Liposomal β-Glucan: Preparation, Characterization and Anticancer Activities

Majed Halwani¹, Zakir Hossain², Mohammad A Khiyami³ and Abdelwahab Omri*²

¹King Saud bin Abdulaziz University for Health Sciences, King Abdullah International Medical Research Center, Riyadh, Saudi Arabia
²Richardson Centre for Functional Foods and Nutraceuticals, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, R3T 2N2, Canada
³King Abdulaziz city for Science and Technology, Riyadh, Saudi Arabia
⁴The Novel Drug and Vaccine Delivery Systems Facility, Department of Chemistry and Biochemistry, Laurentian University, Sudbury, Ontario, P3E 2C6, Canada

Abstract

Context: An assortment of 1,3-β-glucans have been explored for their structural and pharmacological capabilities. The immunomodulatory effects of 1,3-β-glucans have served to fight cancer.

Objectives: The aim of this work was the investigation of the physicochemical characterizations of 1,3-β-D-glucan encapsulation of liposomes and its effects on human lung carcinoma epithelial cell line A549.

Materials and Methods: β-glucan was encapsulated into liposomes composed of different lipids and cholesterol [PC, PC:Chol (6:1), DDAB:DPPC:Chol (4:2:1), DCP:DPPC:Chol (4:2:1), DPPC:Chol (6:1), DSPC:Chol (6:1), DSPC]. The liposomal formulations were prepared by the hydration-dehydration method. The mean diameter of liposomes and the polydispersity index were determined by photon correlation spectroscopy (PCS) with the use of Submicron Particle Sizer. The hemocompatibility of liposome on human erythrocytes was evaluated. The MIT assay was also performed to evaluate the A549 cells viability.

Results: The mean particle size of vesicles loaded with β-glucan varied from 130.20 ± 10.5 to 180.10 ± 11.3 nm. The polydispersity index ranged from 0.66 ± 0.0004 to 0.90 ± 0.08. The encapsulation efficiencies in DDAB:DPPC:Chol, DCP:DPPC:Chol and DPPC:Chol formulations were 17.20 ± 0.08%, 23.60 ± 0.20% and 18.23 ± 0.06%, respectively. The hemolysis rates did not exceed the negative control value by more than 2% and 8% at 10 and 24 h, respectively. These three formulations showed higher growth suppression of A549 cell compared to free β-glucan. This growth suppression of A549 cell by 1 µM doxorubicin was further promoted by liposomal β-glucan.

Conclusion: Liposomal 1,3-β-D-glucan might potentially enhance antitumor activity.

Keywords: β-glucan encapsulated liposome; Stability of liposome; Hemocompatibility; Cytotoxicity

Introduction

Glucans are structurally distinct polysaccharides consisting of D-glucose units linked by (1→3)-β and (1→6)-β glycosidic linkages [1]. Glucans demonstrated to control colon cancer cell proliferation via direct interaction of the glucan with the colon cancer cells and their apoptosis induction [2]. Glucan extracted from mushroom Agaricus blazei stimulated apoptosis or programmed cell death in human ovarian cancer HRA cells [3]. β-glucans in particular those extracted from fungi or yeasts have shown anticarcinogenic effects both in vivo and in vitro [4,5]. Barley β-glucan administration in mice enhanced the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models [6]. Mushroom extract, Antrodia camphorate showed promising therapeutic potential due to its cytotoxic effects against human pancreatic cancer BxPC-3 cell line which include inhibiting cell migration and inducing mitochondria-mediated apoptosis [7]. Mushroom β-glucan and resveratrol lowered the number of metastases in Lewis lung cancer model [8]. As an immunomodulating agent, β-glucan acts through the activation of innate immune cells such as macrophages, dendritic cells, granulocytes, and natural killer cells. This activation triggers the responses of adaptive immune cells such as CD4(+) or CD8(+) T cells and B cells, resulting in the inhibition of tumor growth and metastasis [9]. β-D-glucan regulates breast cancer-relevant gene expression and may be useful for inhibiting endocrine-resistant breast cancer cell proliferation [10]. Beta glucan has the potential to be used as a complementary or adjuvant therapy for improving quality of life in breast cancer patients in combination with cancer therapies [11]. Irradiated β-D-glucan exhibited three human cancer cell lines including Colo-205, T47D and MCF7 growth inhibition through enhancing antioxidant and antiproliferative activities [12].

Liposomes can entrap drugs inside their aqueous interior and/or into their bilayer [13]. These vesicles entrap in their interior volume part of the water phase and consequently can capture and segregate polar molecules. Moreover, because of the physicochemical properties of their constituents, they can also dissolve hydrophobic molecules in their bilayers. The properties of liposomes in addition to the general properties of surfactants, those make them useful for different applications are the structural thermodynamic and kinetic stability on dilution and ability to entrap both water soluble and insoluble substances and deliver them into desired environments. The liposomes are widely used for entrapment of therapeutic agents including a wide variety of drug molecules, especially anticancer drugs, and biomaterials. In addition, liposomal formulations are often proposed for the improvement of the therapeutic action of...
The mean diameter of liposomes and the polydispersity index were determined by photon correlation spectroscopy (PCS) with the use of Submicron Particle Sizer, Model 270 (Nicomp, Santa Barbara, CA, USA). All liposomes were analyzed and Gaussian distribution was chosen based on our facility standard. Polydispersity index of 0.0 represents a homogeneous particles population while 1.0 indicates a heterogeneous size distribution in the liposome preparations.

**Stability of liposomes loaded with β-glucan**

The stability of liposomes loaded with β-glucan was evaluated in PBS and in normal human pooled plasma as we previously described [19]. Briefly, appropriate amount of liposomes was suspended in PBS (pH=7.4) and incubated at 4°C or 37°C with a mild agitation (100 rpm). Likewise, to mimic the physiological conditions, normal human pooled plasma was supplemented with liposomes and incubated at 37°C with a mild agitation. After incubation periods of 0, 0.25, 0.5, 1, 3, 6, 12, 24 and 48 h, samples were removed and centrifuged (18,300 × g for 15 min at 4°C) to remove the leaked β-glucan. The concentrations of β-glucan were determined by Fungitec G test as described above. The stability of Liposomes was evaluated by determining the amount of β-glucan released over a 48 h study period. β-glucan release was expressed as percentage of the concentration of the entrapped β-glucan measured by Fungitec G test.

**Erythrocyte hemolysis assay**

An erythrocyte hemolysis assay was performed according to the method outlined by [20,21]. Whole, heparinized human erythrocytes (provided by Dr. Onuska, St. Joseph’s Hospital, Sudbury ON) were pooled and centrifuged at 700 × g for 10 min at 4°C. The erythrocytes were washed two times, using centrifugation at the above mentioned specifications, with ten times their volume of an ice cold buffer (147 mM NaCl, 6 mM glucose, and 20 mM of Tris-HCl (pH 7.2). After washing, the erythrocytes were re-suspended in twenty times their volume of fresh, ice cold buffer. This suspension was stored at 4°C and was used within ≤ 2 weeks of preparation [20]. To perform the hemolysis assay, a 3% erythrocyte solution was prepared by suspending the appropriate volume of erythrocytes into a hemolysis buffer solution (74 mM NaCl, 147 mM sucrose, 6 mM glucose; 20 mM Tris-HCl (pH 7.2). Erythrocytes were incubated in a shaking water bath, under gentle agitation, at 37°C with liposomes in concentrations ranging from 50-500 µg/mL for up to 24 hrs. After 4, 10 and 24 hour incubation, samples were removed and centrifuged at 1000 × g for 10 min at 4°C. The supernatant was removed and was centrifuged at 15 000 × g for 15 minutes at 4°C. The higher centrifugation force was required to ensure that the liposomes were completely removed from the suspension. This did not result in the sedimentation of hemoglobin [22]. The supernatant was removed and the amount of hemoglobin release was measured spectrophotometrically at 540 nm (reference at 625 nm) [23]. Controls included 3% erythrocytes (representing 0% hemolysis), and red blood cells treated with 1% Triton X-100 (representing 100% red blood cell lysis). A blank consisting of hemolysis buffer alone was also included. The hemolysis buffer blank was subtracted from all measurements, and the % hemolysis was calculated from the following equation:

\[
\%\text{Hemolysis} = \left(\frac{Abs_{\text{Sample}} - Abs_{\text{Control}}}{Abs_{100\%\text{Lysis}}}\right) \times 100
\]

Materials with a hemolysis ratio less than 5% were regarded as hemocompatible [24].

**In vitro cytotoxicity assays**

The cytotoxicity of the β-glucan containing liposomes was evaluated using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The human lung carcinoma epithelial cell line A549 (provided by Dr. Azghani, University of Texas), was cultured in DMEM (Hyclone) supplemented with 10% FBS. Cells
were maintained using standard cell culture procedures, through incubation in a humidified atmosphere at 37°C, 5% CO₂. Cells were passaged at sub-confluency, and all cells used were of late passage. At sub-confluency, the cells were seeded into 96-well plates at a density of 5 × 10⁵ cells/well. In all instances, cells were allowed to adhere overnight, prior to treatment. Next, the cultures were incubated with 5, 10, 15, 20 and 40 mg/ml of liposomal β-glucan for 24 h. The nanoparticles were removed by careful washing with PBS. Then, 200 μl of MTT solution (0.5 mg/ml in PBS) was added and incubated for another 4 h at 37°C. The reaction product was solubilized in 200 μl DMSO and absorbance (Abs) was measured at 570 nm. For all studies, negative controls included untreated cells (media alone) and media containing 50% D-PBS (HyClone) (this would accommodate the maximum amount of PBS that could be released from the empty liposome formulations. H₂O₂ was chosen as a positive control, as it has been shown that H₂O₂ can induce apoptosis in the A549 cell line [25]. The cell viability was calculated using the following formula: cell viability=(Absorbance of sample/Absorbance of negative control) × 100. The viability of untreated controls was normalized to 100%. Cytotoxicity=100- % cell viability. All studies are expressed as the mean of a minimum of three separate experiments, ± SEM in triplicate.

**Data analysis**

The data are expressed as means ± SEM of three independent experiments in triplicates. Comparisons were made by paired Student’s t-test and P ≤ 0.05 was considered significant. For multiple comparisons within and between groups, ANOVA with the two-tailed Dunnett’s test post test analysis was used.

**Results**

**Particle size, encapsulation efficiency and polydispersity index of liposomes**

The mean particle size of liposomes with different lipid composition are varied from 125.90 ± 15.01 to 180.10 ± 11.3 and the encapsulation efficiency was varied from 2.23 ± 0.03 to 23.60 ± 0.2 (Table 1). The encapsulation efficiency of DCP; DPPC: Chol (4:2:1), DPPC: Chol (6:1) and DDAB: DPPC: Chol (4:2:1) were significantly higher (p<0.0001) than other groups. The polydispersity index of the liposomes indicates heterogeneous in nature. The liposomal β-glucan encapsulation efficiency (%EE) of liposome formulations was in the following order: DCP: DPPC: Chol (4:2:1)>DDPC: Chol (6:1)>DDAB: DPPC: Chol (4:2:1)>PC: Chol (6:1)>DSPC:PC>DSPC: Chol (6:1).

**Stability of liposomes loaded with β-glucan**

The stability studies of liposomes loaded with β-glucan were performed in PBS and plasma at 4°C (storage temperature) and 37°C (body temperature). The study was performed for a period of 48 h at intervals of 0, 1, 3, 6, 12, 24 and 48 h. It was evident from the stability data that liposomal β-glucan in PBS and plasma at 4°C was more stable compared to that stored at 37°C. In PBS at 4°C the highest retention was more than 95% for DPPC: CHOL formulation and at 37°C the highest retention was more than 95% for PC: CHOL formulation. However, in plasma the DPPC: CHOL formulation has highest retention of more than 80% at 4°C and 70% at 37°C. Among all, DDAB: CHOL is the only formulation has significantly higher (p<0.0001) retention in comparison to other formulations (Figure 1).

**Hemocompatibility of liposomes loaded with β-glucan**

In order to investigate the toxicity of different liposomal β-glucan formulations, the liposomes with different concentrations were incubated with human erythrocytes for 4, 10 and 24 h. The amount of hemoglobin released was measured spectrometrically and results are indicated in Figure 2A. After 4 h of incubation, no significant hemolysis was observed with different liposomal β-glucan formulations, the hemolytic activities were less than 1%. After 10 h of incubation, PC: CHOL is the only formulation at different concentrations showed significantly hemolysis (p<0.0001) than 1% and all the remaining formulations were less than 1%. Finally after 24 h of incubation, all the liposomal β-glucan formulations exhibited hemolysis range of 8-10% with increasing concentrations of 0–40 µg/mL. Remarkably, DCP; DPPC: CHOL formulation did not follow this pattern as the data showed decreased hemolysis at 24 hr for 10 µg/mL. The general pattern for all liposomal formulations was that the % of hemolysis increased over time, and with increasing concentrations.

**Cytotoxicity of liposomes**

The A549 cell cultures were incubated with 5, 10, 15, 20 and 40 mg/ml of liposomal β-glucan of different formulations for 24 h (Figure 3A) and also tested in the presence of Doxorubicin (Figure 2B). The liposomal β-glucan PC is the only formulation that have obtained highest viability of 80% and DPPC: CHOL formulation showed significantly higher (p<0.0001) growth suppression compared with PC and free β-glucan groups. All the other liposomal β-glucan formulations have obtained viability in between the range of 40-80%. The empty liposomal formulations did not show any toxicity and all cells remain viable. In the presence of Doxorubicin (Figure 3B), the highest values of 70% were obtained for liposomal β-glucan PC formulation and in least 30% for liposomal β-glucan DPPC: CHOL formulation (p<0.0001). In between the range of 30-70% all other formulations in the presence of Doxorubicin has obtained viability. However, all the cells remain viable and did not show any toxicity for empty liposomal formulations. In A549 cells, the half maximal inhibitory concentration (IC₅₀) value of free -glucan was 13.94 µg. The IC₅₀ values of PC, PC: Chol (6:1), DDAB: DPPC: Chol (4:2:1), DCP: DPPC: Chol (4:2:1), DPPC: Chol (6:1), DSPC: Chol (6:1), DSPC liposome were 12.01, 11.71, 12.34, 13.26, 11.07, 9.516, and 10.86, respectively (Table 2).
Discussion

The efficiency of liposomal drug delivery depends on physicochemical characteristics such as size, composition, loading efficiency and stability of liposomes. In the current study, we prepared 5 different kinds of liposomes with different lipids and cholesterol where concentration of cholesterol was optimized one-sixth of lipids. The use of high relative cholesterol amount in the lipid fraction of liposomes increased the drug entrapment percentage, due to the stabilizing effect of this steroid into the lipid bilayers [26]. Since it is known that the presence of cholesterol in the phospholipid bilayer can generally improve the stability of liposomes [27], liposomes containing DPPC: Chol molar ratios of 8:1 and 4:1 has been reduced the leakage but still more than 50% of 5(6)-carboxy-fluorescein (CF) was leaked within the first hour of cross-linking. On the other hand, 90% of CF was leaked from the pure DPPC liposomes within the first hour [28].

Since the glucan retention (%) is temperature dependent, the DPPC: Chol (6:1) in PBS showed more stable at 4ºC, 37ºC and plasma with a mild agitation. At times indicated, samples were centrifuged at 18,300 g for 15 min and β-Glucan concentrations in the supernatants were determined by Fungitec G test. Results are given as means ± S.E.M. of three separated experiments in triplicate. ****P<0.0001.

Table 2: Liposomal β-Glucan: The IC50 values were calculated using sigmoidal concentration-response curve-fitting methods (GraphPad Prizm 6 software) and the logit-log equation. In all cases, best fits were obtained with the coefficient of correlation R² ranging from 0.94 to 0.99.

<table>
<thead>
<tr>
<th>Liposome lipid composition</th>
<th>IC50 (µg)*</th>
<th>R²#</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Glucan</td>
<td>13.94</td>
<td>0.959</td>
</tr>
<tr>
<td>PC</td>
<td>12.01</td>
<td>0.987</td>
</tr>
<tr>
<td>PC:Chol (6:1)</td>
<td>11.71</td>
<td>0.990</td>
</tr>
<tr>
<td>DDAB:DPPC:Chol (4:2:1)</td>
<td>12.34</td>
<td>0.983</td>
</tr>
<tr>
<td>DCP:DPPC:Chol (4:2:1)</td>
<td>13.26</td>
<td>0.976</td>
</tr>
<tr>
<td>DPPC:Chol (6:1)</td>
<td>11.07</td>
<td>0.988</td>
</tr>
<tr>
<td>DSPC:Chol (6:1)</td>
<td>9.516</td>
<td>0.940</td>
</tr>
<tr>
<td>DSPC</td>
<td>10.86</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Note: * The concentration of β-Glucan necessary to produce 50% inhibition of cell growth. # R²=the square of the correlation coefficient.

PC: L-α-Phosphatidylcholine; Chol: Cholesterol; DDAB: Didecylamilammonium bromide; DPPC: 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; DCP: dicetyl phosphate; DSPC: 1, 2-Distearoyl-sn-glycero-3-phosphocholine.
in vivo behavior are highly dependent on liposome size [29]. The range of average size of liposomes was 130.20-180.10 nm which can easily transport to the blood stream through intestinal wall. Liposomes were revealed good hemotolerance in relation to major cellular components of the blood. As a confirmation to RBC counting, hemolysis rate was determined in the presence of the formations. The hemolysis rate did not exceed the negative control value by more than 2% at 10 h. However, the hemolysis rate was around 8% at 24 h. Therefore, the liposomes may be considered non-hemolytic [30,31]. The membrane bilayer became more polar in the presence of DPPC lipids and cholesterol, resulting in increased efficiency of incorporation of the hydrophilic drug [32]. In addition, an increase in the content of edge activator may have led to formation of pores in the bilayer [33].

1,3-β-glucans have been used in combinatorial therapies with antibodies in implanted human tumor xenografts from melanoma, epidermoid carcinoma, breast carcinoma, metastatic lymphoma and daudi lymphoma. The mice had higher survival rates in the presence of 1,3-β-glucans as compared to treatment with antibodies alone [34]. Chen et al. [35] showed that β-D-glucans from the mushroom *Fomes fomentarius* has a direct antiproliferative effect in vitro on human gastric cancer cells in a dose- and time dependent manner. Xie et al. [36] showed that *Ganoderma lucidum* glucan extract inhibits proliferation of SW480 human colorectal cancer cells. These examples demonstrate the versatility of 1,3-β-glucans in cancer inhibition and thus these polysaccharides can be modified to suit the desired application. We found significantly higher growth suppression of

**Figure 2:** A) Hemocompatibility of liposomal β-Glucan formulations. B) % Hemolysis after treatment of human erythrocytes with liposomes for a) 4 hrs; b) 10 hrs; and c) 24 hrs, the amount of hemoglobin released was determined, and expressed as a percent hemolysis ± SEM relative to untreated red blood cells. Results are given as means ± S.E.M. of three separated experiments in triplicate. ****P<0.0001.

**Figure 3:** A. Effect of liposomal β-Glucan formulations on cell viability. A549 cell viability after 24 hours treatment with various concentrations of liposomal β-Glucan in absence. B. In the presence of 1µM Doxorubicin. Results are expressed as a percentage of PBS-treated cells. Concentrations are relative to the amount of encapsulated component in the liposomal β-Glucan formulation. NOTE: The empty liposomal formulations did not show any toxicity and all cells remain viable. ****P<0.0001.
human lung carcinoma epithelial cell line A549 with DPPC: Chol=6:1, DDAB:DPPC:Chol (+)=4:2:1 and DCP:DPPC:Chol (-)=4:2:1 liposome which was encapsulated with β-glucan. Our results showed dose dependent growth suppression. The mechanism could be involved in its inhibitory activity on tumor growth by stimulating the release of tumor necrosis factor TNF-a from monocytes/macrophages [37]. 1,3-β-glucans is highly dependent on the activation of T-lymphocytes-mediated adaptive immunity while down-regulate suppressive immune activity via Glucocorticoid-induced TNF receptor ligand interaction, leading to a more efficient defense mechanism against tumor development [38].

Doxorubicin was also encapsulated with β-glucan in liposome as it is one of the main chemotherapeutic agents [39]. It is effective against many types of cancer, including leukemia’s, lymphomas, breast and ovarian cancers, etc., than any other drug [40,41]. The main advantage of the liposomal doxorubicin is the significant reduced dose-dependent cardiotoxicity compared to the conventional form [42]. We found higher growth suppression of A549 cells with doxorubicin when it was delivered with liposomal β-glucans. Doxorubicin affects the uncontrollable division of cells, by inhibiting the activity of topoisomerase II, which prevents the replication of cellular DNA and RNA [43], thus stopping cancer proliferation. However, doxorubicin has high cardiotoxicity, causing damage to the myocardium. Early effects of doxorubicin administration include acute left ventricular dysfunction and arrhythmias and, with repeated use, dilated cardiomypathy was observed that can eventually lead to drug-induced congestive heart failure and is fatal in approximately 50% of cases [44,45]. Other acute side effects of doxorubicin include neutropenia, nausea, arrhythmias, vomiting and alopecia [46]. Encapsulation of doxorubicin and other anti-tumor drugs in liposomes and micelles (especially polymeric micelles) alters the pharmacokinetics and distribution of those agents and thus reduces their cytotoxicity [47-49].

Conclusion

In conclusion, prepared liposome was hemotolerant, liposome formulation with cholesterol was stable in plasma at both 4 and 37 oC and application of liposomal β-glucans can be an adjunct and application of liposomal β-glucans was increased the anticancer activity of doxorubicin. Liposomal β-glucans can be an adjunct advantage of the liposomal doxorubicin is the significant reduced dose-dependent cardiotoxicity compared to the conventional form [42]. We found higher growth suppression of A549 cells with doxorubicin when it was delivered with liposomal β-glucans. Doxorubicin affects the uncontrollable division of cells, by inhibiting the activity of topoisomerase II, which prevents the replication of cellular DNA and RNA [43], thus stopping cancer proliferation. However, doxorubicin has high cardiotoxicity, causing damage to the myocardium. Early effects of doxorubicin administration include acute left ventricular dysfunction and arrhythmias and, with repeated use, dilated cardiomypathy was observed that can eventually lead to drug-induced congestive heart failure and is fatal in approximately 50% of cases [44,45]. Other acute side effects of doxorubicin include neutropenia, nausea, arrhythmias, vomiting and alopecia [46]. Encapsulation of doxorubicin and other anti-tumor drugs in liposomes and micelles (especially polymeric micelles) alters the pharmacokinetics and distribution of those agents and thus reduces their cytotoxicity [47-49].


