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RESEARCH ARTICLE

Loading Efficiency of Doxorubicin-Loaded Beta-1,3- Glucan Nanoparticles: An Artificial Neural Networks Study

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ABSTRACT

Objective(s): We used artificial neural networks (ANNs) to optimize a preparation of β-1,3-glucan nanoparticles containing doxorubicin (Dox) through investigating the critical parameters influencing the drug's loading efficiency.

Methods: Using an ANNs model, we evaluated the effect of four inputs, involved in preparation of the carrier system, including concentrations of succinic anhydride (Sa), NaOH and polyethyleneimine (PEI) as well as ratio of Dox/Carrier, on loading efficiency of Dox as output parameter, when Dox was conjugated to the carrier (Con-Dox-Glu) or in unconjugated form (Un-Dox-Glu).

Results: The model demonstrated that increasing Sa and PEI leads to reduced loading efficiency, while the effect of NaOH on loading efficiency does not appear to be important in both Con-Dox-Glu and Un-Dox-Glu delivery system. Ratio of Dox/Carrier showed complex effects on loading efficiency: while a certain value was required to provide maximum loading efficiency in Con-Dox-Glu, a different critical value was associated with obtaining minimum loading efficiency in Un-Dox-Glu.

Conclusions: This study demonstrated the possibility of employing an ANNs model to identify the effect of each parameter on loading efficiency and optimize the conditions to achieve maximum loading efficiency in both conjugated and non-conjugated drug delivery system.

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INTRODUCTION

Drug delivery systems based on nano-approaches offer several advantages, such as increased drug loading (due to their large surface area), better bioavailability/ solubility of hydrophobic drugs, extended drug biological life, lesser immunogenicity and possibility for providing controlled release. β-1,3 glucan (Glu) is a carbohydrate-based polymer, extracted from bacteria, mushrooms, yeast or grains with unique properties such as biocompatibility, biodegradability (1) and mucoadhesivity (2) as well as antibacterial and immunomodulatory activity. The polymer has gained lots of popularity for drug delivery purposes(3) Nanoparticles of Glu have been introduced as unique biomaterial platforms, capable of delivering drugs, proteins or nucleic acids

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into cells stably. Glu, due to presence of a hollow cavity in its molecular structure allows efficient absorption and encapsulation of molecules.

One of the most important pharmacokinetic and pharmacodynamics properties of drug-loaded nanoparticles is drug loading efficiency. A successful delivery system should have a high drug loading efficiency (see equation 1).

$$\text{Drug loading efficiency (weight [wt] \%)} = \frac{\text{Mass in preparation}}{\text{Mass in feed}} \times 100\% \quad (1)$$

In essence, drug loading efficiency is determined by mechanism of drug loading and chemical/ physical properties of the carrier (4) Of particular interest is, in case of toxic and expensive drugs, where the drug loading efficiency becomes more important (5, 6). Commonly, drug loading occurs by either adsorption onto carrier particles or entrapping during the formulation (7, 8). Several parameters have been reported to increase loading efficiency of the drug: use of porous carriers, protein nanoparticles, conjugation between the drug and the carrier, carrier-free nanomedicines with no excipient and formation of complex are examples of approaches which have been employed to increase drug loading efficiency (4). Currently, conventional preparation of Doxorubicin (Dox) which is its free form, is being clinically used as an effective and broad spectrum anti-cancer drug (9, 10). Nevertheless, clinical use of Dox is limited due to its important adverse effects such as cardiotoxicity (11). One of strategies to overcome this concern is to develop novel delivery systems which offer promising results via enhancing drug solubility, increasing accumulation in tumor tissues, decreasing systemic toxicity and increasing efficiency (12). Artificial neural networks (ANNs) try to mimic neurons of human brain processes different data (13). ANNs are composed of connected neurons and aim to find patterns in data under study (14). Where a classic statistical analysis may fail to distinguish complex or nonlinear patterns, ANNs are considered as attractive alternatives (15). They are able to produce predictive models in different pharmaceutical applications (16).

In our previous report, we reported a Dox delivery system based on β-1,3-glucan polymer. The nanoparticles were designed through both conjugated and unconjugated Dox to introduce the drug into $HER2^+$ breast cancer cells. The system released conjugated Dox as a function of pH variations [1]. In this study, we used ANNs to determine effect of four independent parameters, namely amount of NaOH, amount of succinic anhydride (Sa), ratio of Dox/Carrier and amount of polyethylenimine (PEI), on loading efficiency on both conjugated) Con-Dox-Glu(and unconjugated (Un-Dox-Glu(delivery system. The present study for the first time evaluates the possible role of theses parameters on Dox loading efficiency.

MATERIALS AND METHODS

Materials

Curdlan (β-1,3-glucan) was provided from Sigma-Aldrich (USA). Doxorubicin hydrochloride was purchased from Ebewe Pharma (Austria) and other reagents/materials used in this study were obtained from Merck chemicals (Germany).

Preparation of carrier (Glu–Sa–PEI)

Products were obtained according to our previous procedure [1]. Briefly, N-succinated form of Glu was prepared by adding Glu (200 mg, MW 18 kDa) to a round-bottom flask in deionized water (DI, 10 ml) and magnetically stirred with succinic anhydride (Sa) and 3 N NaOH overnight at 25 °C. After 24 h, the white solution was dialyzed against DI water and lyophilized at −30 °C.

To facilitate grafting of PEI to Glu–Sa, Sodium Periodate ($NaIO_4$) was used to oxidize Glu–Sa was (30 min, 50 °C, constant stirring). PEI, dissolved in hot DI, was then added to the oxidized Glu-Sa and stirred for 6 h at 70 °C to obtain Glu–Sa–PEI as product.

Afterwards, Dox was encapsulated into the carrier (Glu–Sa–PEI) by either of conjugation or unconjugation methods.

Preparation of conjugated nanoparticles (Con-Dox-Glu)

Solution of Dox in DMSO was added to Glu–Sa–PEI at different concentrations to obtain different Dox/Carrier ratios (0.05- 0.5). The mixture was then added to DI water under constant sonication (amplitude level of 50%) at injection rate of 1mL/min. Obtained dispersion was then lyophilized to be stored for further uses (see supplementary information).

Preparation of unconjugated nanoparticles (Un-Dox-Glu)

To load Dox into the carrier without conjugation, Dox was dissolved in DMSO (0.5 mL) and added to Glu–Sa–PEI (2 mg/mL) at different Dox/Carrier ratios (0.05- 0.5). The obtained dispersion was then dialyzed (MWCO 12 kDa) in a dark place. Obtained nanoparticles were then lyophilized and kept for future works.

Artificial Neural Networks (ANNs) study

In our study, ANNs were used to find relationships of input parameters, including amount of used Sa (200- 230 mg), NaOH (1.3- 2.5 mL) and PEI (110- 150 mg) as well as ratio of Dox/Carrier (0.05- 0.5), on the output parameter (i.e. loading efficiency of Dox) in Con-Dox-Glu and Un-Dox-Glu, using INForm v4.02 (Intelligensys, UK).

In total, 50 samples were prepared based on a random design for the input parameters and Dox loading efficiency was measured for the samples. Afterwards, the data were divided randomly into three data sets: training data (38 data) to train the network and establish probable relations, test data (4 data) to avoid overtraining the network and unseen data (8 samples) to validate the model (see Table 1). Subsequent to modeling process, the training parameters as listed in Table 2 and reported previously (17), were employed to generate response surfaces (in form of 3D graphs) to illustrate relationships between the input and the output parameters. The 3D graphs show relations of two input parameters on the output variable when the remaining input parameters are fixed at their medium value. Coefficient determination (R2) was computed for training, test and unseen data based on equation 2. R2 values closer to unity indicate preferable predictability for the model.

$$R2 = 1 - \frac{\sum_{i=0}^{n}(yi - \dot{y})^2}{\sum_{i=0}^{n}(yi - \bar{y})^2} \quad (2)$$

Where the predicted values by the model and the mean of actual variables are represented by $\dot{y}$ and $\bar{y}$, respectively.

RESULTS

Loading efficiency of Dox in the two preparations was determined and found to be in the range of 46% to 70% for Con-Dox-Glu and 14% to 34% for Un-Dox-Glu. Afterwards, effects of the four input variables (Sa, NaOH, PEI, and Dox/Carrier ratio) on the drug loading efficiency were studied through ANNs modeling. The most suitable predictive model showed R^2 values of 97.5%, 88.8% and 88.9% for training, test, and unseen data, respectively. The model was then employed to evaluate the effect of the input variables on Dox loading efficiency.

Table 1. Unseen data which were employed to validate the obtained model

Sample	Input parameters				Output parameter	
	Dox/Carrier	Sa (mg)	NaOH 3N (mL)	PEI (mg)	Loading efficiency (%)	
					Obtained	Predicted
Con-Dox-Glu	0.05	230	1.5	110	23	26.2
Con-Dox-Glu	0.2	230	1.3	110	22	24.3
Con-Dox-Glu	0.25	220	1.7	130	24	14.4
Con-Dox-Glu	0.5	200	2.0	150	22	31.5
Un-Dox-Glu	0.05	230	1.5	110	51	50.7
Un-Dox-Glu	0.2	230	1.3	110	65	65.3
Un-Dox-Glu	0.25	220	1.7	130	59	69.4
Un-Dox-Glu	0.5	200	2.0	150	57	59.8

Table 2. Training parameters set within the software

Network Structure	Number of Hidden Layers	1
	No. of nodes in hidden layer	5
Back propagation type		QuickProp
Transfer function	Output	Linear
	Hidden layer	Asymmetric Sigmoid

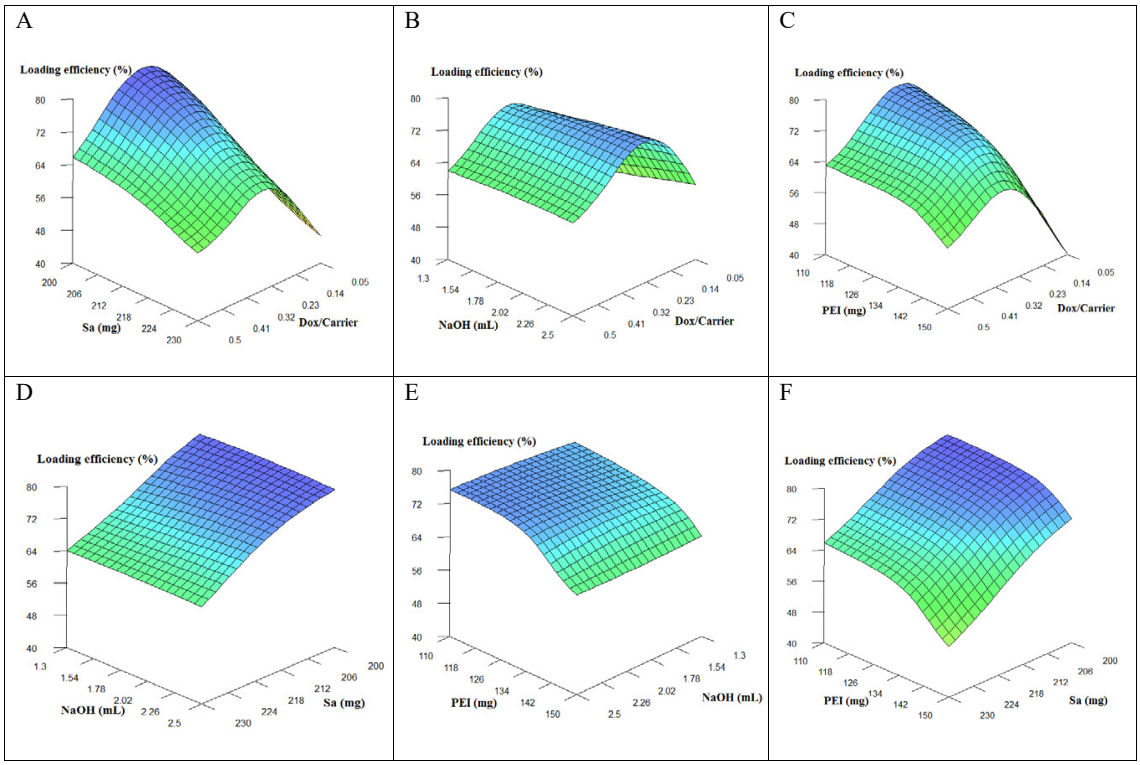

Fig. 1. 3D plots generated by the ANNs model about Dox loading efficiency (%) in nanoparticles containing conjugated Dox (Con-Dox-Glu) as a function of A) amount of Sa (succinic anhydride) and Dox/Carrier ratio, B) amount of NaOH 3 N and Dox/Carrier ratio, C) amount of PEI (polyethyleneimine) and Dox/Carrier ratio, D) amount of NaOH 3 N and Sa, E) amount of PEI and NaOH, F) amount of PEI and Sa

Determination of input parameters affecting Dox loading efficiency in Con-Dox-Glu

Fig. 1. Shows the effect of two input parameters on Dox loading efficiency when the other two variables are fixed at their medium level. Fig. 1A shows the loading efficiency of Dox *vs.* Dox/Carrier ratio and Sa amount (mg) in the Con-Dox-Glu formulation where amount of NaOH and PEI are fixed at medium value (1.9 mL and 130 mg, respectively). The results indicate that addition of Sa causes reduction in loading efficiency. In addition, maximum efficiency can be observed in medium range of Dox/Carrier ratio (i.e., ~ 0.25).

The effect of NaOH amount and Dox/Carrier ratio on loading efficiency is also evident in Fig. 1B, in which amount of Sa and PEI are fixed at 215 mg and 130 mg, respectively. In general, the effect of NaOH on loading efficiency of Dox appears to be negligible. Meanwhile, effect of Dox/Carrier ratio is important with maximum loading efficiency at ~ 0.25.

In Fig. 1C amount of Sa and NaOH are fixed at medium value (215 mg and 1.9 mL, respectively), to determine the effect of Dox/Carrier ratio and PEI on loading efficiency. From the details, the loading efficiency slightly decreases as PEI amount increases from 110 to 150 mg and the dominant effect is from Dox/Carrier ratio which indicates a peak at around 0.25.

In remaining parts of Fig. 1 (D, E, and F), the interactions between other input parameters on the loading efficiency of Dox are shown. As observed, increasing Sa and PEI leads to reduced loading efficiency, while the effect of NaOH on loading efficiency does not appear to be important, findings which have been reported above.

Determination of variables affecting Dox loading efficiency in Un-Dox-Glu

Fig. 2 shows effect of independent variables under study on Dox loading efficiency in Un-Dox-Glu. The graph in Fig. 2A which illustrates the effect of Sa and Dox/Carrier ratio on loading efficiency, indicates that increasing Sa in the formulation decreases the loading efficiency. Ratio of Dox/Carrier shows a complex effect: minimum loading

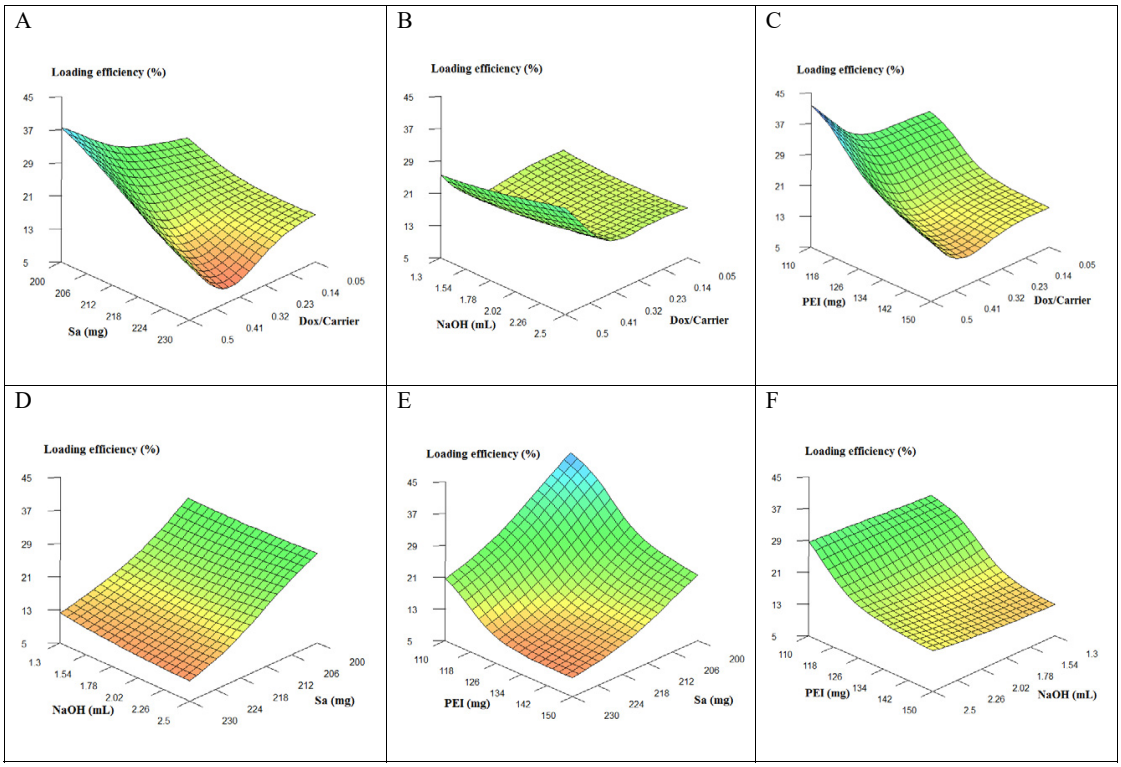

Fig. 2. 3D plots generated by the ANNs model about Dox loading efficiency (%) in nanoparticles containing unconjugated Dox (Un-Dox-Glu) as a function of A) amount of Sa (succinic anhydride) and *Dox/Carrier ratio*, B) amount of NaOH and *Dox/Carrier ratio*, C) amount of PEI (polyethyleneimine) and *Dox/Carrier ratio*, D) amount of NaOH and Sa, E) amount of PEI and Sa and F) amount of PEI and NaOH.

efficiency is observed in Dox/Carrier ratio ~ 0.35 and Dox/Carrier values of above or below this value enhance the loading efficiency. In addition, from the Figure, Sa amount in the formulation is more effective on the loading efficiency compared with Dox/Carrier ratio.

Graph 4B is about the effect of NaOH and Dox/Carrier on loading efficiency where PEI and Sa are fixed at 130 mg and 215 mg, respectively. From the details, the effect of NaOH variation is negligible, while Dox/Carrier shows a small effect on loading efficiency: the lowest efficiency is observed in the medium values (~ 0.35) of Dox/Carrier rati.

Fig. 2C details the impact of PEI and Dox/Carrier where the other parameters are fixed. The Figure indicates that increasing the amount of PEI can reduce the loading efficiency. Also, Dox/Carrier ratio of ~ 0.35 shows minimum loading efficiency. Furthermore, it is evident that PEI is a more influential factor compared with Dox/Carrier ratio.

Figs. 2D, E and F also show that increasing Sa and PEI is accompanied by a decrease in the loading efficiency while NaOH does not influence it, findings which have been mentioned above.

DISCUSSION

In this study, after successful training and validation, the developed models were employed to evaluate the effect of different independent parameters on Dox loading efficiency in two different types of formulations (i.e., a conjugated and an unconjugated form). In conjugated form, Succinic anhydride (Sa) was selected for grafting onto Glu. Dox was conjugated with the carrier through an amide bond between the amine group from Dox and acid groups from Sa (see Supplementary Figure). In unconjugated form, formation of hydrogen bonds between -OH groups of Dox and –NH/-OH groups of the carrier is believed to be the main interaction type in the encapsulation process.

It has already been reported that drug loading efficiency is highly affected by mechanism of loading of the drug: while physical and electrostatic adsorptions usually lead to low drug loading

efficiency, covalent bonds as well as crystals are expected to provide high efficiency of drug loading (4). However, in many cases, physical entrapment of drugs is usually preferred due to its ease of preparation (18). Our results also show that conjugation of Dox to the carrier make high loading efficiency while in unconjugated form, loading efficiency is substantially smaller.

In conjugated form, the ANNs results showed that increasing Sa amount reduced the loading efficiency. Similar finding was obtained for PEI. It is arguable that increasing PEI makes steric hindrance as well as electrostatic repulsion (19, 20) which prevents Dox molecules to become close enough to the Sa chains. Therefore, less loading efficiency is expected. However, we could not find a reason for the effect of Sa of loading efficiency.

Another important factor in determining loading efficiency was Dox/Carrier ratio. An optimum value for the ratio is required to provide maximum loading efficiency. Apparently, above a certain level of Dox, the carrier is not able to load more Dox molecules, thus, loading efficiency becomes less.

Results of unconjugated form were more or less similar to the conjugated form: Increasing Sa decreases loading efficiency. We believe that Sa contribute to covering active sites of the carrier, thus, reduce possibility of the drug interacting with the surface of the polymer. This is in contradiction with a previous report in which Sa made increase in loading efficiency of a succinylchitosan formulation. In the report succinylchitosan exhibited higher loading compared with a liposomal doxorubicin formulation (21, 22). Formation of ionic interactions in addition to other hydrophobic/hydrophobic interactions were claimed to be responsible of Dox loading in this formulation (23).

Our findings also showed that increasing PEI decreases loading efficiency. PEI was added to the nanoparticles to achieve higher transfection efficiency. PEI facilitates interaction of the nanoparticles with negatively charged cell membranes of tumor cells by creating cationic charge on the surface of the nanoparticles (24). Moreover, covalent binding between hydrophobic DOX and hydrophilic PEI helps self-assembly into nanoparticles (25). However, it appears that PEI by steric hindrance or electrostatic repulsion decreases loading of Dox in the preparation, as mentioned above. A previous study indicated that by shifting zeta potential of carrier towards more negative values, loading efficiency of calcitonin (positively charged peptide) increases, due to formation of ionic interactions (26).

The effect of Dox/Carrier ratio on loading efficiency in Con-Dox-Glu was also studied. Increasing Dox/Carrier ratio up to 0.25 leads to increasing Dox loading efficiency, and subsequently loading is reduced. It is arguable that by increasing the carrier content up to a certain level, more active sites are provided for formation of efficient conjugations. However, above a limit, PEI molecules make a positive charge density in the carrier, thus, repel the positively charged Dox molecules (27).

Compared with conjugated form, the effect of Dox/Carrier ratio on loading efficiency was different: at a certain value, minimum loading efficiency was observed while Dox/Carrier ratios above or below this point made the loading efficiency slightly higher. This result may be due to two counteracting effects: while at lower DOx/Carrier ratio, Dox and PEI repel each other due to their positive charges, at higher Dox/Carrier ratio, hydrophobic interactions may overcome the electrostatics repulsion.

CONCLUSION

In summary, a novel β-1,3-glucan nanosystem was prepared as a potential drug delivery carrier of Dox. Using an ANNs model, it was found that the dominant parameters affecting loading efficiency of Dox are concentration of succinic anhydride and PEI.

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The authors report no conflicts of interest in this work.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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