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HYDRODYNAMIC DIAMETER CONTROL IN THE SYNTHESIS OF CHITOSAN NANOPARTICLES: OPTIMIZATION BY EXPERIMENTAL DESIGN

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Abstract

Background: Chitosan nanoparticles are an alternative vehicle for drug formulation and delivery. Objective: This study aims at the synthesis of chitosan/tripolyphosphate nanoparticles through an experimental design 3^2, to evaluate the ideal conditions for obtaining a reduced particle diam- eter with potential for encapsulation of biomolecules. **Method:** The nanoparticles were obtained by ionotropic gelation, in 11 experimental conditions. Statistical Analysis were carried out using the *software Statistics 7*, having as independent variable: the polymer/crosslinker mass ratio and the drip flow rate of the crosslinker. The hydrodynamic diameter of the nanoparticles was the response variable, which was characterized by dynamic light scattering. **Results:** For the opti- mized system the results show that the mass ratio between the reactants influences the particle size obtained, and the mass ratio of Chitosan/TPP between 2.25 - 2.50 is the most indicated for obtaining diameters between 20-50 nm. **Conclusion:** Considering the range of hydrodynamic di- ameters observed in this study, the nanoparticles are well-suited for utilization as active carriers, specifically targeting organs to enhance the effectiveness of therapies.

Keywords: *nanoparticles, chitosan, experimental design, hydrodynamic diameter.*

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1. INTRODUCTION

Biomaterials, such as chitosan polymeric nanoparticles have become prominent in several areas of biomedicine, given their important role as vehicles of pharmaceuticals, in addition to the practicality in the controlled release of drugs, for different therapeutic purposes (Attia et al., 2023). Numerous studies have demonstrated the potential of utilizing this material for tumor treatment (Muresan et al., 2023; Kong et al., 2023). Additionally, it has found applications in pharmaceutical formulations such as

nanosuspensions, nanoemulsions, and nanosprays (Mehmood et al., 2023). In particular, the utilization of chitosan in the development of nanostructures offers distinct advantages compared to other polymers due to its biodegradability (Chen et al., 2023) and biocompatibility. Among several methods for synthe- sizing chitosan nanoparticles, ionotropic gelation has been prominently utilized (Sreena and Nathanael, 2023). This method exploits the protonation of amino groups present in chitosan structure under acidic conditions, thereby making them available for reaction with sodium tripolyphosphate (TPP), an anionic crosslinking agent. Consequently, gelatinous structures are formed. Multiple factors, including the mass ratio between reactants, pH of the medium, reaction time, and agitation, influence the formation and size of these nanostructures (Mekaru, 2023). Numerous studies have explored the impact of mass proportions among the reagents for the proposed method. However, it is important to consider other factors that can influence the reduction of nanoparticle diameters, such as the drip flow rate of the crosslinking agent. Therefore, the objective of the present study is to synthesize chitosan/tripolyphosphate nanoparticles (NP_Chi-TPP) using the ionotropic gelation method and investigate two primary parameters: the mass ratio between the reagents (Chi: TPP) (z_1) and the drip flow rate of the crosslinking agent TPP (z_2), in order to optimize the hydrodynamic diameter of the nanoparticles through the application of an experi- mental design.

2. METHODOLOGY

2.1. Synthesis of NP-Chi-TPP

The nanoparticles were synthesized from the solubilization of low molecular weight chitosan (deacetylation 75-85%) (Sigma-Aldrich) in 1.0% acetic acid solution (v/v), according to literature (Pedroso-Santana and Fleitas-Salazar, 2020). After total solubilization, the pH was adjusted with di- luted NaOH solution up to pH 4. The solution was filtered in a 0.45 µm membrane and then 0.22 µm. An aqueous solution of sodium tripolyphosphate (TPP) was dripped into the filtrated

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chitosan solu- tion, following the mass ratio and flow rate established by the experimental design. The gel pellet of the NP_Chi-TPP obtained were centrifugated at 9,000 rpm for 20 min, followed by the resuspension in ultrapure water and then taken for characterization.

2.2. Characterization of NP_Chi-TPP by Light Scattering

The NP_Chi-TPP were characterized by Dynamic Light Scattering (DLS) using the Malvern Zetasizer particle analyzer to determine their mean hydrodynamic diameter and particle size distribution using a chitosan refractive index of 1.52 and absorption of 0.010. Analysis was performed in triplicate.

2.3. Experimental design for synthesis of NP_Chi-TPP

A factorial design 3^2 was applied, considering the factors: Chi/TPP mass proportions and drip flow as controlled variables, having as response variable the hydrodynamic diameter of the nanoparticles. The experimental design generated 11 experiments (**Table 1**). The statistical analyses and development of

corresponding empirical models for the experimental design were performed with the software *Statistics*,

version 7.0, developed by Stat Soft Inc. A normal probability distribution with a 95% confidence interval was admitted. The objective function (F_{obj}) for parameter estimation (**Equation 1**) was defined based on the least squares method. Where NE is the number of experiments; y^e represents the measured experi- mental value and y^c the value calculated by the quantitative model.

Equation 1

$$F_{obj} = \sum_{j=1}^{NE} (y_i^e - y_i^c)^2$$

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The parameter estimation procedure was performed using the Hooke-Jeeves and Quasi-Newton method to minimize the objective function (Omran et al., 2022). The definition of the empirical model (linear in the parameters) was based on trials, taking into account the main effects of the variables, synergy effects between the variables and quadratic effects of the variables. We started from the reparameterized generic expression, presented by **Equation 2,** in which α_i are the parameters of the model, $z1$ and $z2$ are the independent variables of the model and y is the dependent response variable of the model.

Equation 2

$$y = \alpha0 + \alpha1z1 + \alpha2z2 + \alpha3z1z2 + \alpha4(z12-2/3) + \alpha5(z22-2/3) + \alpha6(z12-2/3)\, z2 + \alpha7z1(z22-2/3) + \alpha8(z12-2/3)\,(z22-2/3)$$

The factors applied in the experimental design were: Chi-TPP mass ratio (minimum level 2.0, central point 2.25 and maximum level 2.5) and TPP drip flow rate (low level 0.5 mL/min; central point 1.0 mL/min and high level 1.5 mL/min). The levels were normalized to -1 (minimum level), 0 (central point) and +1 (minimum level).

Table 1. Experiments performed to determine the hydrodynamic diameter of *NP_Chi-TPP* particles according to the experimental design 3^k, (k=2), with mass ratio and drip flow rate variations.

Run	Mass ratio (Chi/TPP) (z1)	Drip flow rate (TPP mL.min^{-1}) (z2)
1	2.00	0.50
2	2.00	1.00
3	2.00	1.50
4	2.25	0.50
5	2.25	1.00
6	2.25	1.50
7	2.50	0.50
8	2.50	1.00
9	2.50	1.50
10	2.50	1.00
11	2.50	1.00

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3. RESULTS AND DISCUSSION

The hydrodynamic diameter (HD) of the particles was obtained according to the experimental de- sign used. The results obtained in the DLS analysis for the complete set are presented in **Table 2**, where P1, P2 and P3 are the hydrodynamic diameters obtained for the three measurements of each sample in nm, according to the graphs generated (S1) for particle size distribution by number and the HD value corresponds to the mean of the three measurements.

Table 2. Input variables and respective output variables for the experiments corresponding to the experimental design. σ: standard deviation; HD: hydrodynamic diameter and P1, P2 and P3: triplicates.

Rum	z1	z2	Chi/TPP	Q (mL/min)	P1 (nm)	P2 nm)	P3 (nm)	HD (nm)	σ
1	-1	-1	2.00	0.50	37.84	58.77	68.06	54.89	15.50
2	-1	0	2.00	1.00	32.67	32.67	78.82	48.05	26.60
3	-1	+1	2.00	1.50	78.82	78.82	78.82	78.82	0.00
4	0	-1	2.25	0.50	24.36	32.67	58.77	38.60	18.00
5	0	0	2.25	1.00	43.82	58.77	58.77	53.79	8.60
6	0	+1	2.25	1.50	18.17	43.82	50.75	37.58	17.20
7	1	-1	2.50	0.50	28.21	37.84	50.75	38.93	11.30
8	1	0	2.50	1.00	32.67	37.84	37.84	36.12	3.00
9	1	+1	2.50	1.50	21.04	28.21	28.21	25.82	4.10
10	0	0	2.25	1.00	50.75	50.75	50.75	50.75	0.00
11	0	0	2.25	1.00	18.17	18.17	21.04	19.13	1.70

σ: standard deviation; HD: hydrodynamic diameter e P1, P2 e P3: triplicates.

The correlation between the input and output variables of the Experimental Design is represented by the correlation matrix (**Table 3**). The correlation matrix indicates a significant correlation (bold num- ber) between the HD value and the mass ratio between chitosan and TPP. The normalized values of the input variables (z1 and z2) and the corresponding values of the output variable (HD), organized in Table 2, were input to the Statistica software for the prediction of empirical statistical models, in the format presented by Equation 2. The final model obtained (**Equation 3**) presents significant parameters, ex- pressed with their respective parametric errors. The adjustment obtained can be seen in **Figure 1.A**, which presents the diagram that relates the experimentally observed values with the values predicted by the model. The response surface obtained is presented in **Figure 1.B** to illustrate the linear behavior of the model presented.

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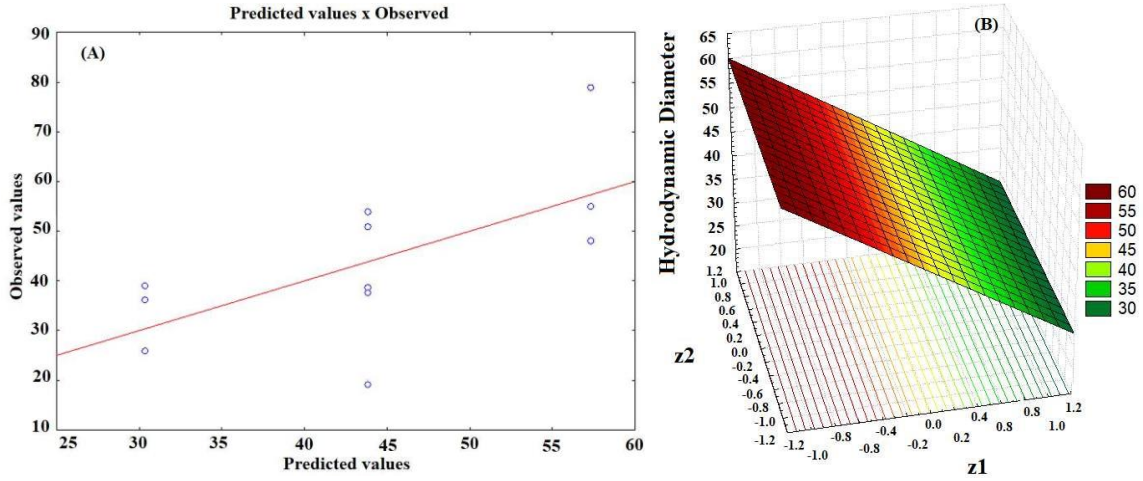
Table 3. Correlation matrix between input and output variables of the experimental design. Where: z1: Chitosan/TPP; z2: Drip flow; HD: hydrodynamic diameter.

	Z1	z2	HD
z1	1,00	0,00	**-0,65**
z2	0,00	1,00	0,08
HD	**-0,65**	0,08	1,00

Final model**:** **HD** = **43,86±3,90 -13,48 ±5,28. z1** **Equation 3**

Figure 1. (A) Diagram of predicted x observed values and (B) Response surface obtained using factorial design of 3^2.



Analyzing **Equation 3 and Figure 1**, it is possible to notice that the variable z2, TPP drip flow, was not considered significant, in which a first-order linear effect was observed with contribution only of the variable z1, mass proportion of Chi/TPP. Thus, it is suggested that, for the analyzed interval, any of the values defined for the TPP drip flow can be used. However, in previous developments, the group ob- tained heterogeneous distributions for the hydrodynamic diameter of chitosan particles when the drip flow was not controlled. As the mass ratio between chitosan and TPP is significant, it influences the par- ticle size obtained, as was also observed by Gao et al. (2022). The response

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surface (**Figure 1B**) presents an inclined plane, since it contains only first-order effects, and it is possible to observe that, according to the increase in chitosan mass content there is a lower value of the hydrodynamic diameter of the chitosan nanoparticles.

Some studies in the literature report the application of nanoparticles for specific application purposes considering the size of the pseudospheres, as reported by Gao (et al., 2022) who used chitosan nanopar- ticles modified with mannose of 150 nm to elaborate a nasal spray, in addition to the diversified use in diagnostic imaging. Rashighi and Harris, (2017) analyzed the application of nanoparticles 6 to 7 nm in probes to obtain images in patients with melanoma.

4. CONCLUSIONS

The diameter control in the synthesis of chitosan nanoparticles allows to direct the region of administration in therapies in the target organism, considering its permeability in the cell. Thus, the study showed the possibility of obtaining nanoparticles without the addition of organic solvents, from a controlled experimental system.

From the parameters defined in this study, loading and release steps of the active should be investigated, as well as their synthesis adaptation to the sterile system. Despite the relative simplicity of the method, there is still a timid and effective preparation of products developed in the pharmaceutical industry in Brazil, which would add value and benefits to its application in drug release control in different therapies.

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Interest conflicts

The authors declare that there are no conflicts of interest. The authors are solely responsible for the con- tent and writing of the article.

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