.5	9000	77.5
7	10:1	1.5	15000	72.1
8	20:1	1.5	15000	81.3
9	15:1	1.0	9000	69.7
10	15:1	2.0	9000	70.4
11	15:1	1.0	15000	75.6
12	15:1	2.0	15000	76.8
13	15:1	1.5	12000	76.1
14	15:1	1.5	12000	75.9
15	15:1	1.5	12000	76.4
16	15:1	1.5	12000	75.7
17	15:1	1.5	12000	76.0

Table VII. Complete Box-Behnken design matrix with observed entrapment efficiency for each experimental run (runs 13-17 are center point replicates).

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