ORIGINAL PAPER

PROCESS OPTIMIZATION USING QUALITY BY DESIGN (QBD) APPROACH OF A GENTAMICIN LOADED PLGA BIOCOMPOSITE

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Abstract. *Osteomyelitis continues to be a major concern when orthopedic surgery is performed. Orthopedic infections have an incidence of 5% to 10% but their management proves to be quite difficult due to both biofilm formation and limited access of the drug to the infected area when systemic treatment is employed. The aim of the study was to optimize the synthesis process of a gentamicin loaded poly(-lactic-co-glycolic) acid (PLGA) based biodegradable composite by varying parameters that affect both efficiency encapsulation and nanoparticle size. Furthermore, a kinetic study was conducted to study the biodegradation process of the polymer. Gentamicin loaded PLGA nanoparticles were obtained using the double emulsion technique which allows the variation of several factors such as gentamycin concentration, PLGA concentration, buffer concentration and stirring speed. Out of the four factors evaluated, gentamicin concentration had the highest impact on both encapsulation efficiency and nanoparticle size. A few relevant interactions between factors were also registered.*

Keywords: *kinetic study; biodegradation; PLGA-gentamicin; full factory design; MODDE.*

1. INTRODUCTION

Poly (lactic-co-glycolic acid) (PLGA) is a polymer approved by the Food and Drug Administration for human studies [1]. It is a synthetic polymer that is both biocompatible and biodegradable. PLGA has a number of biomedical applications as dermic graph and implant material. Furthermore, it is used in the formulation of drug delivery systems in order to achieve targeted delivery [2]. Both drugs (antibiotics, chemotherapeutics, anti-inflammatory medication) [3] and high molecular weight proteins have been encapsulated into PLGA [4].

This wide range of biomedical applications has led to an increased attention towards this polymer and an exponential growth in the number of publications in the last 15 years. PLGA is a lactic acid and glycolic acid copolymer. It may degrade in water or biological fluids as a result of the hydrolytic bond cleavage of the ester groups [1, 5].

PLGA degradation time is low when the number of glycolic units is high. The polymer with a lactic acid: glycolic acid monomer ratio of 65:35 exhibits a degradation time of approximately 60 days [1, 2].

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Literature describes several methods for encapsulating antibiotics into PLGA microspheres. The synthesis method may be selected after considering several parameters such as length of treatment with a specific antibiotic and purpose of biomaterial synthesis. Furthermore, the hydrophilic/ hydrophobic nature of the drug may help to determine which method of synthesis is elected [6].

Methods commonly chosen for synthesizing PLGA-antibiotic biomaterials include solvent evaporation technique and nanoprecipitation. Water in oil in water (W/O/W) double emulsion method is used for encapsulating hydrophilic drugs that include gentamycin whereas the oil in water (O/W) simple emulsion is mainly used for lipophilic drugs [6].

Both antibiotic and surgical treatment are required to treat chronic osteomyelitis which is a severe bone infection mainly determined by Staphylococcus aureus. Treatment of implant related bone infections proves to be very difficult due to low vascularization which leads to a reduced antibiotic quantity available at the infection site [7].

Choosing the correct antibiotic remains empiric as some medical aspects are still poorly understood. Table 1 exhibits some of the antibiotics used and the length of treatment.

Table 1. Antibiotic and route of administration reported in literature as treatment for orthopaedic infections.

Author (reference)	Antibiotic	Bacteria	Length of treatment (number of weeks) /route of administration
Slama et al. [8]	Ciprofloxacin	*Gram-negative*	11/oral
Siebert et al. [9]	Carboxypenicillin/ clavulanic acid	*Staphylococcus aureus*	4/intravenous
Li and Hu [10]	Gentamycin	*Staphylococcus aureus*	Local treatment / Implant
El-Ghannam et al. [11]	Gentamycin	*Gram-negative*	Local treatment / silica-calcium phosphate nanocomposites with gentamycin-based implant
Stemberger et al. [12]	Gentamycin		Local treatment / Collagen-gentamycin biocomposite based implant

Researchers found that the most effective treatment strategy is to use antibiotic loaded biocomposite based implants which contain the drug either attached to the surface or incorporated in bulk [13]. Synthetic polymers such as poly(-methylmethacrylate) or poly(D,L-lactic-co-glycolic acid) [14] and natural polymers that include collagen, cellulose, chitosan [15] have been used either in association or not with hydroxyapatite [16, 17] for the synthesis of such implants. Moreover, implants may include antibiotics such as ciprofloxacin, gentamycin, oxacillin, or vancomycin [18, 19].

The scope of the study was to evaluate and enhance the synthesis process of gentamicin loaded PLGA nanoparticles. The nanoparticles were synthesized using the double emulsion technique. The solvents used and stirring rate determine, in the double emulsion technique, the encapsulation efficiency and particle size [5, 20, 21]. Four variables, namely gentamycin concentration, PLGA concentration, buffer concentration and stirring rate were evaluated in relation to nanoparticle size and encapsulation efficiency. A Full Factory (2 levels) interaction model experimental design was implemented using quality by design (QbD) approach, which is widely promoted by the Food and Drug Administration and the International Conference on Harmonization (ICH) [22, 23]. Furthermore, a degradation study was conducted to evaluate the PLGA 65:35 used for the synthesis of gentamicin loaded nanoparticles.

2. MATERIALS AND METHODS

2.1. MATERIALS

65:35 PLGA was purchased from Merck, Darmstadt, Germany, while gentamycin was acquired from Carl Roth GmbH & Co KG (Karlsruhe, Germany). Dichloromethane (DCM), polyvinyl alcohol (PVA) and hydrochloric acid (HCl) were all purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.2. METHODS

2.2.1. Gentamicin loaded PLGA based implant synthesis

Various quantities of gentamycin (10 to 150 mg, factor X_1) were dissolved in 400 µL 0.5% PVA solution. The pH of the solution was adjusted using monosodium phosphate and disodium phosphate 0.1M - 0.3M, factor X_3, solution W_1. The solution was then added to the oily phase (DCM) which contained a specific quantity of PLGA dissolved (100 to 200 mg, factor X_2). The two solutions were stirred at 4500 rpm using a Heidolph Silent Crusher in order to obtain the primary emulsion (W_1/O). This emulsion was then added to an aqueous phase (W_2) and stirred constantly. The secondary aqueous phase was obtained by dissolving PVA in water at 80 ^oC, resulting in a 1% solution (w/w). The primary solution was injected into the secondary phase. The organic solvent was evaporated by stirring the double emulsion at a rate varying between 500 and 1500 rpm (factor X_4), for 3 to 4 hours at room temperature.

The suspension obtained was submitted to a lyophilization process (freezing at -45°C for 10 hours, void at 0.014 mbar for 12 hours and heated under void 0.014 mbar for 10 hours at 20°C). The composite was then cut into 6 mm diameter discs.

2.2.2. Encapsulation efficiency

The method used for evaluating the encapsulation efficiency was detailed in a previous study [24, 25]. Briefly, 5 mg gentamicin loaded PLGA nanoparticles were suspended in 1 mL borate buffer, pH = 10. Then, the suspension was filtered through a 0.45 µm porous membrane and 0.25 mL solution was mixed with 0.5 mL FMOC-Cl in order to be injected into the chromatographic system. A Thermo Finnigan Surveyor HPLC System with DAD detector was used to conduct the study.

2.2.3. Particle size determination

Particle size was assessed by dynamic light scattering (DLS) using a Brookhaven 90Plus Particle Size Analyzer equipped with a solid-state laser. All measurements were conducted at room temperature.

A Full Factory (2 levels) interaction model experimental design, implemented by Modde software, version 12.1 (Sartorius Stedim Data Analytics AB, Umeå, Sweden), was developed for the process optimization using quality by design (QbD) approach. Four factors (gentamycin concentration, PLGA concentration, pH, stirring speed) were the independent

variables used in the screening step. Two responses (efficiency, size) were the dependent variables (Table 2).

Table 2. Independent and dependent variables of the experimental design.

Variables	Level		
	-1	0	1
Independent variables (factors)			
Gentamycin concentration (X_1)	10	80	150
PLGA concentration (X_2)	100	150	200
Buffer concentration (pH) (X_3)	0.1	0.2	0.3
Stirring speed (X_4)	500	1000	1500
Dependent variables (responses)			
Efficiency (Y_1)			
Size (Y_2)			

The Design of Experiment (DoE) approach allowed us to understand the influence of the variables over the process of gentamicin-PLGA preparation. The fitting of the experimental data with the experimental design was completed through multiple linear regression (MLR) by using the following statistical parameters: R^2, Q^2, validity indicator, reproducibility indicator [26]. R^2 (percent of the variation of every response explained by the model) was measured and a good model is considered when R^2 is large. The value of Q^2 indicates how well the model predicts new data. The validity of the interaction model is measured using Model Validity indicator and it must be larger than 0.25. Reproducibility was assessed to check the variation of the responses under the same conditions, and it must be above 0.85. The condition number was used to evaluate the performance, or our experimental design and it must be less than three for a very good screening design. ANOVA test was applied to check for the model's validity, showing if the variance of the results could be influenced by modifications of the formulation factors, or if it represents a variance determined by experimental errors. Based on the screening investigation, the DoE model was further used to generate a design space for an optimal formulation.

2.2.4. Kinetic biodegradation study - Sample preparation for the degradation study

The PBS solution was obtained by dissolving 8 g sodium chloride, 1.38 g disodium phosphate, 190 mg monopotassium phosphate and 200 mg sodium azide; the pH was adjusted to 7.44 using hydrochloric acid. PLGA films were obtained by dissolving the polymer into DCM under constant stirring for 90 minutes at room temperature. Then, they were poured into a glass mould and dried in an oven. Each disc was then added to a reagent bottle containing 20 mL PBS and maintained at 37°C in an autoclave. The degradation methodology was adapted from Vey et al [27].

2.2.5. Potentiometric investigation

The pH was determined using a Consort multi-parameter Analyser, after careful calibration of the apparatus with three buffer solutions at pH values of 4.0, 7.0 and 10.

2.2.6. Mass loss and water uptake determination

Samples were weighed before being introduced in PBS. Then, the samples were taken out of the solution at predetermined times and weighed after 30 minutes. After the experiment was completed, the samples were removed, washed with distilled water and dried in an autoclave at 100°C for 72 hours.

3. RESULTS AND DISCUSSION

3.1. THE FULL FACTORY INTERACTION MODEL EXPERIMENTAL DESIGN (MODDE)

The matrix of the experimental design containing 19 formulations together with outcomes obtained after performing all the experimental runs are summarized in Table 3. The experiments were conducted in an entirely randomized order, to assure that uncontrolled factors did not influence the outcome.

Table 3. Matrix of the experimental design (X_1 – gentamycin concentration (mg), X_2 – PLGA concentration (mg), X_3 – buffer concentration (mol/L), X_4 – Stirring speed (rpm), Y_1 – efficiency (%), Y_2 – particle size (nm).

Experiment Name	Run Order	X_1	X_2	X_3	X_4	Y_1	Y_2
N1	13	10	100	0.1	500	6	468.7
N2	5	150	100	0.1	500	19	226.4
N3	18	10	200	0.1	500	3	757
N4	8	150	200	0.1	500	12	133.1
N5	2	10	100	0.3	500	5	456.3
N6	6	150	100	0.3	500	17	203.9
N7	4	10	200	0.3	500	4	653.2
N8	19	150	200	0.3	500	15	192.1
N9	15	10	100	0.1	1500	6	384.2
N10	11	150	100	0.1	1500	14	215.5
N11	7	10	200	0.1	1500	1.7	502.7
N12	1	150	200	0.1	1500	13	254.2
N13	14	10	100	0.3	1500	6	360.5
N14	17	150	100	0.3	1500	17	530.1
N15	9	10	200	0.3	1500	5	493.1
N16	10	150	200	0.3	1500	19	120.2
N17	16	80	150	0.2	1000	10	325.4
N18	12	80	150	0.2	1000	9	383.1
N19	3	80	150	0.2	1000	10	361.1

As shown in Fig. 1, the selected model showed an excellent quality for efficiency encapsulation, with R^2>0.9 and Q^2>0.8, and good quality for the size response, with R^2>0.8 and Q^2>0.5. Moreover, a very good reproducibility value was obtained for both responses:

0.992 (for efficiency) and 0.974 (for size). The condition number was 1.08972 (for efficiency) and 1.08973 (for size) with very good screening design for our model.

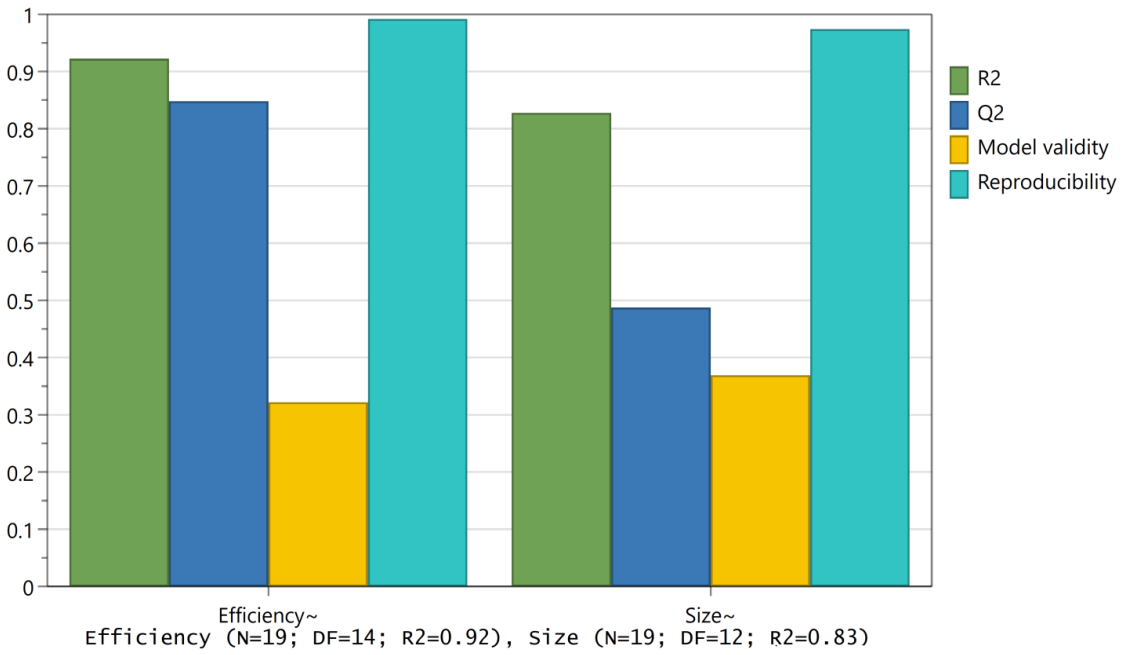

Figure 1. MLR response summary plot.

Regarding all dependent variables, the p-values of the ANOVA test were lower than 0.01 (<0.0001 for efficiency, 0.001 for size) for the model and larger than 0.05 (0.067 for efficiency, 0.08 for size) for the lack of fit. As ANOVA plot shows in Fig. 2, the standard deviation of the regression (first bar) is much larger than the standard deviation of the residuals with its upper confidence level (third bar).

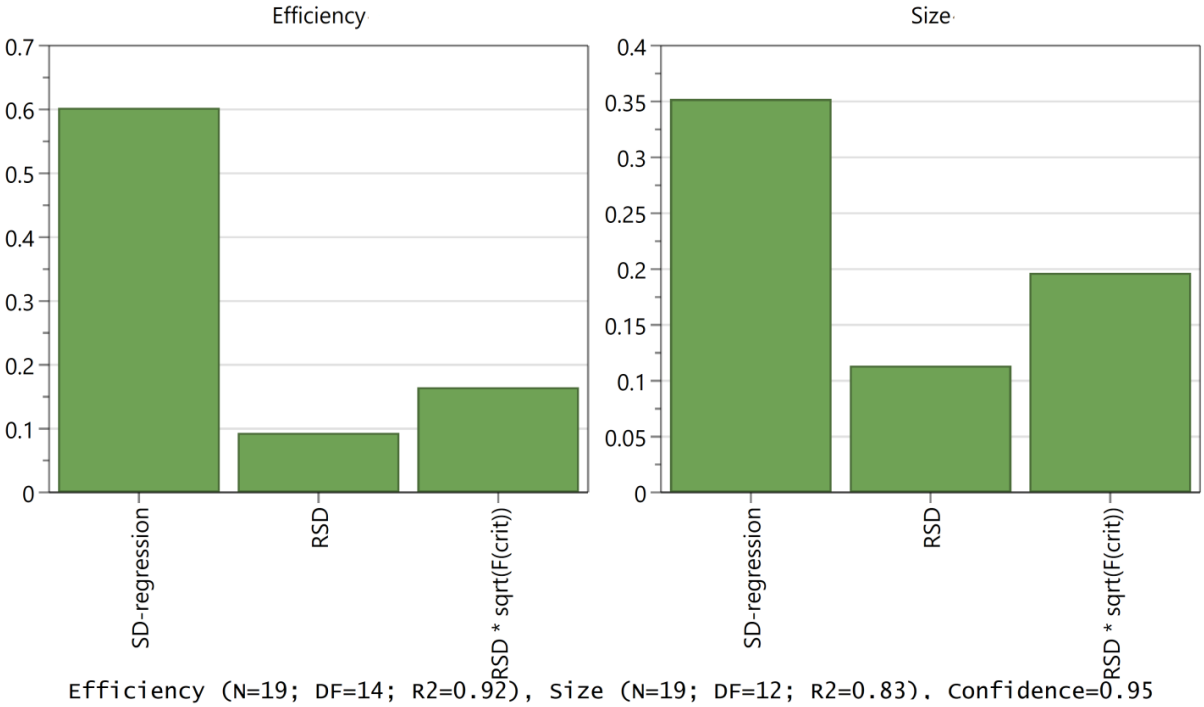

Figure 2. ANOVA plot

The regression coefficients with confidence intervals are displayed in Fig. 3.

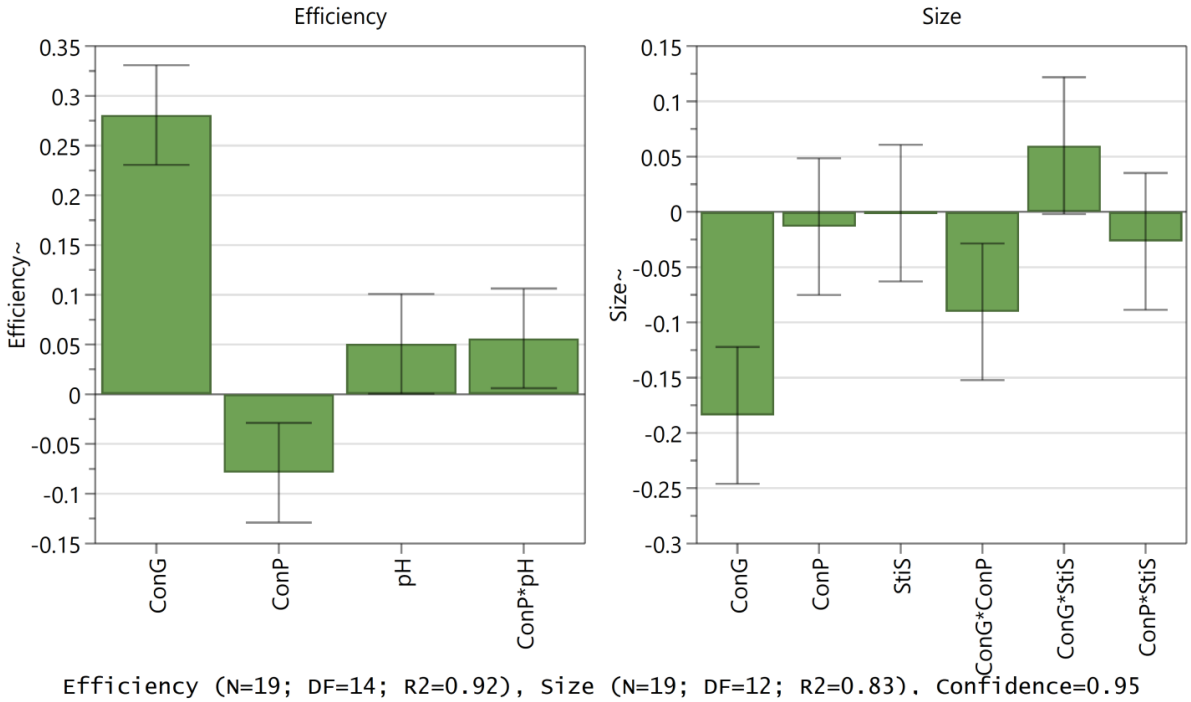

Figure 3. Coefficient plot

As it can be seen, gentamicin concentration influenced both efficiency and size, namely encapsulation efficiency increased with gentamicin concentration and nanoparticle size decreased with gentamicin concentration. Furthermore, gentamicin concentration showed a higher influence on both responses when compared with PLGA concentration. Also, a few statistically relevant interactions between PLGA concentration and pH or gentamicin concentration and PLGA concentration were registered. A moderate interaction between gentamicin concentration and stirring speed was observed for the size model.

The response contour plot for buffer concentration equal to 0.2 and stirring speed equal to 1000 is shown in Fig. 4. It was observed that on increasing gentamicin concentration, smaller size and higher efficiency was achieved.

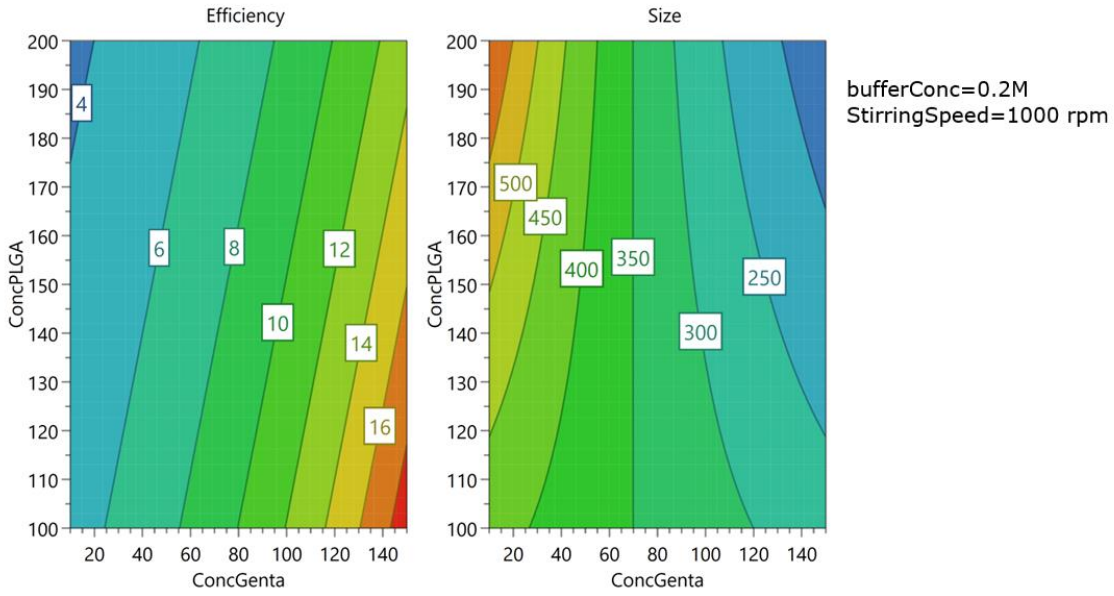

Figure 4. Response contour plot.

Based on the initial investigation screening, the DoE model was further used to predict an optimal formulation and to generate a design space. The target values for efficiency and size were set at 15% and 500 nm, respectively (Fig. 5).

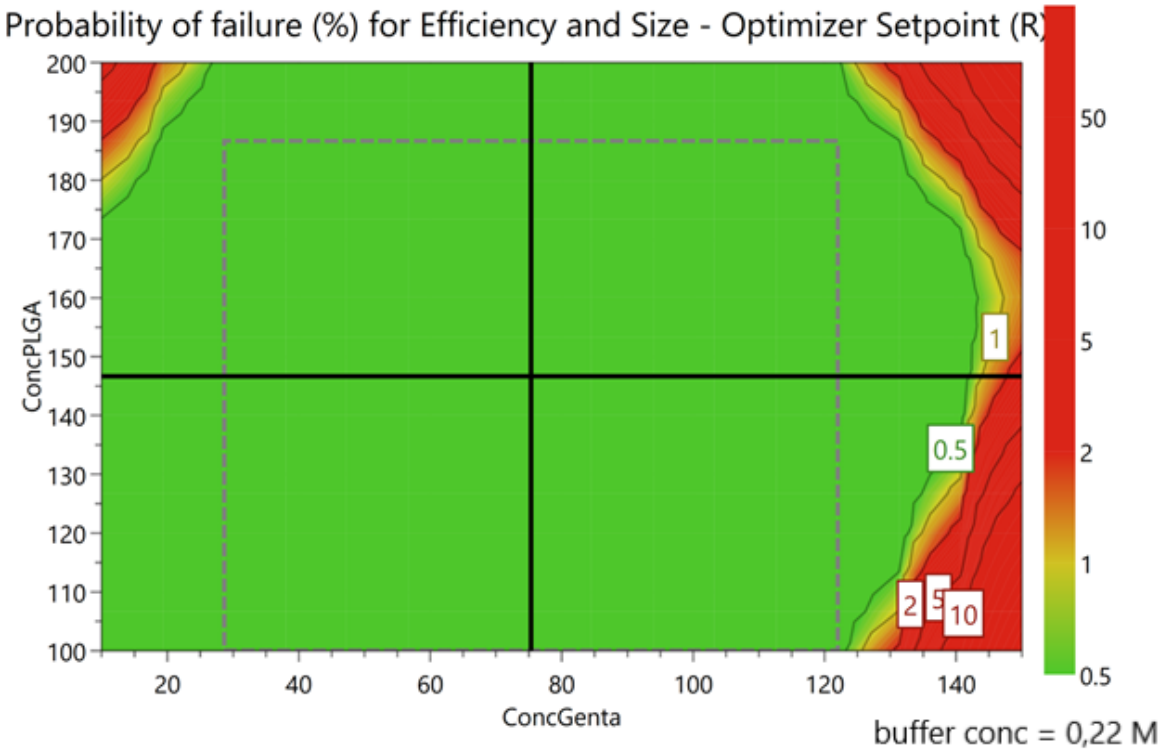

Figure 5. Design space.

As dynamic profile shows in Fig. 6, the results demonstrated that the stirring speed had no discriminative impact on efficiency or particle size. Not the same can be said about gentamicin concentration, PLGA concentration or pH: gentamicin encapsulation efficiency increased when gentamicin concentration and pH were raised, and PLGA concentration was decreased.

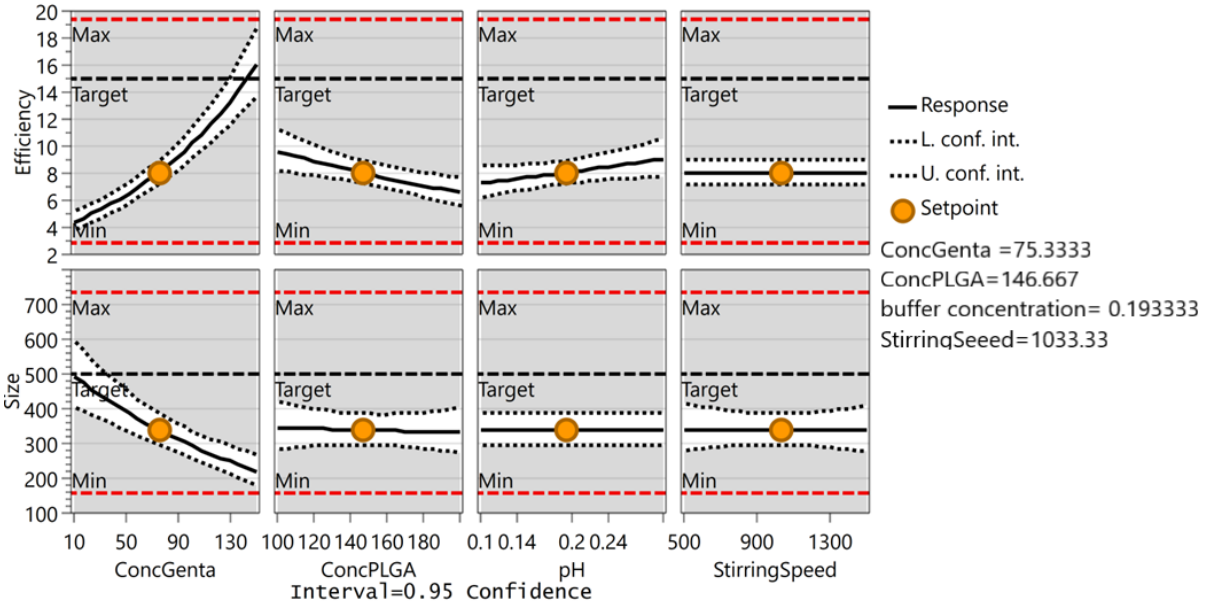

Figure 6. Dynamic profile of the model.

The DoE software was used to predict an optimal formulation. The most robust values were set for 75 mg gentamicin, 147 mg PLGA c, at 0.2 mol/L buffer concentration and 1033 rpm stirring speed. The predicted 8% efficiency and 340 nm particle size, which would be obtained for this set point, must be further tested.

3.2. KINETIC BIODEGRADATION STUDY

PLGA may undergo two degradation processes in PBS, namely a hydrolysis process shown in Fig. 7 and an autohydrolysis process shown in Fig. 8. Ester groups in the structure of the polymer will undergo a hydrolysis reaction. After it begins, the hydrolysis reaction which is the main process that takes place in the first few days may be catalysed by the resultant acids. The pH should rapidly decrease to lower values due to the free acids [28].

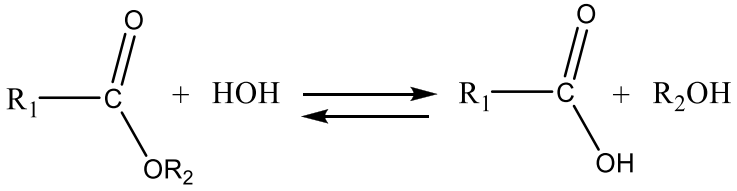

Figure 7. Hydrolytic degradation of PLGA

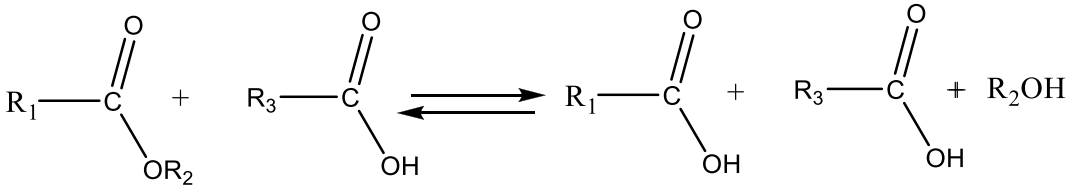

Figure 8. Autocatalytic degradation of PLGA

During the experiments, the samples immersed in the degradation medium acquired a soft appearance at their surface. Furthermore, a thickening of the upper soft polymer layer was observed beginning with the first days of the experiment. Studies indicate that this is determined by the hygroscopic nature of the polymer. The results show the water uptake inside the polymer gradually increasing (Fig. 9A). A diffusion process inside the polymer determines its retention [29, 30].

Fig. 9B displays the mass loss of PLGA 65:35 at certain times during the degradation process. The mass loss is reduced in the first 10 days which correlated with the pH values suggests that at first only a few lactic and glycolic units are hydrolysed. As figure 9B shows, mass loss only reaches 9.5% even though the water uptake inside the polymer is as high as 97%. The volume of the samples exhibits an exponential growth due to water uptake. These results are consistent with other studies which show that for PLGA 50:50 a significant mass loss is observed after 10 days, whereas for PLGA 95:5 it takes 20 days for a significant mass loss to occur [27].

The mass loss increases towards the end of the study, reaching 93% by day 27, which suggests that only 7% of the polymer remained undegraded. The surface of the polymer became very soft; therefore, it was impossible to determine the water uptake after day 12.

The polymer displays a heterogenous degradation with areas more or less accentuated as reported by other studies [30-33].

Fig. 9 C shows how pH is affected at different times during PLGA degradation.

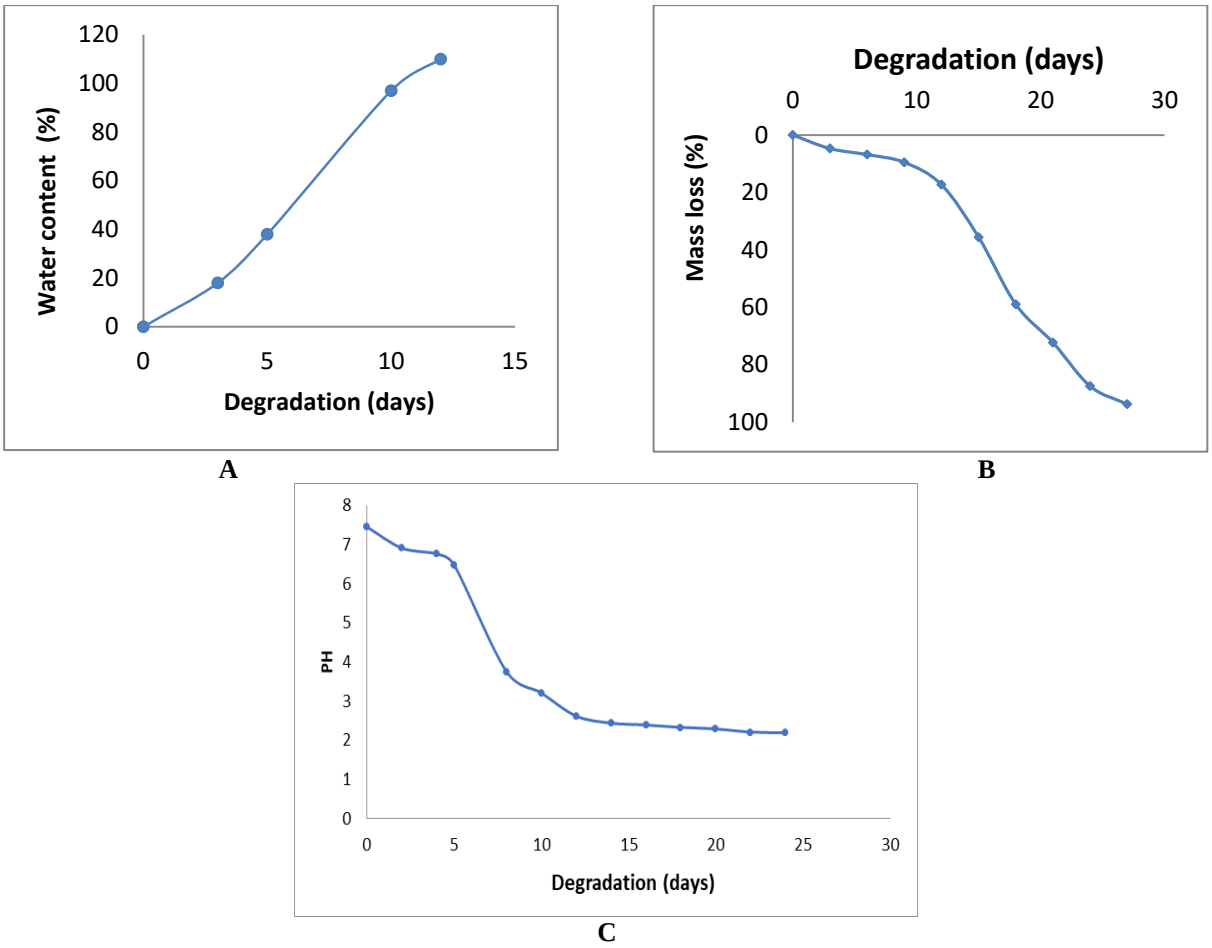

Figure 9. A) Percentage water content of the polymer film as a function of degradation time; B) Mass loss of polymeric film as a function of degradation time; C) Decreasing the pH of the degradation medium depending on the degradation time.

This process is fast; a decrease of the pH value is observed starting the first two days from 7.44 to 6.90. This phenomenon may be explained by the ionization of surface acid groups. They release H^+ ions once they come into contact with water. Moreover, the pH value does not undergo a significant decrease between day three and day five; it almost remains constant. By day eight the pH drops to a value of 3.75, which many be determined by the autocatalysis of the hydrolysis reaction. Then, around day twelve since the beginning of the experiment, the pH continued to slowly decrease. All these variations are determined by the release of lactic acid and glycolic acid in the PBS solution which according to other studies may promote a zero-order kinetic. The significant pH reduction may help preserve the physiological pH after PLGA based biocomposite is inserted. Furthermore, it may also help dissolve drugs, namely gentamicin, that have a better solubility in an acidic medium [35-37].

4. CONCLUSIONS

An increase in gentamicin concentration determined both a decrease in particle size and a better encapsulation efficiency. Moreover, the decrease of PLGA concentration and higher levels of pH yielded a higher encapsulation efficiency. Out of the four factors evaluated, the stirring rate did not significantly impact any of the responses evaluated. The variation of the obtained encapsulation efficiency and size under the same conditions (pure

error) is almost 0. The results showed a two-step degradation process of PLGA in PBS. In the first days the reduced mass loss is correlated with a low decrease in pH, followed by a significant decrease in mass loss starting day 10.

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