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ORIGINAL RESEARCH PAPER

Liposome nano-formulation with cationic polar lipid DOTAP and cholesterol as a suitable pH-responsive carrier for molecular therapeutic drug (all-*trans* retinoic acid) delivery to lung cancer cells

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Abstract

The molecular targeted drug ATRA demands a suitable carrier that delivers to the cancer site due to its poor bioavailability and drug resistance. ATRA, being a lipid with carboxylic acid, has been nano-formulated as a cationic lipo-ATRA with DOTAP:cholesterol:ATRA (5:4:1) and its pH-responsive release, intracellular drug accumulation, and anticancer effect on human lung cancer (A549) cell line analysed. The analysis of the physicochemical characteristics of the developed lipo-ATRA (0.8 μmol) revealed that the size of 231 ± 2.35 d.nm had a zeta potential of 6.4 ± 1.19 and an encapsulation efficiency of 93.7 ± 3.6%. The ATRA release from lipo-ATRA in vitro was significantly ($p \leq 0.05$) higher at acidic pH 6 compared to pH 7.5. The intracellular uptake of ATRA into lipo-ATRA-treated A549 cells was seven-fold higher (0.007 ± 0.001 mg/ml) while only three-fold uptake was observed in free ATRA treatment (0.003 ± 0.002 mg/ml). The lipo-ATRA treatment caused a highly significant ($p \leq 0.001$) decrease in percent cell viability at 48 h when compared with the free ATRA treatment. Overall, the results proved that the developed lipo-ATRA has suitable physicochemical properties with enhanced ATRA release at acidic pH, while maintaining stability at physiologic pH and temperature. This resulted in an increased ATRA uptake by lung cancer cells with enhanced treatment efficiency. Hence, it is concluded that DOTAP lipo-ATRA is a suitable carrier for ATRA delivery to solid cancer cells.

1 | INTRODUCTION

Cytotoxic drugs have been used for decades to kill cancer cells in the body, but the remission of disease and the side effects caused by those drugs are enormous [1–3]. As the occurrence of cancer is growing exponentially annually, a treatment that works at the molecular level to prevent cancer progression and to halt cancer growth is mandatory [4]. One such drug, which impacts molecular mechanisms at different stages of cancer progression such as differentiation, apoptosis, and proliferation, is all-*trans* retinoic acid (ATRA). This drug has been a successful treatment strategy in leukaemia patients; however, it has not been effective in solid cancer treatments [5]. The major reasons behind this are its poor stability, poor reachability to the target site, and resistance to ATRA after continuous exposure [6]. A proper nanocarrier which can provide stability

and compatibility, with less of an immune response could solve this problem [7]. The common toxicity symptoms of ATRA treatment is similar to toxicity of vitamin A overdose such as nausea, headache, skin rash, dryness, itching and bone pain. A clinical case study on ATRA therapy (45 mg/m^2 per day) for APL has reported that the patient developed skin problem, depression and mild liver disorders in prolonged treatment after 28 days [8]. It was concluded that the side effects of ATRA therapy are dose and duration dependent as the toxic symptoms reversed once the therapy was stopped. These toxicities can be overcome by targeted delivery of ATRA by an encapsulated nano-formulation. The combination of ATRA therapy with arsenic trioxide has minimised the toxicity in APL patients and 90%-100% complete remission was achieved [9, 10]. However, resistance to ATRA therapy was also developed in APL cases upon prolonged treatment with an ATRA-arsenic oxide combination, which is a clinical problem [11] that involves systemic and cellular elements. The blood level of ATRA also was found to be decreased in prolonged treatment in an APL patient [8]. A very recent review has highlighted the therapeutic significance of ATRA and the history of nano-formulations used for ATRA therapy so far [12]. Among all the nanoparticles, liposome is the only carrier used for a large number of FDA-approved drugs because of its greater biocompatibility and delivery efficiency. Due to its high rigidity and flexible nature, it is compatible with different types of drugs (hydrophobic and hydrophilic) [13].

It was reported that the characteristics of liposomes such as their shape, size, charge, stability, and pH-dependent release, can be modified with the addition of appropriate helper lipids [14]. Thus, the ratio of liposome components, lipid used and the chemical nature of the drug have a major role in successful targeted drug delivery. The widely used conventional liposome drugs are composed of neutral lipids while very few formulations are composed of negatively charged and positively charged lipids. The drug ATRA is also available as a neutral liposome with DMPC and soybean oil, and is named ATRAGEN in the market. The cationic lipids are mostly used for delivery of ionic drugs, such as nucleic acid drugs for effective entrapment and target-specific intracellular delivery [15–18]. One such cationic lipids, which has been under research and clinical trial in recent years, is 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). DOTAP is too flexible, with higher drug retention ability for negatively charged drugs as per various research findings. It is used for transfection delivery of genes as vectors and its polycationic chemical structure facilitates the intracellular release of nucleic acids [19]. The drug ATRA also has a carboxylic acid group, although it is a hydrophobic lipid by nature. Hence, a cationic lipid-like DOTAP may be suitable for better entrapment and intracellular release. Also, cholesterol at appropriate proportions was proven to maintain the stability of liposome and also reduced immune clearance [20, 21]. However, at a higher ratio, it was shown to collapse its structure and exhibit inappropriate drug release [22].

Conditions like pH, charge, and vasculature vary a lot in the tumour microenvironment compared to normal physiological conditions. The cancer microenvironment exhibits acidic pH due to high glucose metabolism; hence, the liposomal drugs should be able to reach and release drugs at this pH and remain stable at normal physiological conditions [23]. Previous research on the pH-responsive release of liposomal DOX has shown dramatic cancer inhibition and enhanced release compared to conventional PC-based liposome [24]. Mo and his research group found that pH-responsive liposomes not only enhanced the entry to cells but also improved escape from endolysosomes [25]. Achieving good entrapment and sustained gradual release of drugs at the target site in a pH-dependent manner in addition to the EPR effect are the major goals of this study. Many clinical formulations and research studies have used the 5:4:1 ratio for lipo-drug formulations and also proved effective in many FDA-approved cancer drugs [26, 27]. Cationic liposomes are accepted as pH-sensitive drug release nanocarriers as they were used in lipoplex and even the neutral lipids like DOPE is conjugated with cationic lipids due to its membrane destabilising at reduced pH, thereby escaping endolysosomal degradation [28].

It was therefore aimed to formulate lipo-ATRA with DOTAP, cholesterol, and ATRA in 5:4:1 ratio and to analyse the physicochemical properties, such as size, charge, morphology, stability, cytotoxicity and pH-dependent drug release ability, which are suitable for its anticancer properties.

2 | MATERIALS AND METHODS

2.1 | Cell lines and chemicals

DOTAP (SC-208,732) was obtained from Santa Cruz Biotechnology, USA. ATRA (R2625) and cholesterol (C8667) were purchased from Sigma-Aldrich, USA. Foetal bovine serum (FBS), Ham's F-12K (Kaighn's) medium, cholesterol, PBS, chloroform, and tween-80 were from Himedia. PSN antibiotic mixture (15640055) was purchased from Invitrogen. All other chemicals used were of analytical grade. The A549 human lung cancer cell lines were obtained from NCCS, Pune, India.

2.2 | Drug dose–response assay for ATRA on A549 cells

The subcultured A549 cells were taken in 96-well flat-bottom plates (5×10^4 cells/well) and allowed to grow for 24 h at 37°C with exposure to a 5% CO_2 atmosphere. Then, the cells were treated with increasing concentrations of the drug (ATRA) in the range of 0.2–2 μmole, and incubated further for 48 h. Four hours before completion of the incubation period, 20 μl of MTT [3-(4,5-dimethylthiazol-2-Yl) - 2,5-diphenyltetrazolium bromide] (5 mg/ml) was added to all the wells and then, after 4 h of colour development, 100 μl of DMSO was added. The experiment was carried out in triplicate and the untreated cells with DMSO addition served as controls. The spectrophotometric absorbance by the intensity of colour developed was measured at 570 nm using an ELISA reader [29]. The percentage of viability was calculated as follows:

$$\% \text{ Viability} = (\text{O.D of treated})/(\text{O.D of control}) * 100$$

2.3 | Preparation of cationic liposomes

Unilamellar cationic liposomes with ATRA were formulated by a thin-film hydration method as described previously [30] for an IC50 concentration of ATRA (0.8 μmole) on A549 cell line growth inhibition (240.32 μg/ml) and the free liposome was prepared without ATRA. Briefly, the DOTAP, cholesterol, and ATRA at a 5:4:1 m ratio were dissolved in 500 μl of chloroform, and the solvent was removed in a rotary evaporator at 55°C at 150 rpm for 60 min. Then, it was kept in a vacuumed desiccator overnight. The lipid film was then hydrated using 1 ml of 0.2 mol PBS (pH 7.4) by rotating with glass beads and diluted to 20 ml with PBS, before being subjected to ultrasonication at 70 W for 30 s by placing it in an ice bath to form unilamellar vesicles. The formulation was then ultracentrifuged at 35,000 rpm for 30 min at 4°C to separate liposomal ATRA pellets from unentrapped free ATRA. After removing the supernatant, the pellets were suspended in 3 ml of PBS and again subjected to ultracentrifugation. The pellets were then suspended in 500 μl of PBS and stored at 4°C until use (maximum time of 1 month). Then, in order to enhance the stability and to get uniform size, the liposome formulation was subjected to extrusion (5–6 times to and fro) for filtration using an extruder with a 200-nm filter before proceeding to treatment.

2.4 | The entrapment efficiency of liposomal ATRA

The liposomal ATRA pellets in PBS (200 μl) were dissolved in 1800 μl of absolute ethanol, in which ATRA alone is soluble and was ultracentrifuged. Then, the supernatant having released ATRA was dried under nitrogen gas for evaporation of ethanol and the residue was subjected to HPLC analysis for quantification of ATRA [31].

The entrapment efficiency was then assessed by the percent entrapment of ATRA (yield), which was calculated as follows:

$$\% \text{ Entrapment} = \left(\frac{Cl}{Ct}\right) * 100$$

where, Cl is the concentration of ATRA in the liposome, and Ct is the total ATRA added to the liposome during preparation.

2.5 | ATRA quantification by HPLC

The amount of ATRA released from the lipo-ATRA formulation was estimated using reversed-phase HPLC analysis [31]. Briefly, the residue was dissolved in acetonitrile (100 μl) and subjected to reversed-phase HPLC (Shimadzu Prominence HPLC system). The system has LC10AT pumps and a PDA detector, and the sample was injected into the column (Phenomenex Luna C18) of (250 × 4.6 mm with 5 μm particle size) using Rheodyne injector loop. Acetonitrile and water (v/v) in 45:65 ratio was used as the mobile phase to separate ATRA at a flow rate of 1.0 ml/min and the detection was done using a UV detector at a wavelength of 340 nm. The peak for ATRA in the test sample was identified by comparing the retention time (RT) of standard ATRA run separately. Then, from the peak area, the concentration of ATRA was calculated using the calibration standard curve constructed by linear least squares regression analysis for the standard ATRA concentration in the range of 10–50 μg/ml.

2.6 | Physical characterisation studies on lipo-ATRA formulation

2.6.1 | Morphologic analysis of liposome formulation

The shape and morphology were evaluated by SEM and TEM analyses [32]. SEM analysis was carried out by taking 200 μl of liposome preparation, ultracentrifugation to collect the pellets and by suspending it in 25 μl of PBS. This sample was coated in gold stabs and viewed using SEM (Carl Zeiss-Sigma 300 VP, UK). Images were taken for evaluation.

TEM analysis was also carried out for the liposome pellets in 25 μl of PBS by loading a drop of it onto a thin copper grid and then allowing it to air dry overnight. Then, it was viewed using the TEM (FEI Tecnai FEI G^2 F20 Twin, USA) and images were taken at different resolutions.

2.6.2 | Analysis of particle size distribution and zeta potential

The size distribution was evaluated by DLS method (173° scattering angle) for the lipo-ATRA (20 μl diluted to 2 ml with PBS) and also the zeta potential of the liposomal formulations (5 μl diluted to 2 ml with double-distilled water) was analysed using a dynamic light-scattering and zeta potential measurement system (Zetasizer Nano ZS, Malvern Technologies, Herrenberg, Germany) by referring to the literature [33]. The measurements were taken in triplicate for statistical validation.

2.6.3 | Stability study for lipo-ATRA formulation

Thermo-stability analysis upon storage at different conditions was carried out by measuring the released ATRA due to leakage upon storage. This gives the retainability of ATRA inside the liposome during storage [34]. The lipo-ATRA formulation (100 μl in 1 ml PBS) was stored in amber tubes (to avoid oxidative damage to ATRA as it is light sensitive) at different temperatures (−20°C, 4°C, 25°C, 37°C, and 45°C). Then, the ATRA retainability was assessed at 10-day intervals

for 30 days by separating lipo-ATRA pellets upon centrifugation, dissolving in ethanol, drying supernatant with released ATRA, and assaying the ATRA concentration using the HPLC method as described above. The percent retainability was calculated from the ATRA concentration.

The photostability analysis of liposomal ATRA formulation kept in tightly closed amber vials was analysed after UV exposure (320–400 nm) for 6 h at regular intervals of 1 h [31]. Briefly, 100 µl of lipo-ATRA was made up to 500 µl with PBS and irradiated by exposing it to a UV lamp at 50 cm distance. Then aliquots (80 µl) were taken at 1-h intervals, ultracentrifuged to separate intact liposome and ATRA, and the pellets dissolved in ethanol. After drying the supernatant under a stream of nitrogen gas, it was dissolved in 100 µl methanol and analysed for the ATRA level by the HPLC method, as described above. The percent retainability was calculated from the ATRA concentration.

2.6.4 | Study of in vitro ATRA release from lipo-ATRA

The release of ATRA at pH 7.4 and an acidic pH 6 that prevails in the tumour microenvironment was analysed by the dialysis method [35]. The liposomal ATRA (50 µl diluted to 1 ml PBS) and free ATRA in 1 ml ethanol were separately sealed tightly in 12,000 Da molecular weight cut-off dialysis bags and put separately into 50 ml of a solvent system containing ethanol, Tween 80 and 0.1 M PBS (pH 7.4) in 10:15:75 ratio. They were kept in a magnetic stirrer with constant stirring at 37°C for 24 h and the speed was maintained at 100 rpm. The aliquots of 1 ml were drawn at 1, 2, 4, 8, 12, 24 and 48 h intervals, and the transfer medium was replaced with fresh solution. The released ATRA concentration was obtained from spectrophotometric absorption at 350 nm and the calibration curve developed with ATRA at a standard concentration. Then the percent drug release was calculated as follows:

$$\% \text{ Release} = \left(\frac{\text{Drug(rel)}}{\text{Drug(load)}}\right) * 100$$

where, Drug(rel) is the concentration of ATRA released, and Drug(load) is the total ATRA added in the liposome during preparation.

2.7 | Study of the effects of lipo-ATRA treatment on a human lung cancer cell line

2.7.1 | Cell culture maintenance and treatment

Cells were cultured according to ATCC protocol in 25 ml tissue culture flasks with Ham F12-K medium (90%), 10% FBS, and PSN antibiotics (10 ml/L). Then, the cells were seeded in 96-well plates and grown for 48 h. The cells were treated with bare liposomes, ATRA, and lipo-ATRA for 24 h.

2.7.2 | Analysis of intracellular release and accumulation of ATRA

The intracellular release of ATRA by the lipo-ATRA compared to free ATRA treatment was evaluated on A549 cells by referring to the relevant literature with slight modifications [36, 37]. Briefly, the cells (5×10^5 cells/well) were seeded in six-well plates for 24 h at 37°C and 5% CO_2 atmosphere for cell adhesion. Then, the cells were treated with bare liposome, free ATRA (0.8 µmole), and lipo-ATRA (0.8 µmole ATRA), while the untreated cells served as a control and all were incubated for 24 h at the same condition. The cells were then scraped mechanically using a cell scraper to transfer them into microcentrifuge tubes and washed thrice with ice-cold PBS. They were then centrifuged at 1200 rpm for 10 min and the cells were resuspended in 1 ml cold PBS/methanol mixture (50:50 v/v). The cells were disrupted by subjecting them to sonication for 30 min with the addition of 2% zinc sulphate for protein precipitation. Then, this mixture was centrifuged at 6000 rpm for 10 min to remove the proteins. The supernatant was collected and processed for retinoid lipid extraction by vortex mixing with organic solvent (80:19:1 mixture of n-hexane, dichloromethane, and propan-2-ol) for 10 min. The contents were centrifuged at 2000 g for 30 min at 4°C and the organic layer was removed and dried by evaporation using nitrogen gas. The residue was dissolved in 100 µl acetonitrile and injected into a reverse-phase HPLC system for assay of ATRA concentration, as described above. The level of ATRA was then compared among the study groups with an average of three independent tests.

2.7.3 | Cytotoxicity analysis by MTT assay

The viability of cells post-treatment was compared by MTT assay using colorimetric measurement at 570 nm [29]. Cells were cultured in 96-well flat-bottom plates (5×10^4 cells/well) and allowed to grow for 24 h at 37°C in a 5% CO_2 atmosphere. Treatments were carried out in different groups in triplicate with bare liposome, free ATRA (0.8 µmole), and lipo-ATRA (0.8 µmol ATRA) and incubated further for different periods, such as 24, 48, and 72 h. The untreated cells with DMSO in eight wells served as control. Before 4 h of incubation were completed 20 µl of MTT [3-(4,5-dimethylthiazol-2-Yl)- 2,5-diphenyltetrazolium bromide] (5 mg/ml in PBS) were added. Then, the media were removed and 100 µl of DMSO/ethanol (1:1) (v/v) added to dissolve the formazan crystals. After brief shaking at 240 rpm for 15 min, the absorbance was measured at 570 nm using an ELISA reader. The percentage of viability was calculated as follows:

$$\% \text{ Cell Viability} = (\text{O.D of treated})/(\text{O.D of control}) \times 100$$

2.8 | Statistical analysis

All experiments were replicated and the statistical significance was analysed by GraphPad Prism software. The comparative statistical analysis was done by two-way ANOVA and the values are given in mean ± SD values and $p \leq 0.05$ is considered as significant in comparative analyses.

3 | RESULTS

3.1 | Dose–response cytotoxicity of ATRA on a human lung cancer cell line (A549)

The cytotoxicity potency of ATRA at different concentrations on human lung cancer cell line A549 was analysed by MTT assay to discover the dose-dependent action. The percent viability of A549 cell lines decreased upon 48 h of treatment with ATRA in a dose-dependent manner as shown in Figure 1. The IC50 value was found to be 0.78 μmol from the dose–response curve of cell viability. Hence, the concentration of ATRA used for lipo-ATRA formulation and the study of comparative ATRA treatment effects was fixed at 0.8 μmol. The study of intracellular ATRA accumulation in A549 also was performed with this concentration of ATRA.

3.2 | ATRA entrapment efficiency

The liposome formulation with and without ATRA was successfully developed as we could separate the liposome pellets after ultracentrifugation. The concentration of ATRA added in lipo-ATRA was 240.32 μg/ml and the loaded ATRA level was then analysed after dissolving the ATRA in ethanol using HPLC method against the calibration curve developed with a standard concentration of ATRA. In the HPLC chromatogram, the ATRA peak was observed in comparison to standard ATRA in the range of 4–4.5 min (RT value). The peak area obtained was then converted into the concentration using the standard graph and the mean per cent entrapment efficiency was calculated and found to be 93.7 ± 3.6% in this study. This shows that the liposome has the ability to entrap the maximum amount of ATRA with minimal loss.

3.3 | Physical characteristics of lipo-ATRA

The physicochemical analysis revealed that the formulated lipo-ATRA has properties suitable for ATRA delivery at the cancer site. The DLS results revealed the size of the formulated lipo-ATRA as 231 ± 2.35 d.nm and suggest that as the size is around 200 nm, it is suitable for targeted ATRA delivery (Table 1). The morphology analysis by SEM and TEM reveals a distinct complete spherical shape with smooth surface for lipo-ATRA, while constricted coarse sphere shapes below 200 nm are observed for bare liposome formulation, as shown in Figure 2. The zeta potential of lipo-ATRA also varied from bare liposome formulation as given in Table 1 and was found to be lowered from the expected zeta potential of DOTAP formulations. This reveals that the higher concentration of cholesterol had an influence on it. In this study, the zeta potential observed for the DOTAP lipo-ATRA formulation was 6.4 ± 1.19 mV, which is lower than bare liposome and the reason may be the neutralising effect of ATRA drug in this formulation.

3.4 | Stability of lipo-ATRA

The stability of lipo-ATRA formulation at different temperatures was evaluated. The drug entrapment percentage indicates the ATRA retainability in the lipo-ATRA over storage time and its drop was plotted over different time durations, as shown in Figure 3. The lipo-ATRA formulation preserved its stability well in 4°C and 25°C. After 30 days at physiologic temperature (37°C) the lipo-ATRA formulation of 5:4:1 was also highly

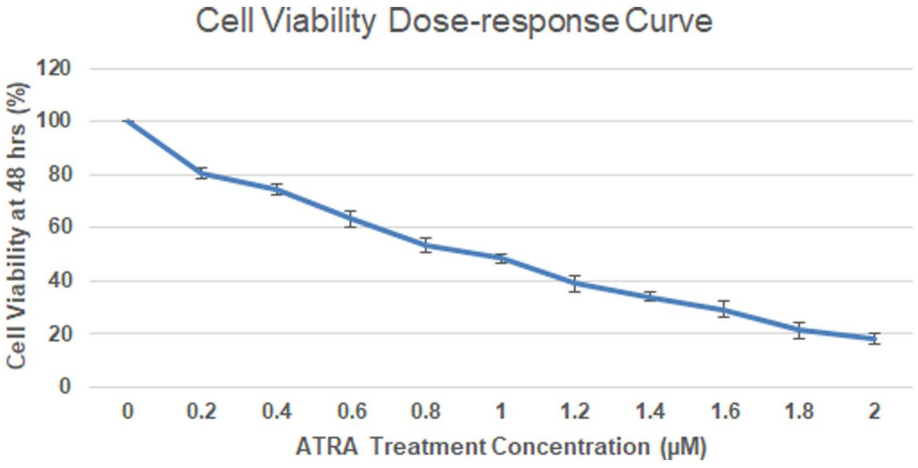

FIGURE 1 Percent cell viability upon ATRA treatment on an A549 cell line. The dose-dependent decrease in cell viability is shown in the graph

TABLE 1 Results of DLS analysis of liposome formulation

Parameters analysed	DOTAP:Cholesterol:ATRA 5:4:1	DOTAP:Cholesterol 5:4
Size (d.nm)	231 ± 2.35	162 ± 2.87
Zeta potential (mV)	6.4 ± 1.19	31.4 ± 2.5

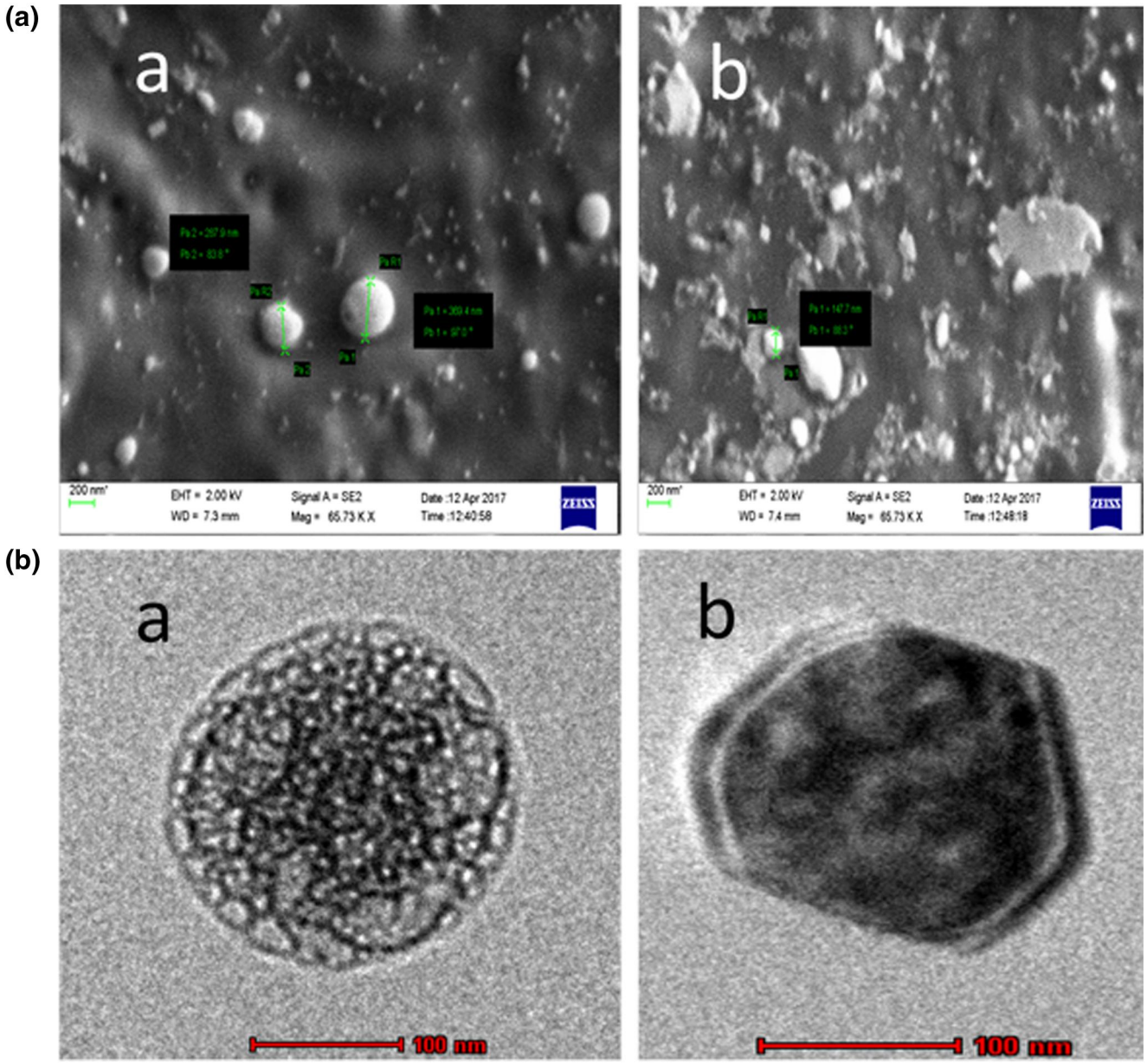

FIGURE 2 Representative images of liposome formulations. (a) SEM images at 2.00 kV with a scale bar of 200 nm: a. The morphology of lipo-ATRA showing a smooth sphere in the size range of ≥200 nm; b. The morphology of bare liposome showing a coarse sphere in the size range of ≤200 nm. (B) TEM images at a scale bar of 100 nm: a. Uniform sphere shape of a lipo-ATRA particle; b. Non-uniform sphere shape of a bare liposome particle

stable with 82.4 ± 2.8% entrapment. The lipo-ATRA decay percent was found to be higher at 45°C as the percent ATRA entrapment dropped drastically.

Similarly, the photostability of the lipo-ATRA formulation under exposure to UV light was observed in terms of percent ATRA retainability up to 6 h at every 1 h. The result indicated that the disruption of lipo-ATRA formulation happened in a slow and steady manner as shown in Figure 4. However, 67.56 ± 2.12 % ATRA retainability was achieved at 6 h of UV exposure.

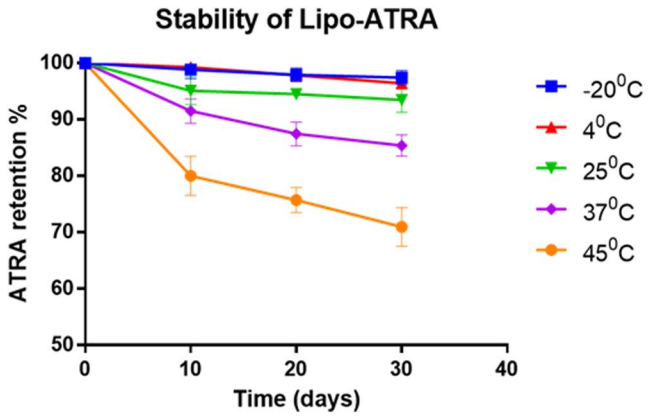

FIGURE 3 Thermostability of lipo-ATRA formulation upon storage. The graph indicates good stability of the lipo-ATRA formulation at −20°C, 4°C, and 25°C. small little drop in stability is shown at the physiological temperature (37°C), while a greater decay of lipo-ATRA is shown at 45°C as the percent ATRA entrapment dropped drastically

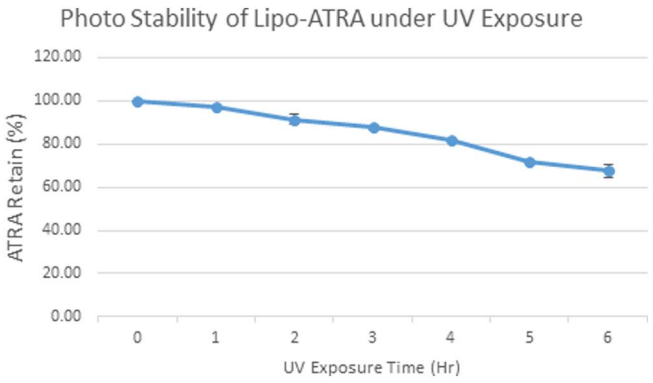

FIGURE 4 Photostability of lipo-ATRA formulation upon UV exposure. The linear graph reveals the slow and steady decrease in percent ATRA retainability due to disruption of lipo-ATRA by UV light. After 4 h of exposure only those above 80% dropped and at 6 h of exposure, above 65% ATRA was retained in lipo-ATRA

3.5 | pH-responsive in vitro drug release of lipo-ATRA

The in vitro drug release profile of lipo-ATRA was determined by the membrane diffusion method and the results are given in Figure 5. After 24 h, a gradual drug release was identified in 5:4:1 lipo-ATRA formulation. Thus, this proves that the gradual release kinetics is maintained in this formulation. In contrast, the free drug showed a burst release as reflected by the increase in ATRA concentration in the release medium. Also, the amount of drug release at pH 6 was found to be significantly ($p < 0.05$) higher than that of at pH 7.4 for lipo-ATRA, while the difference was non-significant for free ATRA release.

3.6 | Intracellular ATRA accumulation potency of lipo-ATRA formulation in a cancer cell line

The level of ATRA in A549 cells with free ATRA treatment increased more significantly ($p \leq 0.05$) than that of the control cells, while it was enhanced with very high significance ($p \leq 0.0001$) in lipo-ATRA-treated cells. The intracellular uptake of ATRA from lipo-ATRA treatment into A549 cells was found to be significantly ($p \leq 0.0001$) increased compared with free ATRA treatment. Thus, the bioavailability of ATRA was augmented up to seven-fold in lipo-ATRA-treated cancer cells, while for the free ATRA-treated cells, it was only improved three-fold (Figure 6).

3.7 | Anticancer effect of lipo-ATRA formulation on A549 cells

In order to compare the cytotoxic effects of DOTAP lipo-ATRA and free ATRA treatments (IC50 concentration) on the human lung cancer cell line (A549), MTT assay was carried out and the results are given in Figure 7. The results suggest that the ATRA loaded in lipo-ATRA (5:4:1) formulation was very effective in inducing cytotoxicity with a very high significant ($p \leq 0.001$) decrease in percent viability at 48 h when compared with free ATRA treatment. However, the difference was only significant ($p \leq 0.05$) at 24 h and highly significant ($p \leq 0.01$) at 72 h, which indicates the steady release of ATRA from lipo-ATRA.

4 | DISCUSSION

The stimuli-responsive drug delivery system using liposomes is under intense research in the field of cancer therapy to promote the site-selective action of effective molecular drugs, and the tumour microenvironment provides suitable stimuli including pH for triggered drug release [38]. A pH-sensitive cationic liposome is considered as an effective carrier in gene therapy [39]. Liposome with dimethylamino propane (DMAP) was reported to be a pH-controlled nano-formulation and has shown enhanced penetration in tumours [40].

In recent years, cationic liposomes have been gaining popularity due to their suitability to encapsulate polar drugs like negatively charged nucleic acid drugs and their effective interaction with cancer cells due to the more negatively charged acidic microenvironment. The cationic liposome of DOTAP with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and cholesterol has been demonstrated to be an effective pH-sensitive carrier in vitro as well as in vivo for co-delivery of drugs such as sorafenib and SiRNA [41]. The folate receptor-targeted liposome formulation with cationic DOTAP used for HuR-siRNA resulted in high-level uptake and cytotoxicity in the human lung cancer cell line H1299 [42, 43].

Since the drug ATRA is also partially a polar lipid compound with an acetic acid group, the authors used the cationic DOTAP lipid for preparation of lipo-ATRA along with cholesterol, which stabilises the formulation. The charge, size,

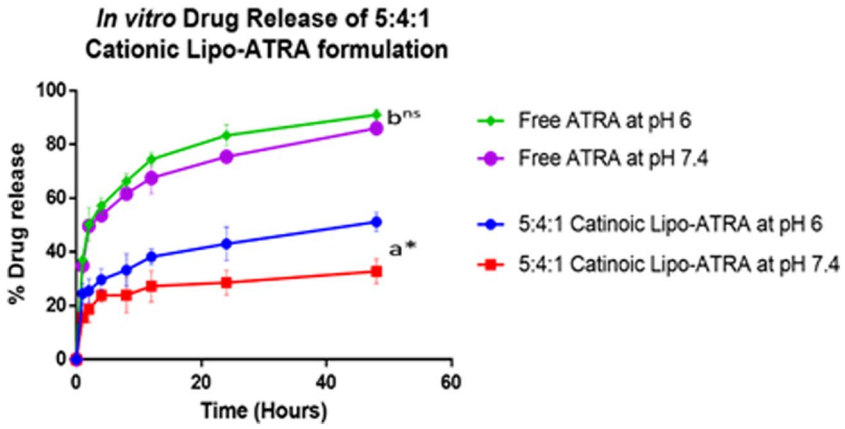

FIGURE 5 Drug release kinetics of lipo-ATRA formulation. a. In vitro drug release of cationic lipo-ATRA at pH 6 versus pH 7.4. b. In vitro drug release of free ATRA at pH 6 versus pH 7.4. ns, Not significant and $*p \leq 0.05$

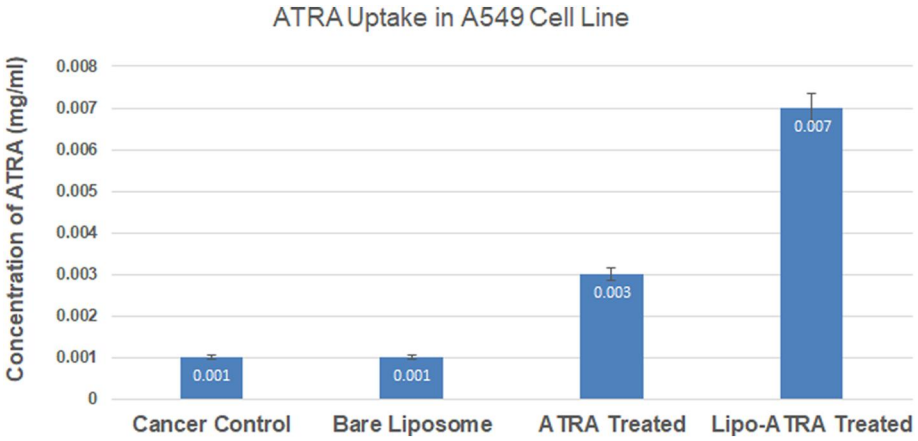

FIGURE 6 Intracellular level of ATRA after treatment with free ATRA and lipo-ATRA. The graph was plotted with the data in mean ± SD. The graph shows the concentration of ATRA calculated from the standard graph: a—ATRA versus lipo-ATRA, b—treatment versus cancer control. $***p \leq 0.0001$ and ns, not significant

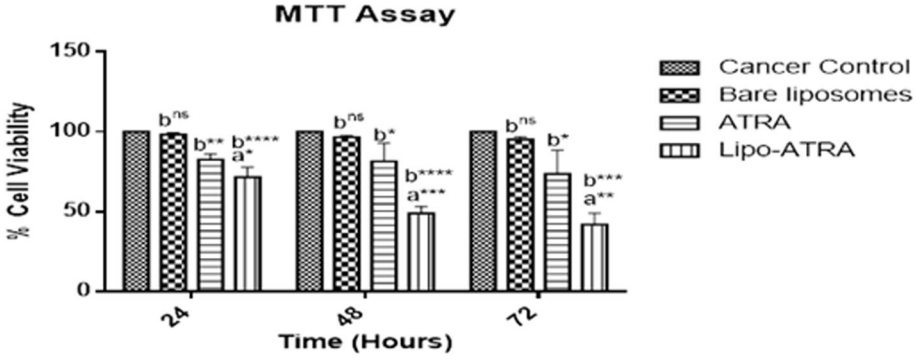

FIGURE 7 Percent cell viability upon treatments by MTT assay: a—ATRA versus lipo-ATRA; b—control versus all groups (significance $****p \leq 0.0001$, $***p \leq 0.001$, $**p \leq 0.01$ and $*p \leq 0.05$)

and proportion of lipids and cholesterol play a major role in the overall liposomal drug efficiency. ATRA is a potent differentiation-inducing first-choice therapeutic agent for APL patients but relapsed due to drug resistance and limited ATRA use [44, 45]. Some studies have highlighted that the addition of arsenic trioxide could prevent molecular relapse in APL [46]. Clinical trials have reported that its use to treat solid cancer is limited due to its poor bioavailability, as described in a recent review [47]. Hence, followed by the use of neutral lipo-ATRA, ATRAGEN^{TM} various lipo-ATRA formulations including pH-responsive nanoformulations have been tried for different solid cancers [48–50]. Since ATRA is a molecular-acting drug, its intracellular delivery by cationic liposomes may be effective in targeted therapy for solid cancers and a few studies have

proven the effect of DOTAP-ATRA formulations on solid cancers [51, 52]. Cationic liposomes like DOTAP normally show very high positively charged zeta potential in the range of +40–60 mV, and are found to be effective at the cancer site for drug delivery [53]. They have also highlighted that the composition of lipids, molar ratio, and the drug interaction influence the zeta potential. At the same time, the higher cationic zeta potential may cause more protein-corona formation in the blood circulation as proteins like apolipoproteins, prothrombin, fibronectin, and vitronectin are anionic in nature. However, this corona formation is unavoidable for all liposomes and it was a problem for cationic lipids in the earlier studies. Nowadays, it is looked at as an opportunity for better targeting and avoidance of immune attack [54, 55]. Hence, the zeta potential charge of liposome formulation has to be optimised to a lower cationic charge.

The difference in the size of lipo-ATRA compared to bare liposomes observed in this study has proven the successful encapsulation of ATRA in these studies. Higher stability was also achieved due to the high cholesterol concentration. The authors have observed a greater percent entrapment (93.7 ± 3.6%) of ATRA and increased stability in this study, as shown in Figures 3 and 4, which may be due to the optimised level of cholesterol used in the lipo-ATRA formulation. Cholesterol plays a major role in stabilising liposomes. A higher concentration leads to difficulty in drug release and the formation of cracks in liposomes and lower concentrations lead to fast degradation. Therefore, the authors believe that an appropriate fraction of the added cholesterol can considerably increase the phase transition temperature of liposomes in neutral conditions and the appropriate ratio of cholesterol can be introduced into the liposome bilayer to increase the well-ordered structure. Thus, liposomes show better stability at neutral conditions. Due to its steroid skeleton, it possesses a rigid structure, which increases the stability of liposomes. However, the mass ratio of cholesterol increases the gap in the hydrophobic chain, which hinders the formation of a highly ordered lipid bilayer [22]. This may be useful in the enhanced release of drugs at the target site when the liposomes fuse with the cell membrane.

The zeta potential of liposome indicates the surface charge of a liposome formulation and a comparative study on different liposome formulations has highlighted the dependence of zeta potential on the pH and molar ratio of lipids used in the liposome formulation [56]. The advantage of cationic liposomes is their efficiency in electrostatic interaction with negatively charged drugs and cell membrane interaction for intracellular delivery of drugs [16, 57]. The zeta potential of the lipo-ATRA formulation observed in this study was 6.4 ± 1.19 mV, which reveals that the concentration of cholesterol, as well as the drug ATRA, might have impacted highly on the zeta potential, which leads to a less positive charge (Table 1). A similar result was attained in an earlier study by Magarker and his team, which showed the correlation between cholesterol content and zeta potential [20]. It was also reported that the substitution of DOTAP with a neutral lipid DOPE or with cholesterol decreases the affinity of blood proteins towards DOTAP [58]. The cationic liposome thus needs an optimum cholesterol level, which can reduce the positive charge of cationic phospholipid and at the same time maintain its positive charge. In this study, the zeta potential of bare liposome was remarkably higher (31.4 ± 2.5 mV) when compared to lipo-ATRA, which indicates that the drug ATRA incorporation also influenced the zeta potential. This may be due to the neutralising effect of the carboxylic acid group of ATRA with cations of DOTAP. Further investigation of these interactions is warranted in future studies. Moreover, the cholesterol level also might have influenced the zeta potential in both bare liposomes and lipo-ATRA, as it is much lower than what was reported by other studies for DOTAP nanoformulations, which are in the range of 40–55 mV.

Liposomes with cationic lipids are prone to binding cells due to an electrostatic interaction with the negatively charged cell membrane (sialic acids and phospholipid head groups). The presence of sialic acid and acidic pH was already reported in previous studies [59, 60]. In this study, a significantly enhanced ATRA release at pH 6 when compared to pH 7 was identified, as shown in Figure 5. This selective drug release on acidic pH reduces the toxicity of the drug to normal cells and increases the accumulation of targeted cancer sites. Obata also reported a pH-responsive release of drugs at cancer sites by both in vivo and in vitro analysis [24]. The seven-fold increase in ATRA level inside the A549 cells treated with lipo-ATRA when compared to free ATRA treatment has proven that the lipo-ATRA uptake by lung cancer cells was more prominent and robust. ATRA is more hydrophilic which makes it repellent from the negatively charged cancer cells, which in turn reduces the drug uptake, but on the other hand ATRA in cationic liposomes can easily enter into the cancer cells and enhances the drug uptake by the cancer cells. Since the cancer cell membrane was loosely packed, the liposomes were easily taken up by the cancer cells compared to the free ATRA.

A study made on the breast cancer cell line MCF-7 has proven that the cationic DOTAP formulation has faster drug uptake/accumulation into cells and has shown a greater (20-fold more than free drug) anti-proliferative action [61]. It has also been highlighted that DOTAP is a very efficient nanocarrier due to its higher stability in aqueous media as well as efficient transport in cancer cells. The pH-responsive interaction and fusion with cancer cell membrane may be the reason behind the improved in vitro treatment efficacy of liposomal ATRA over free ATRA treatment on A549 cells, as observed in this study. Previously improved drug uptake of cationic liposomes was also reported by Ibricevic through in vivo studies [62]. It has also been proven in this study that the ATRA release and accumulation into the A549 cells were significantly higher (Figure 6) as compared to the treatment with free ATRA. This indicates that the treatment with the developed lipo-ATRA formulation in 5:4:1 increased the bioavailability in lung cancer cells when compared to free ATRA treatment. The lipo-ATRA-treated cells showed significantly higher cytotoxicity than the other two groups (Figure 7). This once again proves that the lipo-ATRA accumulated more than the free drug as reflected in the enhanced treatment effect on lung cancer cells. This phenomenon was previously reported by

Ahmed, as an increased apoptosis level was observed with the increased accumulation compared to the free drug [63]. These results revealed that the DOTAP liposomal drug (ATRA) formulated at a ratio of 5:4:1 is a promising drug formulation for targeted lung cancer therapy.

5 | CONCLUSION

The physio-chemical analysis of the cationic nano-formulation of ATRA with DOTAP and cholesterol in 5:4:1 m ratio proved that it can efficiently interact with cancer cell membrane and can provide sustained drug release in an acidic pH-sensitive manner. The carboxylic acid of drug ATRA also might have played a major role in neutralising the cationic nature of DOTAP, resulting in lowered cationic zeta potential, and leading to greater stability at physiologic temperature and pH. The charge reduction also might have been influenced by the higher cholesterol level used in this nano-formulation. However, the acidic tumour microenvironment condition around the cancer cells might have induced the ionic shift, triggering the destabilisation of liposome to favour ATRA release into cancer cells. The authors thus conclude that DOTAP lipo-ATRA is a suitable carrier for ATRA delivery to solid cancer cells.

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