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Stable High-Loading of All-trans Retinoic Acid in Nanoliposomes Achieved Through Optimized pH and Antioxidant Incorporation

Babatunde A. Adeagbo[1,2], Aaron Kahn[2], Oluseye O Bolaji[1], Christopher B. Fox[2,3]

[1]Department of Pharmaceutical Chemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.
[2]Access to Advanced Health Institute (AAHI; formerly Infectious Disease Research Institute, Seattle, United States of America.
[3]Department of Global Health, University of Washington, Seattle, USA

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*Corresponding Author:
Dr. Babatunde Ayodeji Adeagbo
Tel:+2348069019643
Email:badeagbo@oauife.edu.ng

ABSTRACT

Background: All-trans retinoic acid (ATRA) has been shown to have an immunomodulatory activity that could be explored to develop adjuvants for vaccination against intestinal or mucosal infections. However, progress has been limited by the poor chemical stability of ATRA and the high concentrations required. This study aimed to incorporate high-dose ATRA in a previously developed nanoliposome containing Toll-like receptor agonists (TLR) - (GLA) and 3M-052, optimising the excipient composition and the physicochemical stability of ATRA.

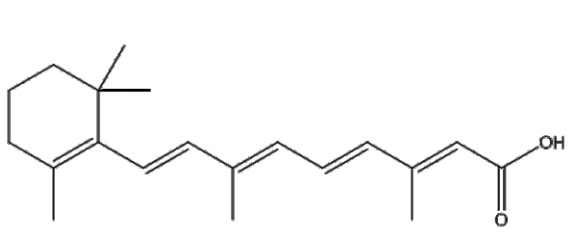
Method: Different ratios of ATRA: lipid, as well as different pH values, were explored to enable the incorporation of up to 3 mg/mL ATRA in the liposomal formulation. A hydrophobic antioxidant, α-tocopherol, was evaluated for its capacity to increase the chemical stability of ATRA within the liposomes. The particle size was determined by dynamic light scattering, and ATRA content was determined by HPLC with a diode array detector. Stability was monitored under normal and stressed conditions.

Results: Maximum ATRA incorporation and nanoliposome stability occurred at low ATRA: lipid ratio, elevated pH, and with the inclusion of α-tocopherol. This study showed that the formulation of ATRA depends on the ATRA: lipid ratio, the pH of the hydrating solvent, and the incorporation of α-tocopherol, which enhances the loading efficiency of ATRA and protects the formulation against oxidation and photodegradation.

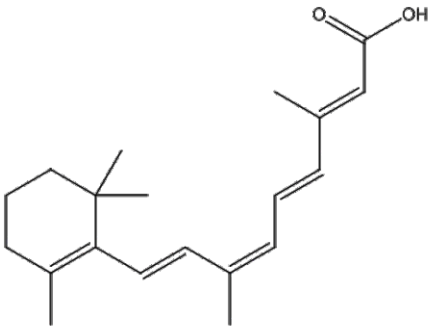
Conclusion: Nevertheless, whereas the optimised liposome formulation remained suitable for TLR7/8 agonist (3M-052) stability, a significant loss in the TLR4 agonist (GLA) content occurred following manufacture, likely due to the high pH condition.

1. Introduction

Retinoic acids (RAs) is the bioactive metabolite of vitamin A and exists as all-trans, 9-cis, 11-cis and 13-cis-retinoic acid. RAs have been reported to have the immunomodulatory capacity to regulate tolerance and immunity at mucosal surfaces[1]. All-trans retinoic acid (ATRA) exerts a multitude of immunomodulatory effects in vivo through binding to retinoic acid receptors and retinoid X receptors[2]. ATRA plays a crucial role in immunosuppression through the induction of regulatory T cells[3] but also primes gut immunity by promoting lymphocyte gut homing and mucosal IgA secretion[4]. ATRA has direct control on CD4+ T cell immunity and differentiation towards Th1/Th17 polarization[5,6] and controls the homeostasis of dendritic cells (DCs) at central lymphoid and mucosal tissues[7].



All trans retinoic acid



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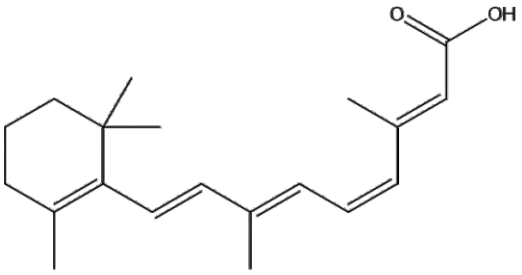
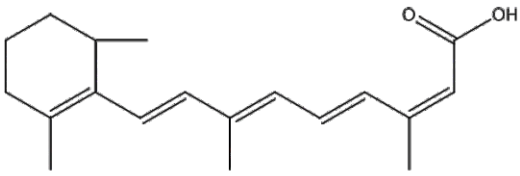


11-Cis retinoic acid



13-Cis retinoic acid

The immunomodulatory capacity of ATRA is being explored to use it as an adjuvant for vaccination against intestinal or mucosal infection[8,9,10,11]. However, challenges in effectively formulating ATRA have been a significant limitation in its use[12]. Following oral administration of ATRA, the plasma concentration is very variable, and this is partly due to the variable absorption in the gastrointestinal tract because of the intraluminal pH and also due to the hepatic first-pass effect, implying metabolism by hepatocytes of ATRA to less active compounds[12]. Parenteral administration is also limited by its poor solubility, as well as chemical instability in physiological solution. [13] Several dosage forms of ATRA, such as oily solutions, emulsions, microemulsions, liposomes and microparticulate systems, have been proposed[11,14].

A combination immunization regimen involving the weekly intraperitoneal injection of ATRA in mice and a mixed intranasal/subcutaneous administration of vaccine antigen with a nanoliposome adjuvant containing synthetic Toll-like Receptor 4 (TLR4), Glucopyranosyl Lipid A (GLA) and 3M-052, a synthetic TLR 7/8 had been reported to stimulate a balanced systemic humoral successfully. Moreover, GLA-3M-052 formulation also induced cellular immune response in addition to a strong mucosal IgA response, to the Entamoeba histolytica Gal/GalNAc lectin LecA antigen[9]. The report also indicated that ATRA injected separately as a solution increased mucosal IgA levels and also appeared to enhance the protective efficacy of the mucosal vaccine from 34% to 55%[9]. Thus, the liposomal TLR ligand adjuvant was shown to be compatible with the use of separately administered all-trans retinoic acid as a co-adjuvant and has the potential to induce a robust mucosal and systemic response towards an amebiasis antigen. Nevertheless, separate administration of ATRA is not ideal as it may result in a complicated immunisation regimen.

Liposomal encapsulation has emerged as a promising strategy to overcome some of the limitations of ATRA solutions by providing a protective carrier for ATRA. Earlier liposomal formulations of ATRA encountered several significant limitations. A primary issue was the limited drug loading capacity and suboptimal encapsulation efficiency, making it challenging to incorporate sufficient ATRA into the liposomes and often requiring large administration volumes[15,16], Furthermore, these formulations frequently exhibited insufficient physical and chemical stability, leading to problems like aggregation, leakage of the encapsulated ATRA, and degradation of both the drug and the liposomal components over time, resulting in a limited shelf life[17,18]. Another major drawback was the rapid clearance by the Mononuclear Phagocyte System (MPS), which reduced the circulation time of the liposomes and their ability to reach target tissues effectively[19]. Finally, challenges in controlling the release profile of ATRA from these liposomes meant that achieving a sustained and targeted drug delivery was difficult[16]. Therefore, if ATRA could be incorporated into a nanoparticle system, a high loading might be necessary to

achieve a therapeutically relevant concentration needed to induce a robust mucosal and systemic response, especially towards an amebiasis antigen at the target site, considering potential issues with stability and cellular uptake. Hence, in this study, we explore the incorporation of high loading dose ATRA into the nanoliposome adjuvant containing GLA and 3M-052 to enhance its chemical stability while maintaining the physical stability of the liposomes. The susceptibility of ATRA to oxidation and photostability in the final formulation was also evaluated.

Materials and Methods:

Glucopyranosyl lipid adjuvant (GLA), 1, 2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC), and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N- methoxy (polyethyleneglycol) -2000] (DSPE-PEG2000), were obtained from Corden Pharma (Liestal, Switzerland). 3M-052 was supplied courtesy of 3M Drug Delivery Systems (St. Paul, MN), and cholesterol was purchased from JT Baker (San Francisco, CA) or Sigma (St. Louis, MO). Tris-hydrochloride and phosphate buffer salts were purchased from Sigma (St. Louis, MO), while glycine was acquired from Janssen Pharmaceuticals (Belgium).

Optimisation of High Concentration Loading of ATRA:

Small Unilamellar Vesicles (SUV) liposomes were targeted in the preparation of our liposomes. The liposomes were prepared by combining Dipalmitoyl phosphatidylcholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000), and cholesterol at a molar ratio of 12:1:7, respectively, along with ATRA, 3M-052 and GLA in chloroform. The chloroform was evaporated for a minimum of 15 hours using a rotary evaporator. Ammonium phosphate buffer (25 mM, pH-5.6) was added to the dried film. After that, it was sonicated in a Crest Powersoic CP230D (Trenton, NJ) ultrasonicator bath at ~60°C for ~2 h to reduce liposome particle size. The final target concentrations of the active ingredients (ATRA, 3M-052, and GLA) were 3 mg/ml, 0.2 mg/ml, and 0.5 mg/ml, respectively. Increased lipid content was employed to vary the ratio of liposomal lipids to ATRA (10:1, 5:1, 3.9:1) using two different buffering systems (1 M Tris–Tris–HCl (pH=9.2) or 0.2M Glycine (pH = 10.2). These formulations were monitored for physicochemical stability and stored at 4°C for two months. Finally, the formulation containing a 1:3.9 ATRA: lipid ratio was alternatively manufactured to include α-tocopherol at a final concentration of 0.2 mg/ml. The resulting formulation was purified using size exclusion gel chromatography (SEC) with Sepharose CL-4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden to separate non-encapsulated ATRA. The formulation was monitored for stability at 4°C for two months.

***Determination of ATRA, GLA and 3M-052 content by HPLC*:**

The concentration of ATRA was determined using a modified high-performance liquid chromatography (HPLC) method, as described by Hsi et al. 2019[20]. The chromatographic system consisted of an Agilent 1100 series liquid chromatography (Agilent Technologies, Palm Alto, USA) fitted with a quaternary pump and a Diode array UV detector (190–900 nm). Chromatographic separation was achieved at 25°C on a reversed-phase Agilent Zorbax XDB C-18 Column (5 µm, 150X4.6mm i.d; Agilent Technologies, Palm Alto, USA), with the mobile phase comprised of methanol % glacial acetic acid (88:12% v/v) at a flow rate of 1.0 mL/min. The effluents were monitored with UV detection from a diode array detector at 343 nm. The formulation was diluted 10-fold with methanol, and 20 µL of the solution was injected and eluted on the C18 column. ATRA eluted at around 9.5min. No interference was observed from the presence of other components constituting the liposome. Under the conditions described, the linear calibration curve of ATRA was obtained in the concentration range of 1-200 µg/mL (r2 = 0.999), and the limit of detection of this assay was 20 ng/ml. The recovery of ATRA in samples was 99.5% in comparison with a methanol solution of ATRA alone. The Relative Standard Deviation Values obtained for both repeatability (intra-day precision) and intermediate precision experiments (inter-day) were less than 3%.

The concentrations of GLA and 3M-052 were determined using reversed-phase High-Performance Liquid Chromatography (HPLC) methods, based on procedures previously described by Abhyankar et al. (2021)[21]. GLA concentration was determined using reversed-phase HPLC coupled with charged aerosol detection (CAD). The chromatographic separation employed an Agilent Zorbax XDB C-18 Column (5 µm, 150X4.6mm i.d; with a gradient elution over 25 minutes at a flow rate of 1 ml/min. Mobile phase A consisted of 75:15:10 (v:v:v) methanol: chloroform: water with 20 mM ammonium acetate and 1% acetic acid. Mobile phase B was a 1:2 (v/v) mixture of methanol: chloroform with 20 mM ammonium acetate and 1% acetic acid. 3M-052 concentration was determined using HPLC with UV detection at a wavelength of 321nm.

Before injection, the formulations were diluted 10-fold in isopropanol containing 0.5% trifluoroacetic acid. Elution was performed on an Agilent Zorbax XDB C-18 Column (5 µm, 150X4.6mm i.d; Agilent Technologies, Palm Alto, USA) using a gradient mobile phase system. Mobile Phase A was 0.1% trifluoroacetic acid in water, Mobile Phase B was methanol, and Mobile Phase C was isopropanol. The gradient program was as follows: initial (85% A, 15% B), 2.5 min (60% A, 40% B), 17.5 min (5% A, 40% B, 55% C), 22.0 min (5% A, 40% B, 55% C), and 22.5 min (85% A, 15% B).

For all the analytes, concentrations were measured approximately 1 month after the manufacturing of the formulations[22].

Determination of particle size, diameter, and polydispersity index (PDI)

The scattering intensity-based mean particle diameter (Z-ave) and polydispersity index (PDI) of ATRA-loaded liposomes were determined by the dynamic light scattering (DLS) method using a Zetasizer Nano ZS (Malvern Instruments- Worcestershire, UK) at a scattering detection angle of 173°C and measured at room temperature. Before the measurement, the liposome was diluted 10-fold in ultrapure water (18.2Ω) to arrive at an adequate scattering intensity.

***Incorporation Efficiency*:**

The amount of ATRA incorporated in the liposome was determined by HPLC after appropriate dilution of each formulation with methanol, performed in triplicate. The percentage of ATRA in the liposome was calculated as % loaded = concentration of ATRA measured in liposome/ initial concentration of ATRA added.

***Stability studies*:**

Selected nanoliposome formulations were subjected to stability studies. The formulations were stored at 4ºC for two months to evaluate the effect of temperature on storage. The stability was determined by measuring ATRA content (HPLC), particle size and size polydispersity (DLS) of the liposomes at monthly intervals.

Photostability:

One millilitre of the nanoliposome (0.5mg/mL GLA; 0.2mg/mL 3M-052) formulation containing 3mg/mL of ATRA with or without α-tocopherol at pH 10.2 was transferred into amber or clear glass vials and exposed to cool white fluorescent and near-ultraviolet lamp for six hours at room temperature. For comparison, a solution of ATRA in methanol at the same concentration was used as a control. The samples (Both control and test) were placed 50 cm from the UV lamp. All the preparations were adjusted to the same ATRA concentration. One hundred microliters of each of the samples were taken at 0, 1, 2, 4, and 6 h, then diluted ten-fold with methanol, while 20 µl of the solutions were injected for HPLC analysis.

Oxidation:

One millilitre of the nanoliposome (0.5mg/mL GLA; 0.2mg/mL 3M-052) formulation containing 3mg/mL of ATRA with or without α-tocopherol was mixed with 1 ml of 30% hydrogen peroxide solution in different amber bottles protected from light, and the reaction mixtures were allowed to proceed at room temperature for 6 hours with intermittent shaking. For comparison, a solution of ATRA in methanol at the same concentration was used as a control. All the preparations were adjusted to the same ATRA concentration. One hundred microliters of each of the samples were taken at 0, 1, 2, 4, and 6 h, then diluted ten-fold with methanol, while 20 µl of the solutions were injected for HPLC analysis.

RESULT

We first evaluated the effect of the ATRA: lipid ratio on liposome particle size and the incorporation efficiency of ATRA. Increasing the ATRA: lipid ratio resulted in a decrease in average particle diameter (Z-ave) and an increase in ATRA loading efficiency (Figure 1). Moreover, we evaluated the effects of different buffer systems (ammonium phosphate (pH 5.6) to Tris-HCl (pH 9.0) and glycine (pH 10.2). Whereas liposome particle size decreased considerably with increasing pH, loading efficiency was maximised in the Tris-HCl pH 9.0 buffer. (Figure 1). Liposomes prepared at pH 9.0 and pH 10.2 exhibited comparable initial particle sizes and ATRA loading efficiencies (Figure 1). However, after two months of storage at 4°C, the particle size of the formulation at pH 9.0 increased dramatically (495%), whereas liposomes at pH 10.2 showed only a slight increase in size (12%) (Table 1). Despite this physical stability at pH 10.2, ATRA content decreased significantly in both formulations after two months (58% at pH 10.2 and 66% at pH 9.0) (Table 1), indicating chemical degradation. The inclusion of α-tocopherol (Vitamin E) during liposome preparation enhanced ATRA

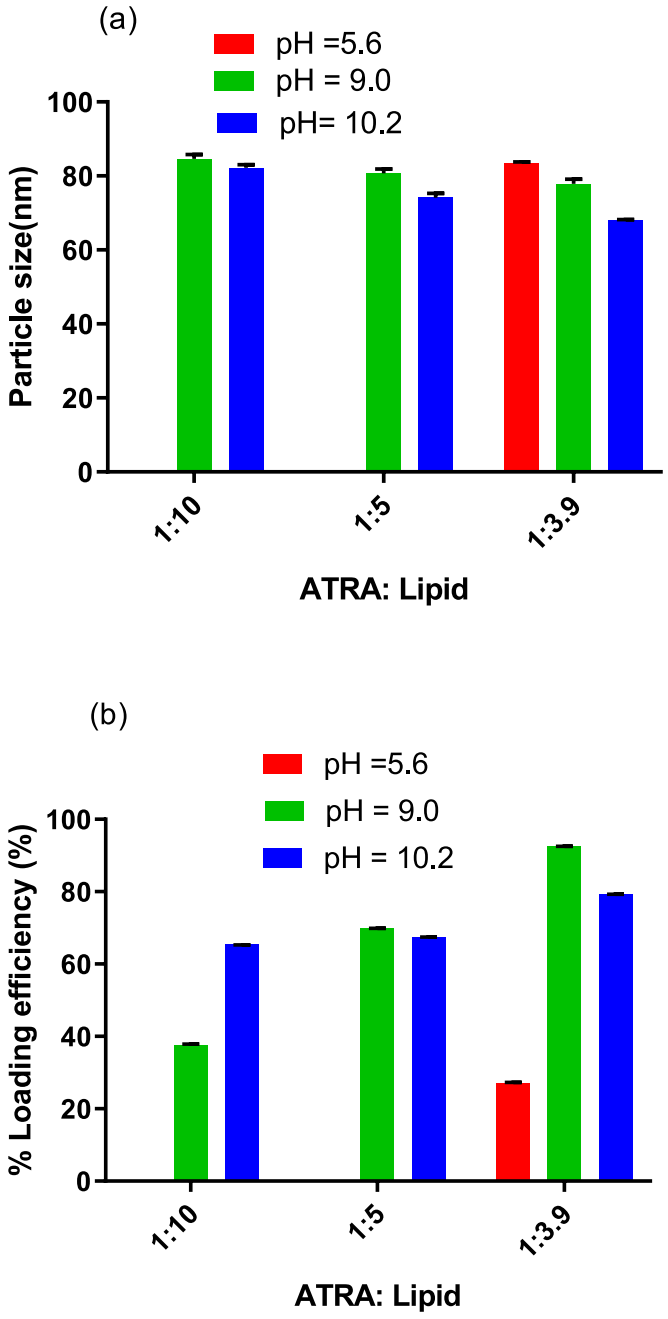


Figure 1: Effects of pH and ATRA to Lipid ratio on the (a) particle size and (b) % incorporation efficiency of ATRA in liposome (mean ±SD, n=6 measurements)

Table 1: Physical and Chemical stability of ATRA loaded liposomes (ATRA/lipid ratio 1:3.9) at 4°C

pH	Particle size (nm)			% ATRA content		
@ 4°C	T0	T 60days	**%Δ**	T0	T 60days	%Δ
9.0	77.85 ± 1.29	463.37 ± 12.20	495.20	92.43 ± 0.11	31.80 ± 0.09	65.60
10.2	68.12 ± 0.12	76.35 ± 3.38	12.08	79.12 ± 0.17	21.33 ± 0.03	57.79

incorporation efficiency to approximately 100% at both pH 9.0 and 10.2 (Figure 2). After two months of storage at 4°C, both formulations containing α-tocopherol demonstrated improved chemical stability of ATRA (Figure 2). However, the formulation at pH 9.0 remained physically unstable, showing significant increases in particle size (160% and 200%) and polydispersity index (PDI) (Figure 3). In contrast, the liposomal formulation at pH 10.2 with α-tocopherol exhibited excellent particle size stability upon storage at 4°C (Figure 3). Exposure of ATRA solution in methanol to UV light for six hours resulted in a 43% loss of ATRA in a clear glass vial, which was reduced to 30% in an amber vial (Figure 4). Liposomal formulation (without α-tocopherol) provided some protection against UV-induced degradation, with 29% and 21% ATRA loss in clear and amber vials, respectively. The inclusion of α-tocopherol significantly enhanced photostability, resulting in only 12% and 2% ATRA loss in clear and amber vials, respectively (Figure 4). Similarly, the liposomal formulation with α-tocopherol offered superior protection against oxidation by hydrogen peroxide. The control ATRA solution in methanol degraded by 88%, and the liposomal formulation without α-tocopherol lost 42% ATRA after six hours. In contrast, the liposomes containing α-tocopherol lost only 14% ATRA under the same conditions (Figure 5). The liposome formulation optimised for high ATRA loading and stability (pH 10.2 with α-tocopherol) showed acceptable recovery of 3M-052 after one month but low recovery of GLA (Table 2), with GLA content continuing to decline over time.

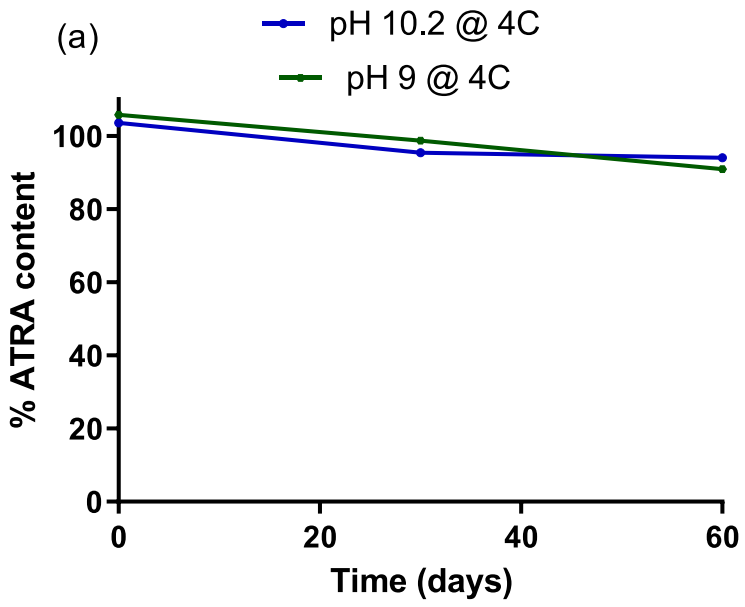


Figure 2: Chemical stability of liposomal ATRA containing α-tocopherol at pH 9.0 and 10.2 over 60 days at 4°C and (mean ± SD, n=3 measurements)

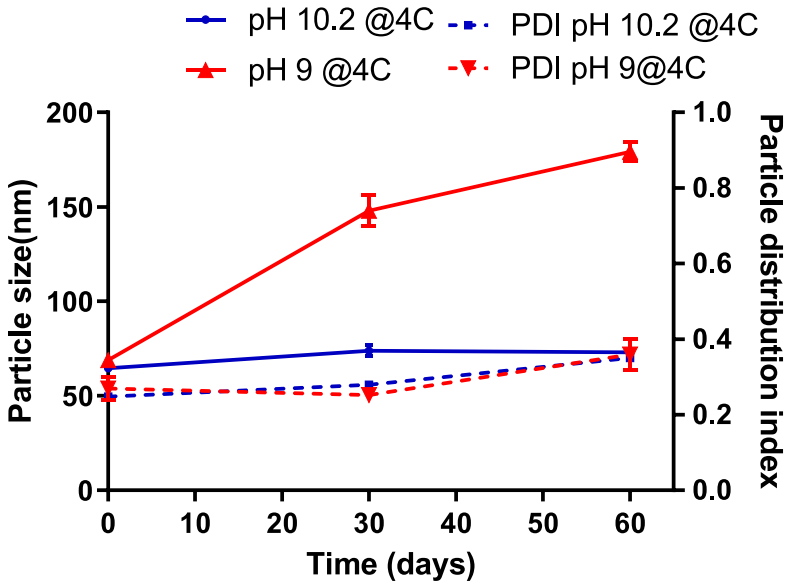


Figure 3: Physical stability of liposomal ATRA containing α-tocopherol at pH 9.0 and 10.2 over 60 days at 4°C (mean ± SD, measurements)

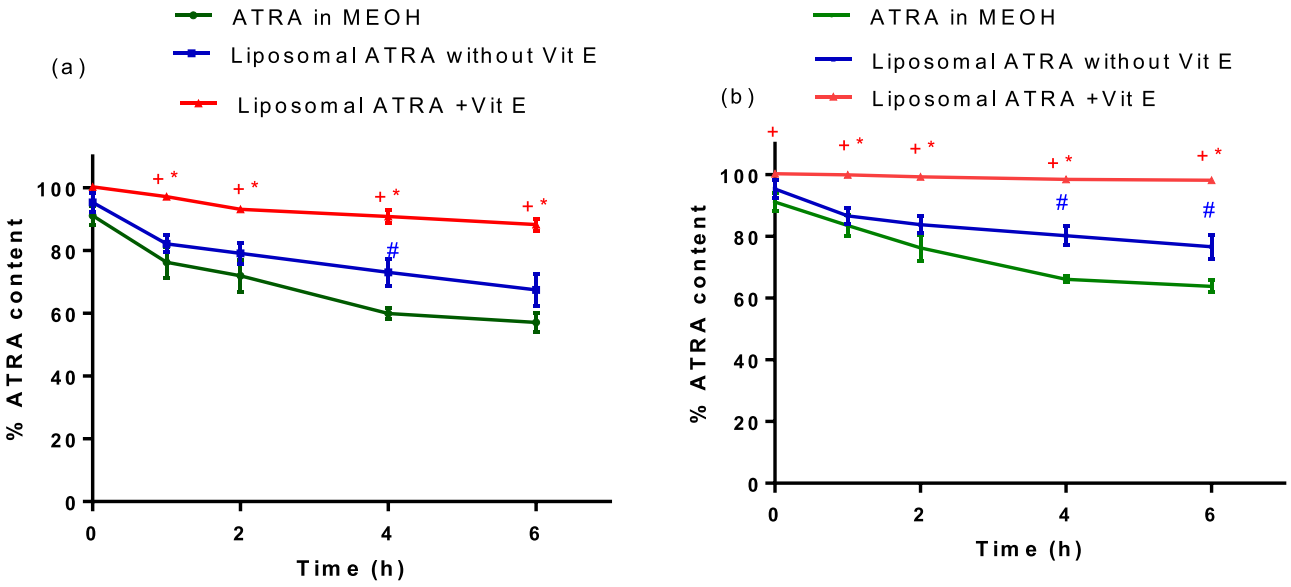


Figure 4: Effect of light on the chemical stability of Retinoic acid loaded liposome by exposing the samples to UV light for 6 h in (a) clear glass and (b) amber glass bottles. (Mean ± SD, n=7 measurements).+ = $p < 0.05$ for ATRA in MeOH VS Liposomal ATRA +Vit E, # = $p < 0.05$ for ATRA in MeOH VS Liposomal ATRA without Vit E, * = $p < 0.05$ for Liposomal ATRA without Vit E VS Liposomal ATRA +Vit E.

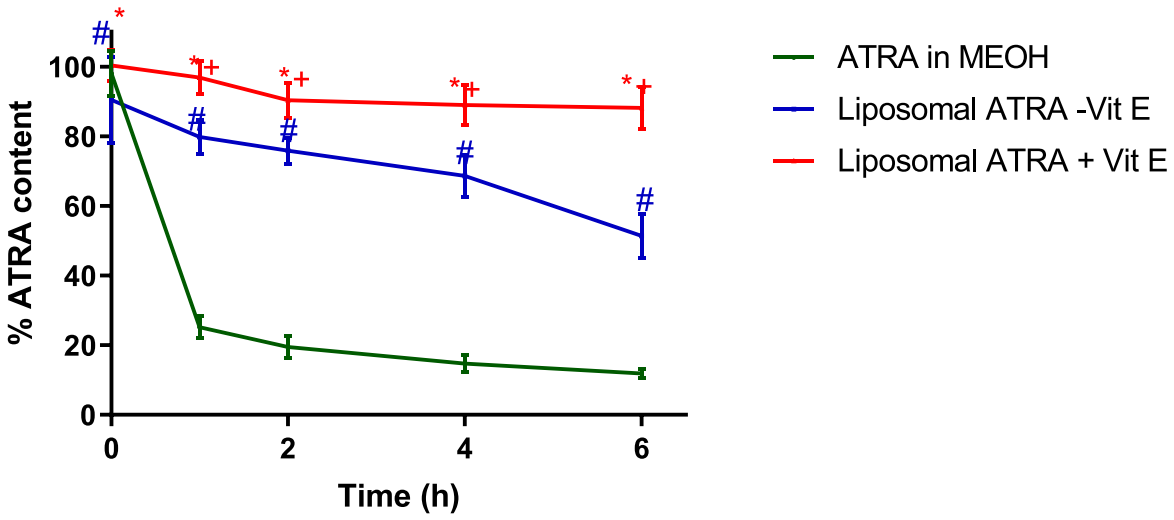


Figure 5: Effect of oxidation on the chemical stability of Retinoic acid-loaded liposome by reaction with hydrogen peroxide for 6 h. (Mean ± SD, n=7 measurements). + = $p < 0.05$ for ATRA in MeOH VS Liposomal ATRA +Vit E , # = $p < 0.05$ for ATRA in MeOH VS Liposomal ATRA without Vit E, * = $p < 0.05$ for Liposomal ATRA without Vit E VS Liposomal ATRA +Vit E.

Table 2: Chemical stability of TLR ligand liposomes (ATRA/lipid ratio 1:3.9) containing ATRA stored at 4°C

Component	Target Concentration (mg/ml)	Measured Concentration (mg/ml)
GLA	0.500	0.305 +/- 0.006
3M-052	0.200	0.196 +/- 0.009
ATRA	3.000	2.603 +/- 0.114

Note: GLA and 3M-052 concentrations were measured approximately 1 month after formulation manufacture; ATRA concentration was measured approximately 2 months after formulation manufacture. Standard deviations shown represent the variation in triplicate measurements of one liposome batch.

Discussion

In the present study, we prepared a nanoliposome adjuvant containing high-loaded ATRA, GLA (TLR4), and 3M-052 (TLR7/8). The liposome was evaluated for its ability to protect ATRA from light- or oxygen-induced chemical instability while maintaining physical liposome stability as measured by average particle size and size polydispersity. The influence of pH on the loading efficiency of ATRA liposome may be related to the chemical behaviour of ATRA in an aqueous solution. ATRA is an amphiphilic [23] compound with a hydrophobic long-chain hydrocarbon and a water-soluble ionizable carboxylic group at its polar end[22]. In non-polar solvents, there is the formation of tail-to-tail dimers of ATRA through self-association[23]. These dimers are considered to be stable through hydrogen bonding between the carboxyl groups of two ATRA molecules[24]. On the other hand, in water at pH 7, self-association takes the form of micelle-like structures with the appearance of turbidity. In contrast, at pH > 7.4, ATRA is known to be soluble in water at 1.5 mM, forming a clear yellow solution. Reduction in the pH of ATRA solution below the critical micellar concentration (2 µmol/L at pH 7) will lead to the eventual loss of the negative surface charge, which stabilises the ATRA micelles. The particles will collide, forming aggregates that precipitate as seen in some of our formulations made at low pH, resulting in low loading efficiency at low pH[24]. Our study demonstrates that the ATRA: lipid ratio and the pH of the hydrating solvent are

crucial factors determining the incorporation of ATRA into liposomes. These findings are consistent with numerous reports in the literature highlighting the significant impact of formulation parameters on liposomal encapsulation efficiency. For example, Rodsamai et al, (2025) and Ko& Lee (2010) explored nanoliposomes as effective carriers for lipid-soluble compounds like Red Palm Oil, rich in carotenoids, and retinol, a retinoid. They demonstrated that optimising the ratio of phospholipid to retinol is crucial for achieving high encapsulation efficiency and improving the storage stability of β-carotene and retinol against oxidation. However, neither article specifically investigates the challenges of achieving and maintaining stability at very high loading capacities for their respective compounds[25,26.] Moreover, the ratio of drug to lipid is a well-documented factor influencing encapsulation efficiency for various types of drugs. Studies have consistently shown that while increasing the drug content relative to the lipid amount can increase the total drug load up to a certain point, the efficiency of encapsulation often declines beyond an optimal drug: lipid ratio.[27] This phenomenon has been observed for both hydrophobic and amphipathic drugs and is often attributed to saturation of the lipid bilayer or aqueous core, drug precipitation, or disruption of the liposome structure at high drug concentrations relative to the available lipid[28,29]. Our finding that maximum ATRA incorporation and stability occurred at a low ATRA: lipid ratio aligns with this principle, suggesting an optimal ratio exists for stable encapsulation within this specific lipid composition[30,31]. Furthermore, the pH of the hydration medium is widely recognised for its significant impact on the encapsulation efficiency of ionizable drugs within liposomes[31,32] Changes in pH can dramatically alter the ionization state and solubility of a drug, thereby affecting its partitioning behavior between the aqueous phase and the lipid bilayer or the internal aqueous compartment of the liposome[33]. For weakly acidic drugs like ATRA, which has a carboxyl group, an elevated pH would increase the proportion of the ionized (deprotonated) form.[34] The ionized form is generally more water-soluble and might interact differently with the lipid membrane compared to the neutral, protonated form, especially if the liposomes contain charged lipids[35,36]. Conversely, conditions favouring the neutral, more hydrophobic form are often preferred for enhancing partitioning into the lipid bilayer of conventional liposomes[36]. Our observation that elevated pH improved ATRA incorporation suggests a complex interplay between the ionization state of ATRA, its interaction with the specific lipids used in this formulation, and the overall liposome structure stability at that pH[37]. While elevated pH benefited ATRA incorporation and 3M-052 stability, it notably impacted GLA content, highlighting the delicate balance required when encapsulating multiple components with potentially conflicting optimal conditions[36]. Moreover, the observation that particle size decreased with increasing pH suggests that the higher pH environment might promote the formation of smaller, potentially more stable vesicles during the preparation process. The physical instability observed in liposomes prepared at pH 9.0 during storage, despite good initial loading, suggests that this pH may not be optimal for long-term vesicle integrity. The dramatic increase in particle size likely indicates aggregation or fusion of liposomes. In contrast, the formulation prepared at pH 10.2 demonstrated better physical stability over the two-month storage period. In earlier studies with retinoic acid and other retinoids, it was shown that heat, light, and oxidants could cause solutions of these compounds to degrade[38]. It was also reported that the inclusion of ATRA in liposomes, nisomes, and solid lipid particles could protect the drug against photodegradation[39]. In an attempt to improve the chemical stability of ATRA, we introduced α-tocopherol (Vitamin E) as a liposome component. The inclusion of α-tocopherol proved to be a crucial factor in enhancing the chemical stability of ATRA within the liposomes, particularly at pH 10.2. The near-complete incorporation of ATRA in the presence of α-tocopherol suggests a synergistic effect, potentially due to the antioxidant properties of α-tocopherol preventing ATRA degradation during the encapsulation process and storage. The continued physical instability at pH 9.0, even with α-tocopherol, indicates that pH 10.2 provides a more favourable environment for maintaining liposome structure in this specific formulation. Thus, the inclusion of α-tocopherol improved the chemical stability of ATRA in liposomal formulation, especially at pH 10.2 when stored at 4ºC[43].

We then investigated the influence of UV light and oxidants on the liposomal ATRA formulation (pH 10.2). The investigation into photostability and protection against oxidation further underscores the benefits of liposomal encapsulation and the added value of α-tocopherol. With the introduction of α-tocopherol, the stability of ATRA in the liposomal formulation was significantly enhanced in both the clear and amber vials. Thus, maximum photostability was achieved in the presence of α-tocopherol. However, in the absence of α-tocopherol, the liposome protected ATRA from photodegradation, most likely due to the protective film surrounding ATRA that is

provided by the phospholipid bilayer in the liposome[40]. Moreover, an additional photostability benefit was achieved by employing an amber glass vial instead of a clear glass vial.

Next, the ability of α-tocopherol to protect the ATRA formulation from oxidation was investigated. The inclusion of α-tocopherol significantly enhanced the protection of ATRA from oxidation. ATRA degrades rapidly in methanol while liposomal encapsulation improves stability, and adding Vitamin E to the liposomes further enhances ATRA stability. α-tocopherol markedly improved the resistance of ATRA to oxidation by hydrogen peroxide, highlighting its role in preserving the drug's integrity. This demonstrates that liposomal encapsulation, especially with Vitamin E, significantly protects ATRA from degradation[46.]. The inclusion of α-tocopherol (Vitamin E), a hydrophobic antioxidant, was found in this study to be important, primarily enhancing the chemical stability of ATRA by protecting against oxidation and photodegradation within the liposomal environment, a known benefit of incorporating antioxidants in lipid formulations[41]. α-tocopherol is chosen as a hydrophobic antioxidant because it integrates into the liposome's lipid bilayer, ideally positioning it to protect the co-localised ATRA and lipids from oxidation and photodegradation. This provides advantages over water-soluble antioxidants, which are less effective in the lipid phase, and may offer a better safety profile compared to some synthetic lipid-soluble alternatives. This specific localisation, combined with its effective radical scavenging activity and favourable safety profile, offers significant advantages over water-soluble antioxidants and some synthetic lipid-soluble alternatives for protecting a hydrophobic drug in a liposomal formulation[42,43].

Finally, we evaluated whether the formulation optimised for ATRA loading and stability remained suitable for the TLR ligands (GLA and 3M-052). The liposome formulation optimised for loading of high amounts of ATRA appeared to remain suitable for the incorporation of 3M-052, but not GLA. The loss in GLA content is apparently due to chemical instability at high pH. The finding that the optimised ATRA formulation was not suitable for the stable incorporation of GLA points to the challenges of co-encapsulating multiple active pharmaceutical ingredients with potentially different stability profiles and optimal loading conditions. It is also likely that vaccine antigen and phospholipid-based excipients will demonstrate reduced stability at high pH values over time, although those parameters were not evaluated here. Extreme pH values are also less desirable in terms of the potential reactogenicity of parenteral injection. Therefore, the high pH during preparation is a processing condition chosen to optimise the chemical state of ATRA for efficient loading and potentially to achieve desirable liposome characteristics like smaller particle size, rather than reflecting the intended physiological environment for administration. Parenteral injections (such as intravenous, intramuscular, or subcutaneous) require the formulation to be at or near physiological pH (approximately 7.4) and osmolarity to avoid pain, tissue damage, haemolysis, and other adverse reactions[42]. Injecting a solution with a pH as high as 9.0 or 10.2 would be highly irritating and potentially harmful to tissues. However, this does not mean that liposomes prepared at high pH cannot be used for parenteral administration. The final step in the manufacturing process for a parenteral liposomal formulation would involve adjusting the bulk suspension to a physiological pH using appropriate buffering agents (e.g., phosphate buffer saline at pH 7.4) and adjusting osmolarity if necessary. The liposomes, once formed and loaded with the drug at the optimal preparation pH, would then be stable within this physiological buffer for injection. The high pH is a transient condition during the liposome formation and loading phase, not the final state of the product administered to the patient. Thus, the formulation developed in this work requires further refinement to optimize usability. Nevertheless, we demonstrated that modulation of pH and inclusion of α-tocopherol promote ATRA incorporation and stability in liposomes,

4.0 Conclusion

In this study, ATRA at a high concentration (3 mg/ml) was incorporated into liposomes containing toll-like receptor ligands (GLA and 3M-052). The ATRA: lipid ratio and pH of the hydrating solvents play a crucial role in the ability to incorporate ATRA into liposomes, with the formulation at pH 10.2 and an ATRA: lipid ratio of 1:3.9 showing improved physical and chemical stability compared to lower pH and higher ATRA: lipid ratios. Liposomes provided some protection from photodegradation and oxidative degradation of ATRA compared to an unformulated methanol solution of ATRA. However, the incorporation of α-tocopherol resulted in significantly enhanced incorporation efficiency and chemical stability of ATRA. Nevertheless, whereas the optimized liposome formulation remained suitable for the TLR7/8 agonist 3M-052, a significant loss in the TLR4 agonist GLA content occurred, likely due to the high pH condition. Future

studies will seek to modify further the liposomes developed here to facilitate stability of GLA (along with 3M-052 and ATRA) at reduced pH values and to evaluate the adjuvant vaccine activity for enteric indications subsequently.

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Author contributions:

Babatunde Adeagbo: Conceptualisation, Methodology, Formal analysis, Writing –original draft preparation; **Aaron Kahn** - Methodology, Formal analysis, Writing –review and editing; **Oluseye Oladotun Bolaji and Christopher Fox** - Conceptualisation, Methodology, Formal analysis, Supervision, Resources, Writing – review and editing.

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