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RESEARCH ARTICLE

# Ratio of Drug/carrier as Dominant Factor in Determining Size of Doxorubicin-Loaded Beta-1,3- Glucan Nanoparticles: A Study using Artificial Neural Networks

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**ABSTRACT**

Particle size is an important parameter in determining many properties of nanoparticles. In this work, nanoparticles of β-1,3-glucan containing doxorubicin (Dox) in conjugated and unconjugated forms (Con-Dox-Glu and Un-Dox-Glu, respectively) were prepared. Then, artificial neural networks (ANNs) were employed to find the impact of different processing/formulation factors on their particle size, which was measured using dynamic light scattering (DLS). The parameters included ratio of Dox/Carrier as well as concentrations of polyethyleneimine (PEI), NaOH and succinic anhydride (Sa). To do so, we prepared fifty samples having different values of the four parameters and measured their particle size. The data were divided randomly into training, test and unseen data. The ANN model demonstrated that in both conjugated and unconjugated forms, the dominant factor determining the particle size is Dox/Carrier ratio. Also, concentration of PEI showed to be important in determining particle size of unconjugated form of the nanoparticles. The remaining parameters indicated no considerable effect on the particle size.

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## INTRODUCTION

β-1,3 glucan (Glu), a biocompatible and biodegradable glucose polymer of the cell wall of the yeasts, acts as a biological response modifier [1]. Glu as a non-digestible carbohydrate with a linear backbone, varies in molecular mass, solubility, viscosity and branching structure. Intestinal microbial flora has been reported to ferment Glu [2-4]. Glu may also be synthesized and functionalized in form of nanoparticles [5]. Glu forms porous, hollow micro/nano-spheres which allow for encapsulation, transport, delivery and release of electrostatically bound payloads. So, it is capable of delivering drugs, proteins and nucleic acids into cells stably.

Currently, use of conventional Doxorubicin (Dox) in clinics is limited because of its important side effects such as cardiotoxicity [6]. To overcome this concern, new nanoparticle systems which offer promising results through increase of drug delivery, decreased systemic toxicity and increased efficiency may be interesting alternatives [7].

An important issue to realize the full promise of nanoparticle-based drug delivery system is finding optimal strategies to achieve desired size to achieve an efficient nanodrug [8,9]. Some physiological and pharmacological effects of nanodrugs such as efficacy of the drug [10] and uptake of the particles [11]

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Table 1. Unseen data used for validation of the obtained model

| | Input parameters | | | | Output parameter | |
|---|---|---|---|---|---|---|
| Sample | Dox/Carrier | Sa (mg) | NaOH 3N (mL) | PEI (mg) | Size (nm) | |
| | | | | | Obtained | Predicted |
| Un-Dox-Glu | 0.05 | 230 | 1.5 | 110 | 370 | 404 |
| Un-Dox-Glu | 0.20 | 230 | 1.3 | 110 | 439 | 490 |
| Un-Dox-Glu | 0.25 | 220 | 1.7 | 130 | 455 | 477 |
| Un-Dox-Glu | 0.50 | 200 | 2.0 | 150 | 530 | 435 |
| Con-Dox-Glu | 0.05 | 230 | 1.5 | 110 | 256 | 251 |
| Con-Dox-Glu | 0.20 | 230 | 1.3 | 110 | 181 | 181 |
| Con-Dox-Glu | 0.25 | 220 | 1.7 | 130 | 200 | 178 |
| Con-Dox-Glu | 0.50 | 200 | 2.0 | 150 | 158 | 181 |

Table 2. Training parameters set within the software

| | | |
|---|---|---|
| No. Hidden Layers | | 1 |
| No. nodes | | 5 |
| Type of back propagation | | Incremental |
| Transfer function | Output | Asymmetric Sigmoid |
| | Hidden layer | Asymmetric Sigmoid |

are dependent on their size.

Our research group has been developing an encapsulating system from β-1,3-glucan polymer to deliver Dox to the tumor site [12]. Artificial neural networks (ANNs) was applied in this study as powerful approach to model non-linear processes of preparation in both conjugation and physical loading approaches [13]. Our technique uses four independent parameters, namely amount of NaOH, amount of succinic anhydride (Sa), ratio of Dox/Carrier and amount of polyethylenimine (PEI). We therefore examined the effect of the four parameters on size of the nanodrug in both conjugated (Con-Dox-Glu( and unconjugated (Un-Dox-Glu( systems. In this study, we used ANNs to determine the possible role of theses parameters on size of the nanodrug.

## MATERIALS AND METHODS

*Materials*

β-1,3-glucan was from Sigma-Aldrich (USA). Dox was obtained from Ebewe Pharma (Austria). All other reagents/ingredients were from Merck chemicals (Germany).

*Preparation of carrier (Glu–Sa–PEI)*

To prepare the carrier, as described previously [12], 200 mg Glu (MW 18 kDa) was added to deionized water (10 ml) and stirred Sa and NaOH (3N) overnight. Afterwards, the obtained solution was dialyzed and lyophilized at −30 °C. Obtained Glu–Sa was stirred with $NaIO_4$ (30 min, 50 °C). PEI was then added to the solution and stirred for (6 h, 70 °C).

*Preparation of Con-Dox-Glu (Dox conjugated to the carrier)*

Dox, dissolved in DMSO, was added to the carrier and added to distilled water (injection rate 1 mL/min) under constant sonication (amplitude 50%). Subsequently, exhaustive dialysis against DI water (dialysis tube, MW cut-off 12 kDa) was used to remove unreacted components (dark conditions for 3 continuous days). and lyophilized for further applications. FTIR (fourier-transform infrared) was employed to verify synthesis of Glu–Sa–PEI conjugated to Dox using KBr pallets at room temperature (Data not shown).

*Preparation of Un-Dox-Glu (Dox loaded into the carrier physically)*

To prepare Un-Dox-Glu, solution of Dox in DMSO was added to Glu–Sa–PEI and dialyzed (MWCO 12 kDa, dark place). The nanoparticles were then lyophilized.

*Artificial Neural Networks (ANNs) study*

To determine relationships between input parameters, including Dox/Carrier ratio (0.05-0.5) as well as amount of NaOH (1.3- 2.5 mL), Sa (200- 230 mg) and PEI (110- 150 mg)) and the output parameter (particle size) in Con-Dox-Glu

and Un-Dox-Glu, using ANNs, INForm v4.02 (Intelligensys, UK), an ANNs package, was used. Fifty samples were prepared and dynamic light scattering (DLS, Zetasizer Nano, Malvern, UK)was employed to measure the particle size. No dilution was made prior to size measurements. Then, 38 data were chosen randomly as training data for network training purposes. Four data were used to avoid overtraining (test data) and the remaining data were considered as unseen data to validate the model as described previously [14]. Subsequently, using training parameters given in Table 2 and in our previous study [8], response surfaces were produced to study relationships between the inputs/output data. The response surfaces indicate relationships of two inputs with the output parameter when the remaining input parameters are fixed.

## RESULTS

A Back propagation neural network was utilized to model the data. After modeling, the optimum showed $R^2$ values of 88.4, 97.6 and 90.1% for the training, test and validation data, respectively, was used to train the data.

Figs. 1 and 2 summarize the influence of the four inputs on the particle size in Con-Dox-Glu and Un-Dox-Glu formulations, respectively.

*Determination of variables affecting particle size in Con-Dox-Glu*

Fig. 1A shows the effect of Sa amount (mg) and Dox/Vehicle ratio in Con-Dox-Glu nanoparticles on the particle size when the other two variables (NaOH and PEI) are fixed at their medium levels (i.e. 1.9 mL and 130 mg, respectively). It is evident from the figure that increasing Dox/Vehicle ratio decreases the particle size considerably, while Sa appears not to be important in general. According to Fig. 1B, in given fixed medium levels of Sa and PEI (215 and 130 mg, respectively), the particle size decreases with increase in Dox/Vehicle ratio while NaOH does not affect the particle size. In Fig. 1C, the amounts of Sa and NaOH are fixed at medium values (215 mg and 1.9 mL, respectively) to evaluate the effect of Dox/Vehicle *ratio* and PEI on particle size. The details also show that *increasing the* Dox/Vehicle *ratio* leads to smaller sizes while variation in PEI does not change the particle size. As illustrated in Fig. 1D, 1E and 2F, NaOH, PEI and Sa do not appear to have important effects on size.

*Determination of variables affecting particle size in Un-Dox-Glu*

Fig. 2 shows the effects of the four input variables (Sa, NaOH, PEI, and Dox/Vehicle *ratio*) on the particle size in Un-Dox-Glu formulation. As shown in Fig. 2A, maximum particle size is observed in Dox/Vehicle ratio of ~ 0.3. Dox/Vehicle values of above or below this value reduce the particle size. In addition, increasing Sa amount decreases the particle size. From Fig. 2B, the highest particle size is around Dox/Vehicle ratio of ~ 0.3 and the size reduces above or below this value. Moreover, increasing NaOH has shown no important effect on nanoparticles' diameter. Fig. 2C indicates similar results to those of 2A and 2B for effect of Dox/Vehicle ratio on the particle size. In addition, based on Fig. 2C, increasing PEI is associated with decreased particle size. Also from fig. 2D-F, when the other two parameters are fixed at a medium level, only PEI affects the particle size.

## DISCUSSION

In recent years, Glucan-based nanoparticle systems have received much attention in cancer immunotherapy [15]. Size of nanoparticles is particularly important in cancer therapy through affecting the pathway of cellular uptake and its renal clearance [16]. Furthermore, particle size can particularly affect the properties of anti-cancer nanomedicines (e.g. biodistribution, adverse effects and circulation time) [17-19]. Thus, the size of the nanoparticle must be fine enough (below 300 nm) to escape scavenging and clearance by the reticuloendothelial system (RES) or circulating macrophages and exhibit better therapeutic efficiency by enhancing residence time in the blood [20,21].

This study aimed at developing a predictive ANNs model to evaluate parameters which may influence the particle size in two newly developed Dox formulations (Con-Dox-Glu and Un-Dox-Glu) in which β-Glucan was used as carrier.

Our results indicated that Dox-conjugated nanoparticles have smaller sizes compared to those obtained by physical loading (unconjugation) method [22]. The diameters obtained for Con-Dox-Glu and Un-Dox-Glu were in the range of 170-320 nm and 320-530 nm, respectively.

The results clearly indicated that in Con-Dox-Glu, only Dox/Vehicle ratio affects the particle size. We believe that, at the ranges studied, more Dox molecules contribute to better interactions within

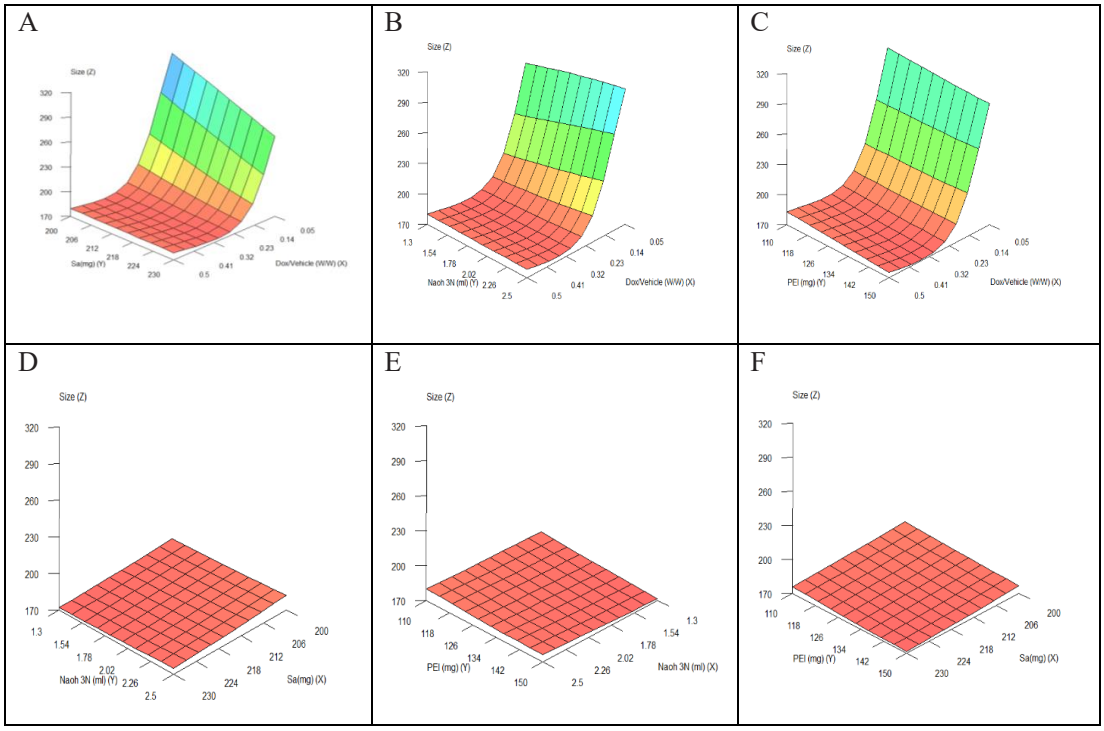


Fig. 1. Response surfaces generated by the model illustrating particle size (nm) of nanoparticles containing conjugated Dox (Con-Dox-Glu) as a function of A) amount of Sa (succinic anhydride) and Dox/Vehicle ratio, B) amount of NaOH 3 N and Dox/Vehicle ratio, C) amount of PEI (polyethyleneimine) and Dox/Vehicle ratio, D) amount of NaOH 3 N and Sa, E) amount of PEI and NaOH, F) amount of PEI and Sa

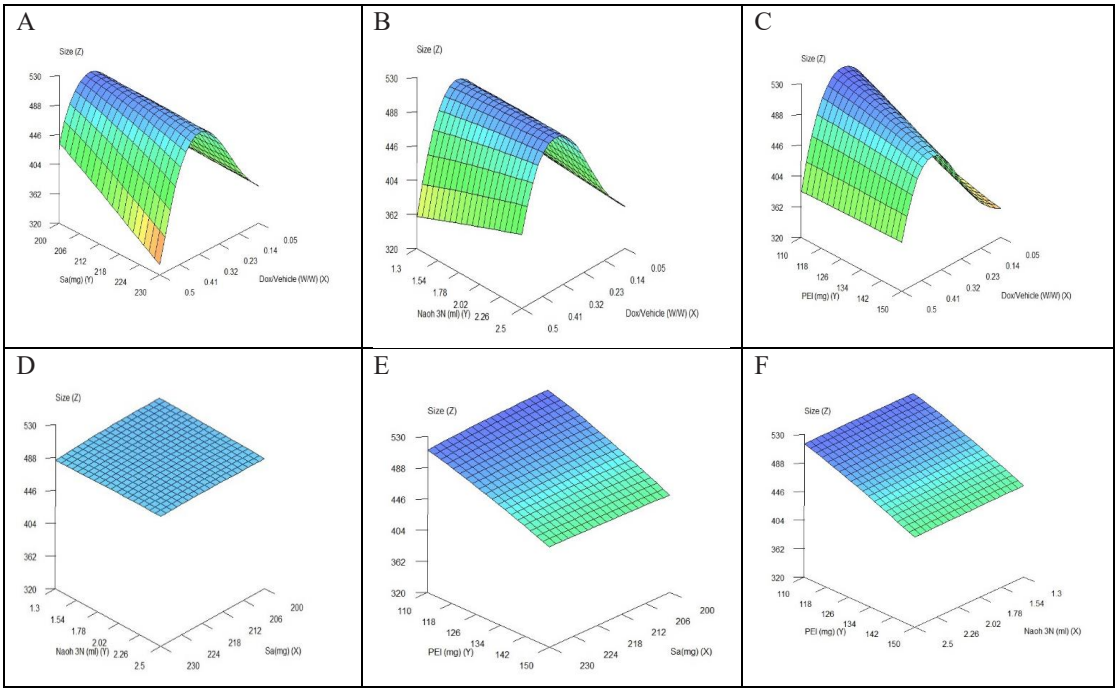


Fig. 2: Response surfaces generated by the model illustrating particle size (nm) of nanoparticles containing unconjugated Dox (Un-Dox-Glu) as a function of A) amount of Sa (succinic anhydride) and Dox/Vehicle *ratio*, B) amount of NaOH and Dox/Vehicle *ratio,* C) amount of PEI (polyethyleneimine) and Dox/Vehicle *ratio*, D) amount of NaOH and Sa, E) amount of PEI and Sa and F) amount of PEI and NaOH.

the nanoparticles, thus, smaller particles are formed.

The results of studying the unconjugated form indicated that the particle size slightly reduced by increasing the amount of PEI in formulation. The positive charges on the surface may make particles repel each other and prevent aggregation via electrostatic stabilization. With regards to role of PEI, this high molecular weight polymer effectively creates a loose coating layer around the nanoparticles and inhibits their agglomeration. Agglomeration increases the size of particles and simultaneously decreases the ratio of surface-to-volume. Therefore, increasing PEI in the formulation decreases particle size due to its protective and stabilizing properties [23,24]. In a previous report, higher concentrations of PEI in superparamagnetic iron oxide nanoparticles enhanced stabilizing activity of the polymer and lowered size of the particles and the clusters [25].

Our findings also showed that when Dox/Vehicle ratio is less than ~3, increasing the ratio increases the size, while above this point further increase in the ratio makes the size smaller. We believe that at Dox/Vehicle ratio < 3, by increasing the ratio, more Dox molecules enter each particle, thus, the particles become larger. However, when this ratio exceeds 3, the particles' charge become positive enough to provide repelling force within the particles, thus, they become smaller.

## CONCLUSION

This paper designs an artificial neural networks model to evaluate the effect of input experimental data including Sa, NaOH, PEI and Dox/Vehicle ratio on particle size of two new Dox formulation. ANN outputs showed that Dox/Vehicle ratio was the dominant factor affecting particle size in both conjugated and unconjugated forms of the vehicle containing Dox.

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## CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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