*IFSCC 2025 full paper (**IFSCC2025-1155**)*

"Breakthrough of lipoplex for the treatment of dermatological diseases"

Ainhoa Díaz de Pedroviejo[1], Paula Vela Cristobal[1], Sandra Freitas-Rodríguez[2], Ana Mª Coto García [1], Rebeca Alonso Bartolomé[1]

[1]R&D, Nalon Innova SL, Oviedo, [2]R&D, Nanovex Biotechnologies SL, Llanera, Spain

1. Introduction

RNA-based therapies have primarily been used in research settings. However, the COVID-19 pandemic demonstrated the potential of this technology through the development and deployment of mRNA vaccines. This breakthrough opened new avenues for exploring RNA technologies across various fields [1].

RNA interference (RNAi) is widely used for gene silencing and drug development due to its remarkable specificity, potent efficacy, minimal side effects, and simple synthesis methods. Among the most studied strategies is the use of small interfering RNAs (siRNAs) as therapeutic agents, which are particularly suitable for the treatment of skin diseases. The skin is the most accessible organ for siRNA, and clinical trials have already been conducted for conditions such as psoriasis, pigmented dermatosis, and androgenic alopecia. However, one of the main challenges in its clinical application is the difficulty of siRNA in penetrating the skin barrier and reaching target cells to exert its gene-silencing effect [2]. This limitation is due to RNA being a negatively charged macromolecule, which causes electrostatic repulsion with the cell membrane, thereby hindering cellular entry. Therefore, the development of effective transdermal delivery systems has become a key focus for enhancing the therapeutic efficacy of siRNA in the treatment of skin diseases [1,9].

An ideal vehicle for the transdermal delivery of siRNA should preserve its physical and chemical properties, and among the various delivery systems available, liposomes stand out for their ability to encapsulate and protect nucleic acids [2]. To overcome these limitations, delivery systems based on cationic liposomes have been developed. These systems not only enhance overall stability but also promote electrostatic interaction with RNA and cellular membranes, thereby facilitating more efficient skin penetration [4].

The objective of this study is the rational design and development of a delivery system based on cationic liposomes complexed with a custom-designed siRNA (lipoplexes) [4] for

topical or transdermal administration. The siRNA has been specifically engineered to target tyrosinase, a key enzyme in the melanogenesis pathway. By silencing tyrosinase expression, this system aims to effectively inhibit melanin synthesis, achieving a depigmenting effect. The approach holds significant potential for application in both pathological and physiological contexts where melanogenesis is dysregulated, such as in melasma, post-inflammatory hyperpigmentation, and vitiligo [3].

To achieve this goal, an in-depth characterization study of the lipoplexes was conducted using advanced analytical techniques. These included: dynamic light scattering (DLS); zeta potential (ZP); nanoparticle tracking analysis (NTA); HPLC-UV-Vis; AF4-MALS-ICP/MS and gel electrophoresis; and in vitro skin absorption assays [5–7]. Finally, although these are preliminary results that still require validation, a delivery system compatible with cosmetic ingredients has been successfully developed. The system is capable of complexing siRNA in a stable manner over time and demonstrates biological efficacy on its molecular target.

2. Materials and Methods

Preparation of Cationic Liposomes

Cationic liposomes were prepared using the thin-film hydration (TFH) method, as previously described in the literature [5–7]. The lipids used phosphatidylcholine (PC), cetyltrimethylammonium chloride (CTAC), polysorbate 80 (Tw80), and cetyl alcohol (CetylOH) were weighed and dissolved in ethanol in various proportions. The organic solvent was then removed by rotary evaporation under vacuum (using an IKA RV 3 rotavapor), forming a uniform lipid film on the wall of a round-bottom flask at room temperature. The resulting lipid film was hydrated with PBS buffer at pH 7.0 for 30 minutes and subsequently homogenized using a Polytron PT-2500 for 5 minutes.

Lipoplex Synthesis

Lipoplexes were prepared by mixing empty liposomes with RNA previously dissolved in nuclease-free PBS buffer. The mixture was incubated by vortexing at room temperature, using different liposome:RNA ratios.

Physicochemical Characterization

Particle size, polydispersity index (PDI), and zeta potential (ZP) were measured by dynamic light scattering (DLS) using a NanoZS90 and Zetasizer Ultra (Malvern). Particle concentration was determined using Nanosight LM10 (NTA).

Gel Retardation Assay

The amount of RNA bound to the liposomes was assessed by agarose gel electrophoresis. Free RNA and lipoplexes were separated by electrophoresis on a 1.5% agarose gel containing 0.01% ethidium bromide, run at 85 V for 20 minutes. The gel was visualized using a Quantum-ST4 3026-WL/26M transilluminator.

Lipoplex Purification and Analysis

A commercially available RNA labeled with a fluorophore was used for this assay. Free RNA was separated from RNA associated with liposomes by ultrafiltration using tubes with membranes made of polysulfone (PES) and cellulose, with different molecular weight cut-offs. Both free RNA and lipoplex samples were centrifuged for 5 minutes at various relative centrifugal forces (G). The resulting fractions were then analyzed and quantified using asymmetric flow field-flow fractionation coupled with multi-angle light scattering and ICP-MS detection (AF4-MALS-ICP/MS).

AF4-MALS-ICP/MS

The Asymmetric Flow Field-Flow Fractionation (AF4) system used was an AF2000 MT instrument (Postnova Analytics, Germany). The AF4 system was online coupled to an Agilent 1260 Infinity MWD UV/Vis absorbance detector (Agilent, Germany); a Multiangle Light Scattering (MALS) PN3621 (Postnova Analytics Inc., Germany); and an ICP-MS/MS instrument (Agilent 8900, Japan). The integration of the fractographic peaks was performed using MassHunter software (Agilent) [8]. This system allowed the detection of RNA via the sulfur signal from the fluorophore, while liposomes were identified based on the phosphorus (P) signal originating from phosphatidylcholine.

In vitro dermal absorption assays

Reconstructed human skin models (16 mm discs) from MatTek were used in this study. After application of the prototypes onto the reconstructed skin, supernatants were collected after 2 days to assess the amount of RNA released into the medium. Skin samples were then harvested after 5 days to evaluate RNA penetration and retention within the tissue. RNA was subsequently extracted from both types of samples. The RNA content in the administered test product was quantified using UPLC/MS/MS.

3. Results

3.1. Development, Characterization, and Stability of Scalable Liposome-Based RNA Carriers

The first step involved the preparation of empty liposomes using the thin-film hydration (TFH) method, was optimize the lipid composition by varying molar percentages of phosphatidylcholine (PC) (23–80 mol%), cetyltrimethylammonium chloride (CTAC) (7–19 mol%), polysorbate 80 (Tw80) (1–70 mol%), and cetyl alcohol (CetylOH) (18 mol%). Once the formulation was defined, the process was scaled up using increasing concentrations of lipids to evaluate whether higher concentrations could be used without affecting particle properties.

As shown in Table 1, the synthesis method yielded liposomes with sizes below 150 nm and a polydispersity index (PDI) below 0.2, even at lipid concentrations up to 400 mg/mL. These results demonstrate that the process is scalable while maintaining desirable physicochemical characteristics.

Table 1. Particle size and polydispersity index (PDI) of empty liposomes synthesized at different precursor concentrations (measured by DLS).

Concentration (mg/mL)	Size (nm)	PDI	Particle Concentration (p/ml) *order of magnitude*
5	83	0.126	10^{12}
50	96	0.160	10^{13}
200	125	0.096	10^{14}
300	120	0.137	10^{14}
400	122	0.152	10^{14}

To further confirm particle characteristics, formulations were analyzed using nanoparticle tracking analysis (NTA), which corroborated particle size and provided particle concentration estimations. As shown in **Figure 1**, the formulation prepared at 5 mg/mL yielded particles of ~80 nm and a concentration in the range of 10^{12} particles/mL. Higher precursor concentrations (up to 400 mg/mL) enabled the production of highly concentrated formulations (~10^{14} particles/mL) while maintaining monodispersity.

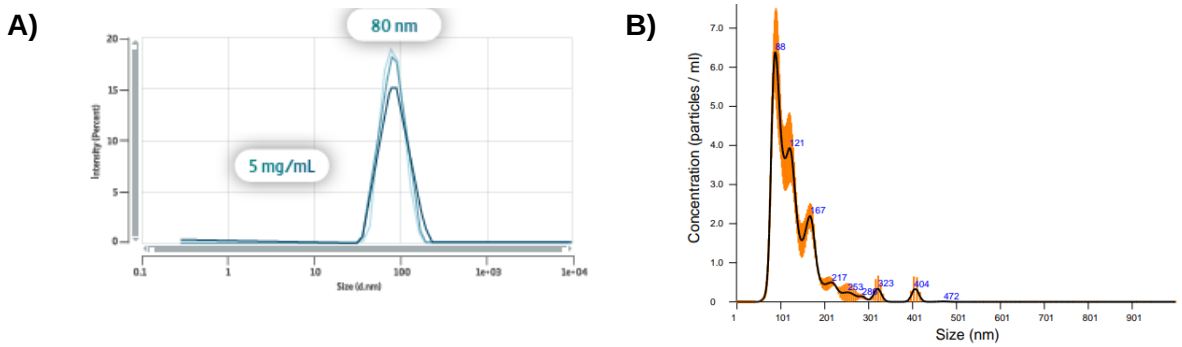

Figure 1. (a) Analysis example of lipoplex by DLS; (b) Size distribution and concentration of lipoplex by NTA.

Once the liposomes were synthesized, lipoplexes were formed by electrostatic complexation with RNA at various weight ratios. The resulting conjugates were characterized in terms of particle size and zeta potential to assess formulation stability.

As shown in Figure 2, Lipoplex A exhibited a stable particle size of approximately 80 nm across all tested RNA:liposome ratios. However, zeta potential measurements revealed differences depending on the amount of RNA used. Increasing the RNA content led to a progressive neutralization of the positively charged liposome surface, with charge saturation achieved at a particle:RNA ratio of approximately 50:1.

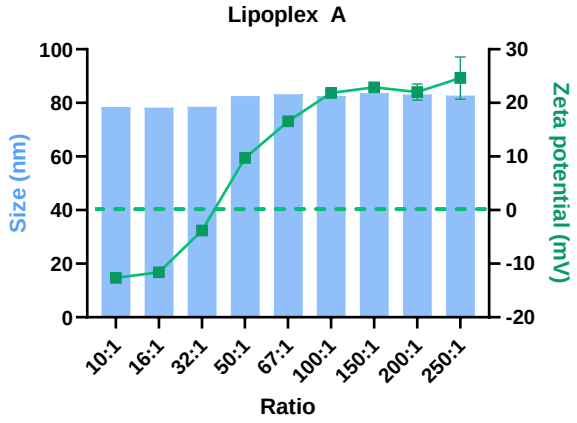

Figure 2. Particle size distribution and surface charge (zeta potential) of Lipoplex A as a function of the RNA:liposome ratio.

3.2 Quantification of RNA Complexation by Gel Electrophoresis

In addition to zeta potential analysis, gel electrophoresis was used to assess the degree of RNA complexation with liposomes and to confirm the stability of the lipoplex formation. While zeta potential provides an estimation of surface charge, it does not distinguish between RNA electrostatically bound to the surface and RNA simply adsorbed.

Gel electrophoresis is a standard method for analyzing RNA–liposome interactions by exploiting differences in electrophoretic mobility. Free RNA, empty liposomes, and lipoplexes display distinct migration patterns, and the use of fluorescent staining (e.g., ethidium bromide) allows the specific detection of RNA within the gel.

To disrupt electrostatic interactions between RNA and liposomes and evaluate the amount of unbound RNA, two different intercalating agents were tested. Initial experiments evaluated liposome:RNA ratios ranging from 10:1 to 250:1, based on prior zeta potential data. However, this range was later expanded to better observe saturation behaviour and improve differentiation between bound and free RNA.

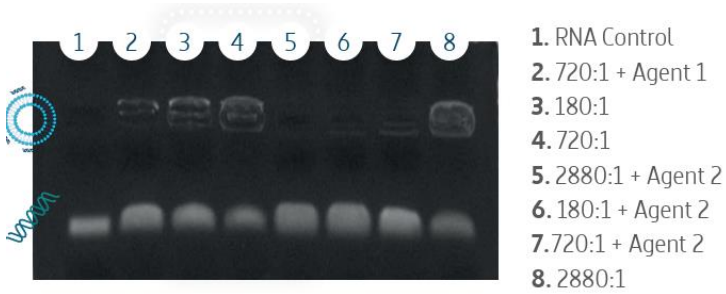

Figure 3. Electrophoretic analysis of lipoplexes using two different agents to disrupt electrostatic interactions.

As can be seen, in Figure 3, a comparison of lanes 3, 4, and 8 with lanes 5, 6, and 7 shows that Agent 2 successfully disrupted RNA–liposome binding, as evidenced by the appearance of a more intense band corresponding to free RNA. In contrast, Agent 1 was ineffective, as no significant increase in the free RNA band was observed (e.g., lane 2 vs. lane 5). To validate

these findings, the experiment was repeated using a higher incubation ratio (2880:1), which further confirmed the superior performance of Agent 2 in disrupting electrostatic interactions.

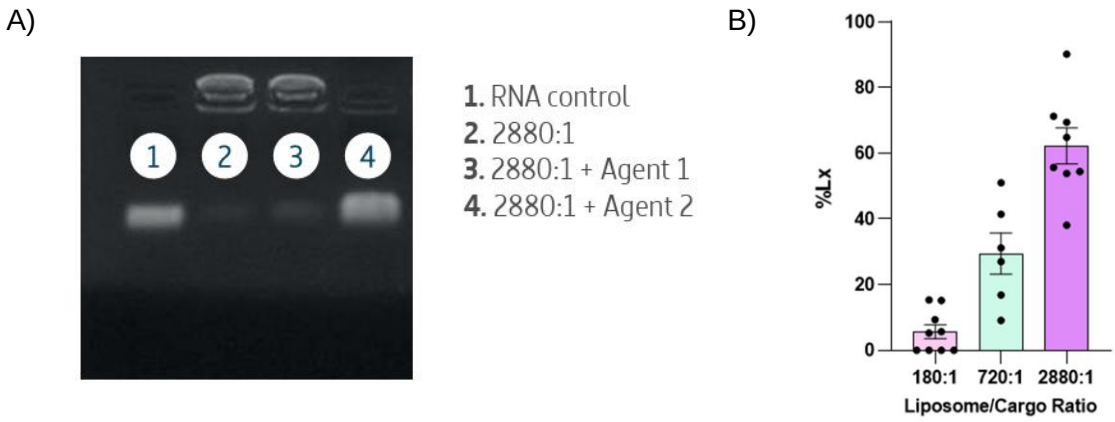

Figure 4. (a) Comparative gel analysis of disruption agents at high liposome:RNA ratio (2880:1); (b) Quantification of RNA complexation (%) at different ratios using ImageJ.

Quantification of gel images with ImageJ revealed that, under the condition with the highest liposome:RNA ratio tested, approximately 60% of the RNA remained associated with the liposomes, indicating a high level of complexation at this ratio.

3.3. Purification and Quantification of Lipoplex by AF4-MALS-ICP/MS.

To further quantify the degree of RNA complexation and assess purification strategies, an assay was performed using one of the previously tested liposome:RNA ratios, incorporating fluorescently labeled RNA to facilitate tracking.

The lipoplex formulations were analyzed using asymmetric flow field-flow fractionation (AF4) coupled with multi-angle light scattering (MALS) and inductively coupled plasma mass spectrometry (ICP-MS). The phosphorus (P) signal, corresponding to phosphatidylcholine (PC) in liposomes, was used to trace the vesicles, while RNA was tracked through its fluorescence signal and later through sulfur (S) content via ICP-MS. The AF4-MALS-ICP/MS analysis indicated that approximately 40% of the RNA remained uncomplexed, i.e., not associated with liposomes.

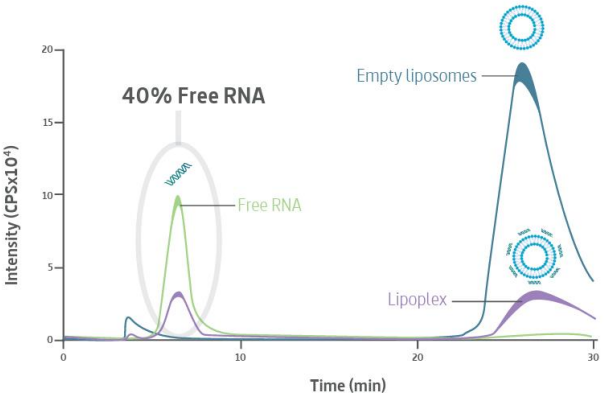

Figure 5. AF4-MALS-ICP/MS analysis showing the elution profiles of fluorescent RNA (green line), lipoplexes, and empty liposomes (purple line), traced by the phosphorus (P) signal associated with phosphatidylcholine.

To evaluate the effectiveness of a purification step, centrifugal ultrafiltration units with different molecular weight cut-offs (MWCO: 10 kDa and 30 kDa) were tested. The objective was to separate free RNA from the lipoplexes, with the expectation that liposomes would be retained while unbound RNA would pass through the filter. ICP-MS was used to monitor both components: phosphorus (P) for liposomes and sulfur (S) for RNA. The results revealed that although free RNA was successfully eluted, a notable loss of material occurred in both membrane types, with 20–30% of total content lost. This loss included both liposomal and RNA signals, suggesting partial retention or non-specific binding to the filter membranes.

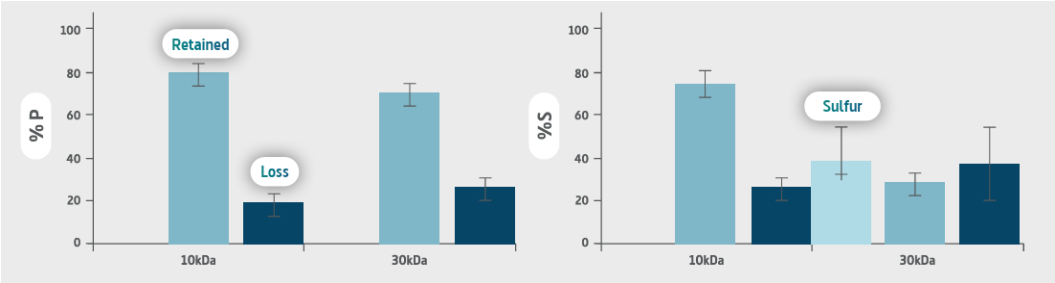

Figure 6. ICP-MS characterization of fractions obtained after centrifugal ultrafiltration using membranes with 10 kDa and 30 kDa cutoffs. Phosphorus (P) tracks liposomes, and sulfur (S) tracks RNA. Light blue: retained fraction; dark blue: non-specific binding; lighter blue: S signal of eluted RNA of non-specific binding.

3.4. In Vitro Skin Absorption and Efficacy Assays

To assess the ability of the lipoplexes to deliver RNA across the skin barrier and induce a biological effect, in vitro skin penetration studies were conducted using human reconstructed skin models. As shown in **Figure 7a**, the quantification of siRNA in the culture medium 2 days after topical application revealed marked differences among the tested prototypes. Lipoplex B exhibited the highest release of RNA into the supernatant, indicating lower retention within the tissue. In contrast, as observed in **Figure 7b**, analysis of the skin samples at day 5 showed

that lipoplex A led to greater RNA retention in the skin, consistent with its lower extracellular release (**Figure 7a**). These complementary results suggest that lipoplex B exhibits more efficient delivery and retention within the skin layers.

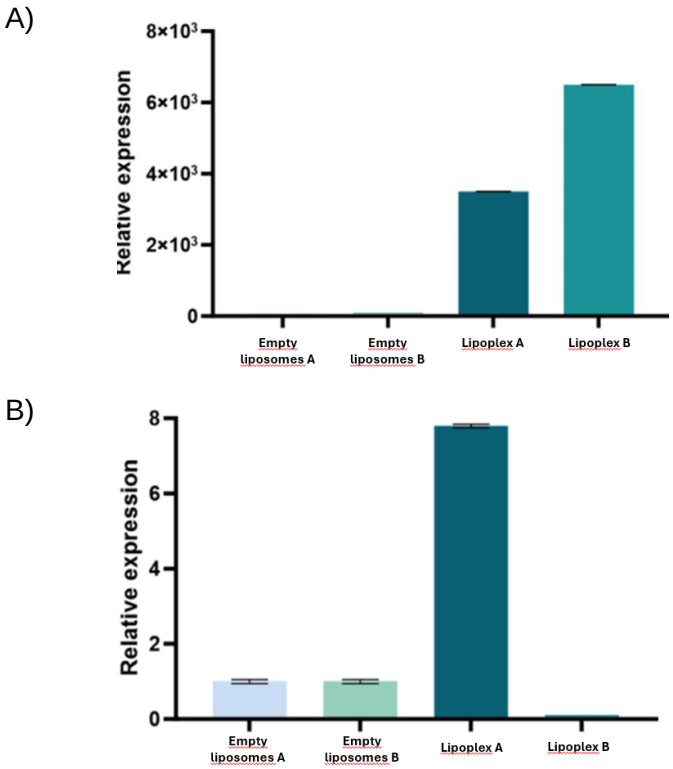

Figure 7. Evaluation of RNA presence following skin exposure: (a) RNA concentration in supernatant after 2 days; (b) RNA content retained in skin tissue after 5 days for each prototype.

In line with the objective of developing a delivery system capable of achieving localized RNA activity in the skin, the next step was to assess the biological activity of the delivered RNA. Specifically, the expression of tyrosinase mRNA – a key enzyme in the melanin synthesis pathway - was quantified by quantitative real-time PCR (qPCR) using the comparative ΔΔCt method. The analysis was performed on skin samples collected at days 5 and 9 post-induction of hyperpigmentation. A conventional depigmenting cosmetic active was used as a reference control.

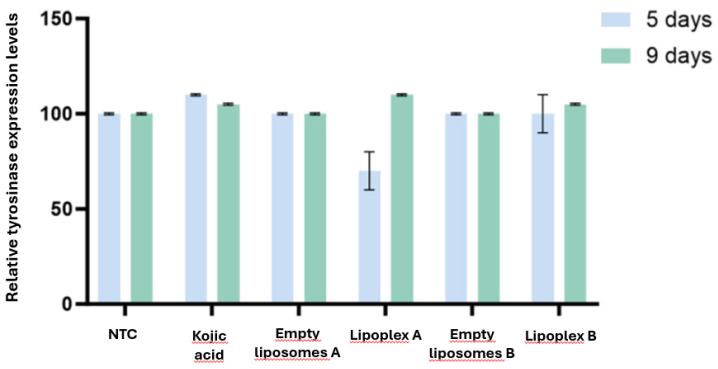

Figure 8. Relative tyrosinase mRNA expression levels in skin treated with different lipoplex formulations compared to corresponding empty controls.

The results indicate that lipoplex A was the only formulation capable of significantly reducing tyrosinase mRNA levels, suggesting effective cellular uptake and functional activity of the encapsulated RNA. This finding supports its potential as a candidate for targeted depigmenting treatment through RNA-based therapeutics.

4. Discussion

The results obtained in this study demonstrate the successful development of a liposomal delivery system for RNA using the Thin Film Hydration (TFH) method. The synthesized nanoparticles were thoroughly characterized by dynamic light scattering (DLS), showing particle sizes below 150 nm and a polydispersity index (PDI) lower than 0.2, indicative of a homogeneous and stable population.

The formulations also exhibited high physicochemical stability, particularly in terms of particle size. As expected, surface charge increased progressively with higher liposome:RNA ratios, reflecting effective surface saturation by the negatively charged RNA. These findings are consistent with previous studies, such as that by Dorrani et al., which reported similar trends in zeta potential as a function of the loading ratio [6].

Regarding complexation efficiency, approximately 60% of the RNA was successfully associated with the liposomes. This was validated through complementary analytical techniques including gel electrophoresis and inductively coupled plasma mass spectrometry (ICP-MS). Particularly, the AF4-MALS-ICP-MS system proved to be a powerful tool to distinguish and quantify the RNA incorporated into the lipoplexes, in line with prior reports [8] employing other nanoparticle system.

It has been demonstrated that purification using centrifugal ultrafiltration presented limitations. Both RNA and liposomes were partially retained in the membrane, likely contributing to the 20–30% loss observed in the process (Figure 7). These findings suggest the need to optimize the purification strategy, either by modifying membrane cutoff properties, exploring alternative materials, or considering entirely different purification approaches to minimize undesired retention and improve yield.

The in vitro skin assays confirmed the ability of the lipoplexes to cross the epidermal barrier. Among the prototypes, lipoplex A demonstrated superior retention within skin layers and was also the only formulation capable of reducing tyrosinase mRNA expression, suggesting a potential biological effect consistent with the intended depigmenting application.

Future work will focus on expanding the characterization toolbox by incorporating additional RNA quantification methods, such as fluorescence-based assays (e.g., RiboGreen), and further investigating the mechanisms of cellular uptake. Subsequent studies will aim to correlate mRNA modulation with corresponding protein-level changes and determine the precise location of RNA accumulation within the skin.

5. Conclusions

This study reports the successful development of two distinct liposomal RNA delivery systems with precisely controlled physicochemical properties—namely particle sizes ranging from 80 to 150 nm and low PDI values. These formulations constitute a versatile and scalable platform for the encapsulation and delivery of RNA molecules via electrostatic interactions.

A comprehensive characterization using advanced analytical tools (DLS, gel electrophoresis, AF4-MALS-ICP-MS) confirmed the integrity and consistency of the delivery systems. Importantly, these platforms have demonstrated their capability to protect RNA, facilitate penetration into reconstructed human skin, and exert biological activity, as evidenced by the down-regulation of tyrosinase mRNA in an ex vivo hyperpigmentation model.

These findings represent a significant step forward in the design of RNA delivery vehicles for dermatological and cosmetic applications, providing a promising approach for topical RNA-based therapies targeting specific pathways such as pigmentation.

6. References

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