J Nanopart Res (2024) 26:27
https://doi.org/10.1007/s11051-023-05913-0

BRIEF COMMUNICATION

Design of a liposome with the property to induce CD16 on cervical cancer cells with a purified spermidine-cholesterol

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Received: 2 October 2023 / Accepted: 13 December 2023

Abstract The purpose of the study is to investigate the use of a purified cationic lipid for fabricate liposomes capable of inducing the appearance of CD16 receptors on the membranes of cervical carcinoma cells. The methods used in this study are chemical synthesis of purified spermidine-cholesterol, liposome fabrication, UPLC-MS analysis, cell culture, and flow cytometry. This study reveals the construction of a liposomal system with a highly purified spermidine-cholesterol with CD16 inducing capacity on cervical cancer cells. We were able to synthetize a highly purified spermidine-cholesterol to fabricate a liposomal system capable to induce CD16 on cervical cancer cells.

Keywords Spermidine-cholesterol synthesis · Liposome · CD16 induction · Cervical cancer · Drug delivery · Nanobiomedicine

Summary.

We significantly enhanced the purity and efficiency of spermidine-cholesterol cationic lipid synthesis, and confirmed using UPLC-MS that the product was highly purified. We then constructed a liposome with this lipid, which formed an 80 nm nanoparticle with preserved and enhanced biological activity against cancer, as evidenced by its induction of CD16 on cervical cancer cells. Finally, we discuss the importance of using pure products in liposomes in pharmaceutical formulations for drug delivery and cancer treatment.

Teaser: Construction of a 80 nm liposome with the property to induce CD16 receptors on cervical cancer cells using a purified spermidine-cholesterol.

Introduction

The application of nanomaterials in the pharmaceutical field offers an innovative landscape that spans from enhancing drug absorption and administration to formulating novel active principles and optimizing existing pharmaceutical forms [1, 2]. This progress is reflected in the remarkable surge of pharmaceutical patents linked to nanotechnology and its integration into drug delivery systems over the last two decades [3, 4]. Liposomes as nanosized vesicles composed of natural or synthetic amphiphilic lipids present significant advantages which include increased dissolution surface, ability to target specific sites,

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Published online: 30 January 2024

modulation of active principle pharmacokinetics, and controlled localized release at the site of action [5]. Although amphiphilic cationic lipids do not exist in nature, their ability to transport acidic molecules such as DNA and RNA has driven research since the late twentieth century [6, 7]. Initially, these lipids exhibited varying levels of toxicity [8, 9], and to overcome this limitation [10], a development of a cationic lipid using cholesterol chloroformate and spermidine, a natural polyamine, resulted in spermidine-cholesterol that demonstrated a higher efficiency and lower toxicity in nucleic acid transfection [11, 12]. Since then, several polyamines for translocation protocols have been developed to introduce genetic material into the cells [13–16]. The use of cationic liposomes with different composition of cholesterol, spermine, and spermidine has shown excellent combination for this type of cellular translocation [17].

A cationic liposome using spermidine-cholesterol was constructed and shown to possess the ability to induce the expression of membrane receptors in cervical cancer cells [18, 19]. When IL-2 was encapsulated, its interaction with the IL-2 receptor on cancer cells produced an in vitro and in vivo apoptotic effect [20, 21], opening the possibility to use these liposomes in anticancer treatments. Nevertheless, the spermidine-cholesterol used presented low purity and poor yield. With the recommended guidelines to use pure molecules to construct nanoparticles for human applications [22], this work was intended to design a new synthetic method to increase not only the purity of spermidine-cholesterol that is not available commercially but also to increase its yield. On the other hand, to evaluate whether a liposome constructed with this cationic lipid conserved its cellular membrane inducing properties on cervical cancer cells.

Methodology

Spermidine-cholesterol synthesis

Our method for the synthesis of non-purified spermidine-cholesterol used spermidine (Sigma-Aldrich S2626) dissolved in chloroform (Sigma-Aldrich 650498), followed by addition of a dissolution of cholesteryl chloroformate (Sigma-Aldrich C77007) also in chloroform. Then, it was placed in an ice bath and allowed to react for 1 h with agitation. Subsequently, chloroform is removed using a rotary evaporator, and after recovering the organic phase, the residue is washed until a viscous yellowish product is obtained. There is no evidence that more than 60 ppm of residual chloroform remains in our formulation in accordance with the toxicologically acceptable parameters (< 60 ppm) [23, 24].

UPLC-MS analysis of spermidine-cholesterol

The analysis of samples by UPLC-MS is performed using a Thermo Scientific Quantis Plus instrument with direct infusion at a rate of 0.05 mL/min in positive electrospray ionization mode. Mass-to-charge ratio relationships in the range of 100 to 1500 m/z + are searched. The dissolution medium used for the samples is the following: methanol (40%)–acetonitrile (40%)–0.1% acetic acid aqueous solution (20%) (Sigma-Aldrich). Samples at room temperature were prepared at a concentration of 1000 µg/mL.

Liposome fabrication with spermidine-cholesterol

Liposomes are prepared by mixing egg yolk phosphatidylcholine (Sigma-Aldrich P2772) and spermidine-cholesterol in a 1:1 ratio, followed by the addition of chloroform and agitation to obtain a homogeneous mixture. The solvent is evaporated using a stream of nitrogen. Subsequently, a sonication process of three cycles of 5 s each with 10 s rest is performed, followed by resuspension in phosphate-buffered saline (PBS) in 2 mL vials.

Transmission electron microscopy

For the negative-stained electron microscopy, the liposomes were suspended in PBS and diluted 1:1 with 4% aqueous uranyl acetate (Sigma-Aldrich) on carbon-Formvar-coated grids (Sigma-Aldrich) and incubated for 1 min. The grids were drained on absorbent tissue, allowed to air-dry, and examined in a JEOL-JEM-1010 transmission electron microscope (JEOL USA, Inc.).

INBL cell culture

Human solid tumor cell line INBL established in our laboratory from one cervical carcinoma and

kept in tissue culture for 30 years was routinely sub-cultured at 37 °C with 5% CO2 in RPMI 1640 medium (Microlab) supplemented with 10% fetal calf serum (Hyclone).

Biological activity evaluation

One million INBL cervical cancer cells were incubated for 30 min with liposomes fabricated with spermidine-cholesterol. Cells are gently detached to preserve their integrity and incubated with a monoclonal antibody against CD16 (FcRIII receptor) conjugated with PE (sc-19620) (Santa Cruz Biotechnology) at 1 µg per million cells. Labeled cells were analyzed using a Cytek Aurora flow cytometer with a 488 nm laser to excite the PE marker. The resulting data are processed using FlowJo v10.9 analysis software.

Results

Synthesis and yield

We used a variation of our method to synthesize non-purified spermidine-cholesterol by the addition of 0.1 M NaOH that neutralized acidity form hydrochlorides due to HCl generated during substitution. Briefly, spermidine is previously dissolved in chloroform; separately, a solution of cholesterol chloroformate in chloroform is prepared. The cholesteryl chloroformate is dissolved in chloroform and placed in an ice bath. The spermidine solution is added dropwise with constant and vigorous stirring and the reaction maintained for 2 to 3 h at the same temperature. To the reaction mixture a 0.1 M sodium hydroxide solution is added and allowed to stir for another hour. After separating the phases, the organic phase is dried, and the solvent is removed using a rotary evaporator. The residue is washed until a white, slightly yellowish, mucoid product is obtained.

With this new method for synthesis of spermidine-cholesterol yielding products in their neutral form, a significantly increased yield from 36 to 70% was obtained measured by comparing the total weight of the reagents in milligrams to those of spermidine-cholesterol.

UPLC/MS analysis

Once an important increase in the yield of spermidine-cholesterol was obtained, we used UPLC combined with mass spectrometry (MS) to evaluate its purity. Control samples with our method of production of non-purified spermidine-cholesterol contained the monopronated species of spermidine-cholesterol with an m/z+of 558.47, a species that is probably a heavy isotope of spermidine-cholesterol with an m/z+value of 559.51, also spermidine-dicholesterol (969.82) and cholesterol ether (755.59) (Fig. 1a). Our results for the new method revealed that no dimers or higher molecular weight polymers were identified, suggesting a pure molecular structure with a 1:1 molar ratio between spermidine and cholesterol (Fig. 1b). The observed mass fragment (369.29) is not part of the product mixture, it is a fragment formed from spermidine-cholesterol in the mass spectrometer. It is important to mention that in this positive electrospray ionization mode we did not capture neutral spermidine-cholesterol with its exact calculated mass of 557.49.

To evaluate if, with an increase in the reaction time in the spermidine-cholesterol synthesis for both methods, we could further increase its purity, we made other batches with an extended reaction time of 18 h. Using our control method, spermidine-cholesterol is observed in its mono (558.47) and deuterated form (559.51) and its fragment (369.31), also spermidine-dicholesterol (969.82) without observing the formation of cholesterol ether (Fig. 1c). When we analyzed the reaction product with our new method, we found mono (558.47) and deuterated form (559.51) and its fragment (369.31) without observing the formation of spermidine-dicholesterol and cholesterol ether (Fig. 1d).

Liposome size, complexity, and biological activity evaluation

Once a pure spermidine-cholesterol was obtained, we proceeded to construct liposomes to evaluate their induction capacity for CD16 receptors on the membrane of INBL cells. By flow cytometry, the liposomes presented a fairly uniform size and complexity characteristics (Fig. 2a and b), and by electron microscopy, they presented an approximate size of 80 nm (Fig. 2c).

Our results also showed by flow cytometry that the liposomes with purified spermidine-cholesterol produced a significant CD-16 induction on INBL cells

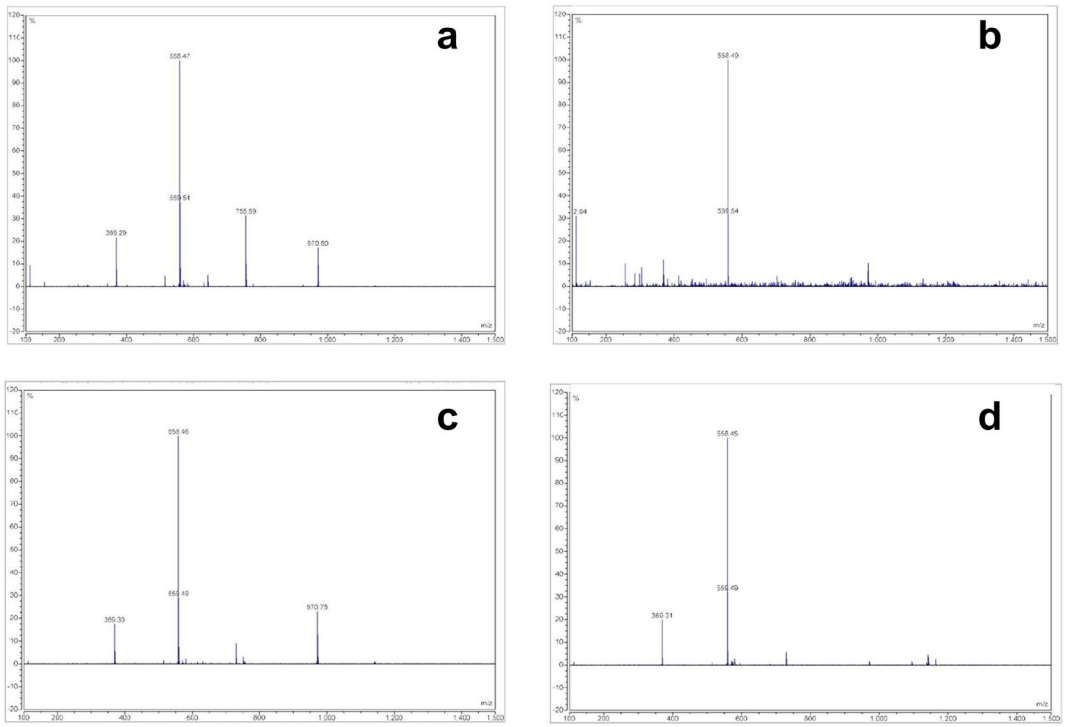

Fig. 1 UPLC-MS products. **a** Our synthesis method for non-purified spermidine-cholesterol (Control). Picks corresponded to spermidine-cholesterol monoprotonated (558.47), deuterated form (559.51), spermidine-dicholesterol (969.8262), cholesterol ether (755.59), and fragmentation of spermidine-cholesterol (369). **b** Our new synthesis for purified spermidine-cholesterol with only one predominant pick of monoprotonated (558.47) or deuterated form (559.51) spermidine-cholesterol. **c** Synthesis with 18-h reaction time for our method for non-purified lipid. In addition of the spermidine-cholesterol peaks, monoprotonated (558.47) or deuterated form (559.51), two more peaks appear spermidine-dicholesterol (969.8262) and fragmentation of spermidine-cholesterol (369). **d** Synthesis with 18-h reaction time for purified lipid. In addition of the spermidine-cholesterol peaks, monoprotonated (558.47) or deuterated form (559.51) a new peak appears at 369 corresponding to fragmentation of this molecule

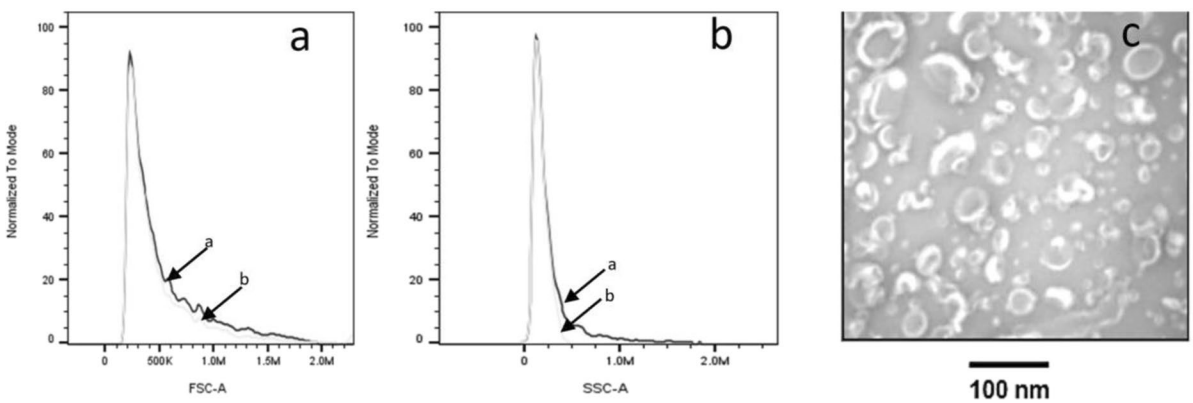

Fig. 2 Liposomes size and complexity. **a** Flow cytometry of liposomes size: (a) with non-purified spermidine-cholesterol, (b) with purified spermidine-cholesterol. **b** Flow cytometry of liposomes complexity: (a) with non-purified spermidine-cholesterol, (b) with purified spermidine-cholesterol. *X*-axis shows size or complexity in logarithmic scale and *Y*-axis shows liposome number. **c** Transmission electron microscopy of liposomes × 75,000

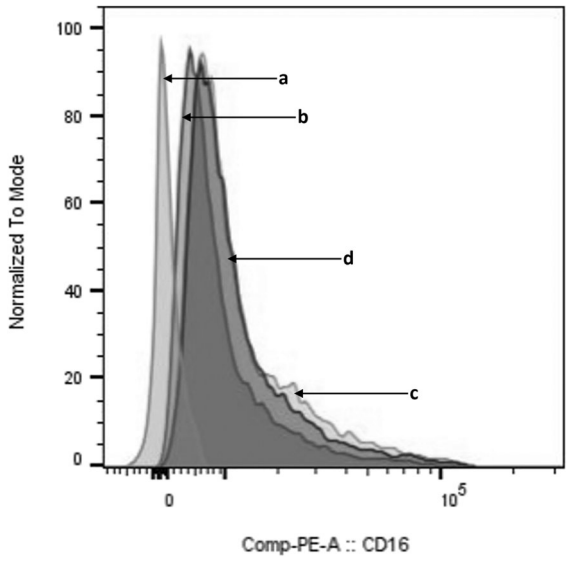

Fig. 3 Flow cytometry analysis of INBL cells cultured in the presence of liposomes for 30 min: (a) autofluorescence (MFI, 981 ± 176), (b) basal CD16 expression in INBL cells (MFI, 10,865 ± 124), (c) CD16 expression after induction with the liposome formulated with non-purified spermidine-cholesterol synthesis (MFI 15, 364 ± 111), and (d) CD16 expression after induction with liposomes formulated with purified spermidine-cholesterol (MFI, 14,288 ± 111)

compared to the already high induction normally presented on those cells and similar to that with the non-purified spermidine-cholesterol liposomes (Fig. 3).

Discussion

It has been published that for pharmaceutical products intended for medical applications, their purity is highly recommended [25]. Spermidine-cholesterol liposomes were shown to have an anticancer effect in vitro [23] and ability to translocate genetic material into cells [24, 26]. Spermidine-cholesterol is not available commercially and thus has to be synthetized locally. If the purity of this cationic lipid is not guaranteed even though the other components are pure, the overall purity of the liposome can be put in doubt [27–29]. In fact, the spermidine-cholesterol obtained by our old synthetic method presented by UPLC/MS several contaminants. We optimized the synthesis by using sodium hydroxide to neutralize the synthesis acidic medium and obtained that not only its purity was significantly increased but also a far better yield. We observed that liposomes constructed with this purified spermidine-cholesterol induced a significant increase in the appearance of CD16 receptors on INBL cervical carcinoma cell membranes similar than that published for the non-purified components, thus producing an ideal nanoparticle to continue applications for cancer treatment. Our liposome not only represents an innovation that can have pharmaceutical applications for the treatment of cancer and for transfecting genetic material into cells but also could be useful in other diseases. For example, we have applied this technique to deliver gel formulated liposomal D-Penicillamine for rheumatoid arthritis (Patent pending MX/a/2021/014147).

Conclusion

We were able to synthetize a highly purified spermidine-cholesterol to fabricate a liposomal system capable to induce CD16 on cervical cancer cells.

Funding The authors are grateful for the support of PAPIIT IN216521 and PAPIIT IN 222623 grants, from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México.

Declarations

Conflict of interest The authors declare no competing interests.

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