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Original Article

Stealth lipid coated aquasomes bearing recombinant human interferon-α-2b offered prolonged release and enhanced cytotoxicity in ovarian cancer cells

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ABSTRACT

Purpose: In present investigation, recombinant human interferon-α-2b (rhINF-α-2b) loaded aquasomes were prepared, optimized and overlaid with PEGylated phospholipid to offer prolong release and high therapeutic index against ovarian cancer, SKOV3 cells.
Methods and results: Central Composite Design (CCD) and Response Surface Methodology (RSM) were employed to calculate the optimized conditions, 1:3 core to coat ratio, sonication power of 12.5 W and time of about 55 min for preparation of aquasomes. Consequently, rhINF-α-2b-Py-5-P-Aq.somes exhibited higher protein loading capacity and retained structural conformations of rhINF-α-2b, as compared to rhINF-α-2b-Cellob-Aq.somes, rhINF-α-2b-Tre-Aq.somes and rhINF-α-2b-Core ($CaHPO_4$). Further, optimized rhINF-α-2b-Py-5-P-Aq.somes was superimposed with phospholipid-PEG_{2000} to prolong the release pattern of rhINF-α-2b from aquasomes. The rhINF-α-2b-core ($CaHPO_4$) released 97.3% of protein in 1 h, while 95.3% of rhINF-α-2b was released by rhINF-α-2b-Tre-Aq.somes in 4 h. Concurrently, rhINF-α-2b-Cellob-Aq.somes and rhINF-α-2b-Py-5-P-Aq.somes released 96.2% and 97.8% of rhINF-α-2b respectively in 6 and 8 h. Ultimately, rhINF-α-2b-Py-5-P-Aq.somes-P-PEG_{2000} displayed evidence of its prolonged release pattern and released 98.1% of rhINF-α-2b in 336 h. FT–IR and XRD substantiated the involvement of vigorous intermolecular hydrogen bonding and amorphous geometry in rhINF-α-2b-Py-5-P-Aq.somes. In last, rhINF-α-2b-Py-5-P-Aq.somes-P-PEG_{2000} exhibited the ~ 4.55, 1.92, 2.3, 2.8, and 3.84 fold reductions in IC_{50} as compared to free rhINF-α-2b, rhINF-α-2b-Py-5-P-Aq.somes, rhINF-α-2b-Cellob-Aq.somes, rhINF-α-2b-Tre-Aq.somes and rhINF-α-2b-Core ($CaHPO_4$), respectively.
Conclusion: Therefore, rhINF-α-2b-Py-5-P-Aq.somes-P-PEG_{2000} warrant further in depth *in vitro* and *in vivo* antitumor study to scale up the technology for clinical intervention.

1. Introduction

Recombinant human interferon-α-2b (rhINF-α-2b), a single non-glycosylated USFDA approved cytokine is recommended to co-administer with cisplatin to elicit cytotoxicity against platinum resistant ovarian cancer cells [1]. In addition, rhINF-α-2b induces mitochondrial-mediated apoptosis in cancer cells, including activation of caspases-3 mediated pathway [2]. Chemically, rhINF-α-2b is a polypeptide chain of 165 amino acids [3]. However, short serum half-life (2–6 h), upregulation of P-glycoprotein mediated multi-drug resistance (P-gp-MDR), low therapeutic index, fluctuated plasma level, and rapid proteolytic degradation hamper the systemic drug delivery [2,4–6]. This necessitates the administration of rhINF-α-2b in a sustained release pattern for successive chemotherapy.

Parenteral route of administration is the most common method for delivery of therapeutic proteins; however, it does not enhance the half-life of proteins *in vivo* [7]. Furthermore, poly(ethylene glycol) mediated stealth nanocomplex of rhINF-α-2b, despite

Abbreviations: rhINF-α-2b, Recombinant interferon-α-2b; Tre, Trehalose; Cellob, Cellobiose; Py-5-P, Pyridoxal-5-phosphate; PVA, Poly(vinyl) alcohol.

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excellent release profile is suffered with immunogenicity, antigenicity and low mean residence time [8,9]. Therefore, a stable drug delivery system is required for safe homing of rhINF-α-2b to gain maximum clinical benefits [2,6,10].

Several attempts have been made to vesiculize rhINF-α-2b for clinical intervention by using nanoparticles and microparticles [11–13]. Aquasomes are self-assembled ceramic nanoparticles whose surface can be non-covalently modified with carbohydrates [14]. Instrumentally, aquasomes congregate through non-covalent bonds, ionic bonds and Van der Waals forces [15]. Polysaccharide coating provides glassy molecular layer that adsorbs therapeutic protein without alteration in three-dimensional conformations. In addition, ceramic core superimposed with carbohydrates enhanced the cellular uptake in cancer cells [16,17]. Hence, aquasomes have been extensively investigated for delivery of both small and high molecular weight, pharmaceuticals [18,19]. However, aquasomes do not tender sustained release of proteins due to adsorption rather than encapsulation phenomena. Therefore, in present investigation, rhINF-α-2b was adsorbed on to the different polysaccharide coated self-assembled ultrafine ceramic nanoparticles. Process parameters that influence the synthesis of aquasomes were optimized by using Central Composite Design (CCD) [20] and Response Surface Methodology (RSM) computational techniques [21]. The optimized nanoformulation was then superimposed with phospholipid-PEG_{2000} (PEGylated phospholipid) to induce the sustained release pattern. It is reported that PEGylated phopholipid retains the pharmacological efficacy of protein, escapes macrophage uptake, and helps to attain high blood level [22,23]. The unique property of PEGylated phospholipid coating is that it allows release of therapeutic component from the drug delivery system through diffusion mechanism [23]. Aquasomes were characterized for particle size, zeta potential, protein loading capacity, *in vitro* release, protein stability and cytotoxicity against human ovarian cancer cell line, SKOV3.

2. Materials and methods

2.1. Materials

Recombinant human interferon-α-2b (rhINF-α-2b, Molecular weight ~ 17 Kda) was obtained as a gift sample from Reliance Life Sciences, Navi Mumbai, India. Trehalose (Tre) and cellobiose (Cellob) were obtained from Loba Chemie, New Delhi, India. Pyridoxal-5-phosphate (Py-5-P) was purchased from Central Drug House, New Delhi, India. All other chemicals used were of highest analytical grade.

2.2. Cell culture

Human ovarian cancer cell line, SKOV3 was maintained in 95% air and 5% CO_2 atmosphere at 37 °C using DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum. All experiments were performed with asynchronous cell populations in exponential growth phase (24 h after plating) [24].

2.3. Experimental design for optimization

The core ($CaHPO_4$) to coat (polysaccharide) ratio was optimized by Central Composite Design (CCD) [20] to get Response Surface Methodology (RSM) [21] that selected an object, noticed the contributing factors and investigated the relationship between responses and factors. CCD enabled several independent variables to be investigated at the same time using a relatively small number of experiments, while RSM optimization analyzed the interactions between variables. Therefore, CCD-RSM defined the interactions between factors, avoided unwanted experiments and optimized the results [25]. Our preliminary investigation indicated that variables, such as core to coat (mg) ratio, sonication power (W) and sonication time (min) were the main factors that influenced the particle size of nanoformulations. A CCD model was used to statistically optimize the factors that affected the particle size of aquasomes. For each factor, experimental range was selected on the basis of previous investigations and probability of preparing the aquasomes at extreme values. The value range of variables were core:coat ratio (x_1): 1:1 to 1:5 (mg), sonication power (x_2): 5–25.11 (W), and sonication time (x_3): 20–113.86 (min). The design consisted of 20 runs (8 factorial points, 6 star points and 1 centre point) and 5 replicated runs (centre points) that yielded total 20 experiments (Table 1). The purpose of replication was to estimate the experimental errors and increase the accuracy. Each experimental run was repeated thrice ($n = 3$). Star points represented the extreme values (low and high) for each factor in the design and allowed estimation of second-order effects. Star points were at some distance, alpha, from the centre, based on the properties desired for design and number of factors in the design. Alpha in coded units was the axial distance from the centre point and made the design rotatable. A rotatable design provides equally good predictions at points equally distant from the centre, a very desirable property for RSM [21].

Table 1
Optimized conditions calculated by central composite design (CCD) and response surface methodology (RSM) for preparation of aquasomes.

Runs	Core:coat (mg) (X_1)	Sonication power (W) (X_2)	Sonication time (min) (X_3)	Particle size (nm)
1	1	20	20	70.6
2	3	–0.113	55	135.8
3	3	12.5	55	90.1
4	1	5	90	125.7
5	5	20	90	130.4
6	3	12.5	55	90.4
7	3	12.5	-3.86	138.4
8	–0.36	12.5	55	82.3
9	6.363	12.5	55	100.4
10	1	20	90	94.2
11	3	12.5	55	91.6
12	5	5	90	115.4
13	3	12.5	55	92.1
14	3	12.5	55	90.8
15	5	5	20	117.2
16	3	25.11	55	88.4
17	1	5	20	98.4
18	3	12.5	113.86	79.3
19	5	20	20	100.2
20	3	12.5	55	91.1

Core: $CaHPO_4$; Coat: polysaccharide (Trehalose; cellobiose and pyridoxal-5-phosphate).

A design matrix comprising 20 experimental runs was constructed and responses were modelled by the following reduced linear model (Eq (1)):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b \quad (1)$$

where y is the measured response associated with each factor level combinations; b_0 is the intercept; b_i's (for $i = 1$, 2 and 3) are the linear effects, the b *ii* are the quadratic effects, the b *ij*'s (for i, $j = 1$, 2 and 3, $i < j$) are the interactions between the i_{th} and i_{th} variables. The statistical analysis was performed by using the software Design Expert (Version 8.0.7.1), where analysis of variance (ANOVA) was significant, when $P < 0.05$. An F-test was used to determine whether there was an overall regression