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Master's Degree Industrial Nanobiotechnologies for
Pharmaceuticals

**pH-Responsive Isoprenoid-AMP Conjugates with Anti-TB
Activity Forming Polymeric Nanoparticles Via
Nanoprecipitation**

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1. INTRODUCTION

1.1 Tuberculosis

Tuberculosis (TB) remains a critical global health and safety threat in the 21st century. According to World Health Organization (WHO) Global TB Report 2025, an estimated 10.7 million people fell ill with the disease, with a global incidence rate of 131 cases per 100,000 population and 1.23 million recorded deaths. Although the absolute number of people falling ill has decreased slightly over the years, progress still falls far short of the “End TB Strategy” targets. TB is one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent¹. The disease is caused by the bacillus *Mycobacterium tuberculosis* (Mtb) and is spread via inhalation of aerosolized particles from infected individuals (e.g., by coughing). About a quarter of the global population is estimated to have been infected with Mtb with the ability to transition into a dormant state (latent TB) where infected individuals display no obvious symptoms until later in life^{1,2}. Approximately 10% of latent infections progress to active TB².

Mtb is an intracellular pathogen. Once inhaled, it travels to the lower respiratory tract. In the pulmonary alveoli, it interacts with alveolar macrophages and gets phagocytosed. Within alveolar macrophages, Mtb evades the immune response by preventing acidification of the bacterial vacuole, preventing maturation of their intracellular compartment to a phagolysosome, and by inhibiting the apoptosis and autophagy of infected macrophages. Lungs affected by the disease form granulomas, a hallmark of pulmonary TB. The granuloma contains a cheese-like center (known as caseum), derived from necrotic macrophages and is a key barrier to drug delivery. The caseous core is avascular, hypoxic and lipid-rich³.

The current standard of care for TB is a 6-month regimen for chemotherapeutic agents known as 2HRZE/4HR. This involves two months of isoniazid (H), rifampicin (R), pyrazinamide (Z), and ethambutol (E), followed by four months of isoniazid and rifampicin. Recent guidelines also allow for a shorter 4-month regimen (2HPMZ/2HPM) using rifapentine and moxifloxacin for individuals aged 12 and older, and for children with non-severe disease⁴. Though largely effective, these chemotherapeutic regimens have several constraints like significant side effects, toxicity, poor compliance to long treatment duration and emergence of resistance^{2,3}. There is a need for exploring therapeutic alternatives in order to combat these challenges.

1.2 Antimicrobial Peptides

Antimicrobial peptides (AMPs) constitute a highly conserved class of small, naturally occurring peptides that function as essential components of innate immunity across plants, insects, amphibians, mammals, and microorganisms. They exhibit broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi, parasites, and viruses. Most AMPs are short—typically 10–60 amino acids—and strongly cationic, due to lysine and arginine enrichment. This positive charge enables selective electrostatic interactions with negatively charged microbial membranes. Although less common, anionic AMPs such as dermcidin and kappacin also exist; these peptides often rely on metal cofactors like zinc to coordinate with microbial surfaces. A defining feature of AMPs is their amphipathic architecture, in which hydrophobic and hydrophilic domains are spatially segregated. This configuration ensures solubility in aqueous environments while enabling insertion into lipid bilayers, ultimately facilitating membrane permeabilization⁵.

1.2.1 Structure of AMPs

AMPs exhibit structural diversity, adopting a range of three-dimensional conformations that fall into four classical families: *linear α -helical peptides*, which remain largely unstructured in aqueous environments but fold into amphipathic helices upon encountering lipid membranes; *β -sheet peptides*, whose rigid architectures are stabilized by intramolecular disulfide bonds; *extended or linear peptides*, which lack conventional secondary structure and are enriched in residues such as tryptophan and proline; and *mixed α/β peptides*, which incorporate both helical and sheet elements within a single chain.

Beyond these categories, cyclic peptides, lasso peptides, and thioether-bridged structures have also gained attention for their enhanced proteolytic stability and unique topologies⁵.

1.2.2 Mechanisms of Action

AMPs exert antimicrobial effects through dual mechanisms: membrane-targeting disruption and intracellular interference.

Membrane-Targeting Mechanisms

Cationic AMPs initiate membrane disruption by binding electrostatically to anionic components of microbial envelopes, including lipopolysaccharides in Gram-negative bacteria, teichoic acids in Gram-positive bacteria, and negatively charged phospholipids. As they

accumulate on the membrane surface and reach a critical local concentration, these peptides undergo conformational rearrangements that enable insertion into the lipid bilayer, ultimately compromising membrane integrity through one of two well-established biophysical mechanisms. In the *toroidal pore model*, peptide insertion induces pronounced curvature of lipid headgroups, generating transient lipid-lined pores that allow uncontrolled ion flux. In the *carpet model*, peptides remain aligned parallel to the membrane, progressively coating its surface until a threshold concentration triggers detergent-like disintegration and micellization of the bilayer. Together, these mechanisms illustrate how AMPs exploit electrostatic attraction, amphipathic structure, and cooperative membrane interactions to rapidly destabilize microbial membranes⁶.

Intracellular Mechanisms

Some AMPs can traverse microbial membranes without causing immediate lysis, enabling them to access intracellular targets and disrupt essential biological processes that are critical for cell survival. Once inside the cytoplasm, these peptides interfere with protein biosynthesis by binding to ribosomal subunits or translation complexes, thereby halting elongation or initiation. Others act on molecular chaperones such as DnaK, preventing proper protein folding and promoting widespread aggregation of misfolded proteins. Several AMPs also compromise nucleic acid integrity by cross-linking DNA, degrading RNA, or inhibiting enzymes involved in nucleic acid synthesis and topology. In addition, certain peptides inhibit key proteases required for microbial physiology or host–pathogen interactions, further destabilizing cellular homeostasis. Finally, some AMPs disrupt cell division machinery—such as by binding to FtsZ or interfering with septation—ultimately arresting proliferation and leading to cell death⁵.

1.2.3 Therapeutic Limitations and Pharmacokinetic Barriers

Despite their strong antimicrobial potency, the clinical translation of AMPs is constrained by several pharmacological and safety limitations. Most AMPs composed of L-amino acids are highly vulnerable to rapid proteolytic degradation by host and bacterial enzymes, resulting in extremely short serum half-lives and poor systemic stability. Their activity is further compromised by physiological conditions *in vivo*, where monovalent and divalent cations such as Na⁺, Mg²⁺, and Ca²⁺ can neutralize peptide charge and diminish membrane binding, while high salt concentrations, iron-induced structural disruption—as observed for histatin

5—and fluctuations in pH collectively reduce antimicrobial efficacy. In addition, many AMPs exhibit dose-dependent cytotoxicity due to excessive hydrophobicity or strong cationicity, which can lead to unintended interactions with mammalian cell membranes, causing hemolysis, epithelial damage, and other off-target effects. Together, these factors highlight the need for structural optimization and delivery strategies to improve AMP stability, selectivity, and therapeutic viability^{5,7}.

1.3 Nanoparticles for drug delivery

Nanoparticles (NPs) have become central to modern drug delivery because their extremely small size and high surface-area-to-volume ratio give them physicochemical and biological behaviors that differ significantly from bulk materials. In nanomedicine, they are generally defined as structures smaller than 100 nm in at least one dimension, a scale that allows them to interact with biological systems in ways that conventional therapeutics cannot. Their ability to carry drugs, protect them from degradation, and release them in a controlled manner has made NPs an essential platform for improving therapeutic precision and reducing systemic side effects.

Therapeutic NPs can be broadly grouped into nano-structured and nanocrystalline systems, each offering distinct advantages depending on the drug and the intended application. Among nano-structured materials, polymer-based NPs form one of the largest and most versatile classes. These systems use natural or synthetic polymers to create biocompatible and biodegradable carriers capable of transporting a wide range of therapeutic molecules. Dendrimers, for example, are highly branched polymers with internal cavities that can trap drugs and surface groups that can be chemically modified. Polymeric NPs exist either as nanocapsules, where the drug is enclosed within a polymer shell, or as nanospheres, where the drug is dispersed throughout the polymer matrix. Polymeric micelles self-assemble into small spherical structures with a hydrophobic core that solubilizes poorly water-soluble drugs and a hydrophilic shell that improves circulation time. Other polymeric systems include polymer–drug conjugates, which alter drug pharmacokinetics through covalent attachment, and protein-based NPs such as virus-like particles and self-assembled protein cages.

The advantages of NPs help overcome several physiological barriers that limit the performance of many biological/biotechnological drugs. Encapsulating sensitive biomolecules within polymeric carriers protects them from rapid enzymatic degradation and prolongs their

circulation time. Their design can also incorporate pH-responsive components that stay stable at physiological pH but release their cargo in acidic environments such as endosomes or diseased tissues, ensuring that the drug is delivered precisely where it is most effective. By concentrating therapeutics at target sites rather than distributing them broadly, NPs reduce systemic toxicity and improve the therapeutic index, allowing lower doses to achieve meaningful clinical outcomes. This principle is well demonstrated by liposomal formulations of toxic antimicrobial agents, such as liposomal amphotericin B, which significantly decrease organ toxicity while preserving strong therapeutic activity⁸.

1.4 Polymer Drug Conjugates

Polymer Drug Conjugates (PDCs) are advanced nano-medicine constructs where one or more therapeutic agents—such as small molecules, peptides, or proteins—are covalently bound to a polymeric carrier backbone, typically via a specialized linker. They offer distinct advantages, including the ability to tailor the physicochemical properties of drugs, improve pharmacokinetic and bioavailability, and enable controlled drug release mechanisms for targeted delivery.

In a previous study⁹ of the research group, the same platform was used by designing a PDC entirely of natural and bioactive components. It utilized an FDA-approved, water-soluble, and biodegradable polymer “ ϵ -Poly-L-Lysine” (PL) as the hydrophilic polymer backbone to conjugate hydrophobic isoprenoid drug “Farnesal”. The resulting system (Far-PL) was made by pH-responsive imine bonds (Schiff bases), without the need for additional chemical linkers. These imine linkages are extremely sensitive to acidic environments, remaining stable at a physiological pH of 7.4 but undergoing rapid hydrolysis at pH levels below 6.5. This property was specifically engineered to trigger drug release within the acidic tumor microenvironment and intracellular compartments (like endosomes)⁹. The PDC platform presents a promising approach for tackling limitations in AMP drug delivery.

1.5 Farnesal

Farnesal (C₁₅H₂₄O) (Far) is an acyclic sesquiterpenoid aldehyde (isoprenoid) produced through the oxidation of farnesol by NAD(P)⁺-dependent farnesol dehydrogenases. It naturally originates from diverse biological systems; as a critical intermediate in the biosynthesis of insect juvenile hormone III (JH III), as a plant secondary metabolite in essential oils, and forms during downstream isoprenoid recycling in microbial and mammalian systems^{10,11}.

As an amphiphilic sesquiterpenoid, Far exhibits potential antimicrobial activity. It intercalates into microbial lipid bilayers, leading to disruption of cell membrane integrity, and causing intracellular leakage. Furthermore, its lipophilic nature and aldehyde functionality allow it to impair bacterial and fungal growth, inhibit biofilm formation, and induce intracellular reactive oxygen species (ROS) production. These properties make it a sought-after candidate for antimicrobial therapy¹².

2. AIM

This thesis is a proof-of-concept study in the larger context of developing polymeric NPs for delivery of antimicrobial peptides with stimuli responsive release in alveolar macrophages for the treatment of Pulmonary TB. Such a system could address several constraints to current chemotherapeutic treatments like toxicity, long duration of treatment and consequently patient compliance. The objectives of my work were: *i)* To conjugate Far with two potential antituberculosis peptides (ATBP1 and ATBP2), via imine bond, to form conjugates (Far-ATBP1 and Far-ATBP2) that have improved safety and anti-TB activity, *ii)* To prepare and characterize polymeric NPs containing these conjugates that show pH-responsive dissociation.

3. MATERIALS AND METHODS

3.1 Materials

ε-Poly-L-Lysine HCl (Average MW 3500–4500 Da) was obtained from Biosynth Carbosynth (Bratislava, Slovakia), and Farnesal (mixture of isomers) was obtained from Sigma–Aldrich (Steinheim, Germany). Ammonium Chloride was obtained from BASF SE (Ludwigshafen, Germany). Mowiol 4–88 [Poly(vinyl-alcohol) MW $\approx$ 31000 Da], Triethylamine (TEA, $\geq$ 99 %) and Deuterated Acetic acid (Acetic acid-d₄, 99.50 %) were obtained from Sigma–Aldrich (Steinheim, Germany). Deuterated Methanol (Methanol-d₄, 99.80 %) was obtained from Deutero GMBH (Kastellaun, Germany), and Tetramethyl silane – TMS (for NMR) was obtained from TCI Deutschland GmbH (Eschborn, Germany). Methanol – HPLC grade (Ph. Eur., 99.90 %) was obtained from VWR International (Rosny-sous-Bois Cedex, France). Acetone (97 %) and Ethyl Acetate (97 %) were sourced in-house from Saarland University (Saarbrücken, Germany). Amicon Ultra-4 centrifugal filters (100 kDa MWCO) were purchased from Sigma–Aldrich (Steinheim, Germany). Spectra/Por 7 Dialysis Membrane (Pre-treated RC Tubing, MWCO: 1 kDa) was obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany).

Research Center Borstel (Leibniz Lung Center, Germany) has synthesized and is investigating two antituberculosis peptides, with molecular weights 2464 Da (ATBP1) and 2405 Da (ATBP2). They act on Mtb cell wall. The compounds are linear 20-mer peptides. ATBP1 contains five lysine (Lys) units, a tryptophan (Trp) unit and a free N-terminal (present as hydrochloride). ATBP2 contains 4 Lys units, a tryptophan (Trp) unit and a free N-terminal (also present as hydrochloride). The complete structure and mechanism of action of both AMPs are kept discreet, as studies are on-going and yet to be published.

For cell viability, A549 cells (human adenocarcinoma alveolar epithelial cells) were obtained from DSMZ (Braunschweig, Germany, No: ACC 107). RPMI-1640 medium, trypsin/EDTA, Dulbecco's phosphate buffered saline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Triton X were purchased from Sigma–Aldrich (Steinheim, Germany).

3.2 Synthesis of Farnesal-Poly-Lysine 30 (Far-PL30)

In Far-PL30, the amount of Far to NH_2 in the repeating units of PL is commensurate to NH_2 :Far molar ratio of 1:0.3. The synthesis method has been adopted from the previous study⁹. In 1 mL methanol, 20 mg of PL (5.3×10^{-3} mmol $\times$ 30 NH_2 in each monomer = 15.8×10^{-2} mmol NH_2) is dissolved and then 11.5 μL Far (4.7×10^{-2} mmol) is added. The solution is left stirring for 24 h at room temperature.

3.3 Preparation of Far-PL30 NPs

Far-PL30 NPs were prepared by three different preparation methods, adopted from the previous study⁹.

Self-assembly (SA): Thin-film hydration method is employed. 300 μL of Far-PL30 (26.6 mg/mL) in methanol is transferred into a round bottom flask. Using rotavapor (R-100, Buchi, Essen, Germany), methanol is removed and a thin-film is obtained. Then 1 mL of Milli-Q water is added and the flask is gently shaken till all film appears to have been homogenized (NPs formed). The contents of the flask are transferred to a pre-labelled eppendorf.

Nanoprecipitation (NA): 100 μL Far-PL30 (26.6 mg/mL) in methanol is diluted with 900 μL of acetone (2.66 mg/mL), this makes the organic phase (1 mL). The organic phase is then added dropwise to 1 mL of 2 % polyvinyl alcohol (PVA) aqueous solution. The final solution (2 mL) contains 1.33 mg/mL Far-PL30 and 1 % PVA. This is left under mild stirring overnight at room

temperature to allow evaporation of the organic solvent (NPs formed). The remaining contents are transferred to a pre-labelled eppendorf.

Nanoemulsion (EM): 160 μ L Far-PL30 (26.6 mg/mL) in methanol is diluted with 640 μ L of ethyl acetate (5.32 mg/mL), this makes the organic phase (0.8 mL). In a glass vial, aqueous phase (1.6 mL) is prepared by mixing 0.8 mL 2 % PVA aqueous solution and 0.8 mL 0.001 % Triethylamine (TEA) aqueous solution. Organic phase is added into aqueous phase under stirring, making the final solution with 1.77 mg/mL Far-PL30, 0.66 % PVA and 3.33×10^{-4} % TEA. This is left under mild stirring for 10 min before forming EM using an ultrasonic homogenizer (Bandelin Sonopuls HD 3100, Bandelin, Berlin, Germany) for 30 s at an amplitude of 37 %. Finally, 2 mL of Milli-Q water is added, and the solution is again left under mild stirring overnight at room temperature to remove organic solvents (NPs formed). The remaining contents are transferred to a pre-labelled eppendorf.

To remove unutilized materials (e.g. PVA, Far or PL), the resulting NPs prepared with SA, NA, and EM methods (Far-PL30-SA, Far-PL30-NA, and Far-PL30-EM, respectively) are purified using Amicon Ultra-4 centrifugal filter (100 kDa MWCO) at x5000 g, for three cycles of 10 min each, at 20 °C in 0.001 % TEA (to preserve imine bond). Finally, the NP suspensions are characterized and freeze-dried (at -110 °C and 0.200 mBar) for further analysis.

3.4 Synthesis of Farnesal-AMP Conjugates

Farnesal-ATBP1 (Far-ATBP1): The synthesis scheme is similar to the previous study⁹. Three different reaction mixtures were prepared (Far-ATBP1-40, Far-ATBP1-66, and Far-ATBP1-200) by changing the molar ratio between the available NH_2 groups of ATBP1 and Far (6:2.4, 6:4, and 6:12, respectively) in the reaction. In Far-ATBP1-40, 5 mg of ATBP1 (20.3×10^{-4} mmol x 6 primary NH_2 in each peptide = 121.8×10^{-4} mmol NH_2) is dissolved in 1 mL methanol- d_4 and then 1.24 μ L Far (50.7×10^{-4} mmol) is added. The solution is to be left stirring for 24 h at room temperature. In Far-ATBP1-66, 2.8 mg of ATBP1 (68.4×10^{-4} mmol NH_2) and 1.12 μ L Far (45.7×10^{-4} mmol) are added. The reaction mixture of Far-ATBP1-200 contains 2.8 mg of ATBP1 (68.4×10^{-4} mmol NH_2) in 1 mL methanol- d_4 along with 0.78 μ L acetic acid- d_4 (136.8×10^{-4} mmol), which is then mixed with 3.33 μ L Far (136.8×10^{-4} mmol) and the solution is left stirring for 24 h at 37 °C.

Farnesal-ATBP2 (Far-ATBP2): The NH_2 :Far molar ratio in reaction mixture was 5:10 (Far-ATBP2-200). In 0.5 mL methanol- d_4 , 1 mg of ATBP2 (4.16×10^{-4} mmol x 5 primary NH_2 in each

peptide = 20.8×10^{-4} mmol NH_2) was dissolved and then 0.24 μL acetic acid- d_4 (42.1×10^{-4} mmol) was added. Finally, 1.03 μL Far (42.3×10^{-4} mmol) was added. The solution was left stirring for 24 h at 37 °C.

3.5 Washing of conjugates by Dialysis

Conjugate in methanol solution is transferred into a pre-treated RC tubing (MWCO: 1 kDa) and washed for 4 h with 100 % methanol as dialysate under constant stirring. Afterwards the dialysate is replaced with 50 % methanol, and the system is left overnight under constant stirring. The dialysate is replaced with Milli-Q Water for 2 h under constant stirring and this step is repeated once more. The resulting residue in the dialysis membrane bag is collected, characterized, and then freeze-dried (at -110 °C and 0.200 mBar) for further analysis. To increase the amount of conjugate in residue post-dialysis, ionic strength of the dialysate is increased by using 20 mM NH_4Cl ¹³. NH_4Cl is a volatile salt and is categorized as GRAS material by FDA (not cytotoxic).

3.6 Proton – NMR Spectroscopy (^1H -NMR)

Conjugate solution in methanol- d_4 is measured with Bruker Ultrashield-500 NMR Magnet 500 MHz (Bruker Corporation, Billerica, USA) using Tetramethyl silane (TMS) as an internal standard.

3.7 Infrared Spectroscopy (IR)

Sample powder is placed on the clean and dry crystal surface of PerkinElmer Spectrum-400 FT-IR. Transmittance is recorded over the wavenumber range of 4000 cm^{-1} to 650 cm^{-1} .

3.8 Preparation of Far-AMP Nanoprecipitated (NA) NPs

Far-ATBP1-NA: A freshly synthesized Far-ATBP1 batch (in methanol) is diluted with acetone to reach a concentration of 1 mg/mL and a volume of 1 mL. This organic phase is added dropwise to 1 mL of 2 % PVA aqueous solution (aqueous phase). The final solution (2 mL) contains 0.5 mg/mL Far-PL30 and 1% PVA. This is left under mild stirring overnight at 500 rpm, at room temperature, to allow the evaporation of the organic solvents.

Far-ATBP2-NA: The preparation protocol is similar Far-ATBP1-NA, however a Design of Experiment (DoE) approach to the NP formulation is applied. The software “*DoE: Web application for Design of Experiments (Camillo Melzi – GitHub)*” is used to perform a design of experiment study. A fractional factorial design is applied to study the influence of three

factors at 2 levels (2^{3-1}) i.e. concentration PVA aqueous solution (1 % and 2 %), volume fraction of organic solution, i.e. FOS, to the total volume (0.33 and 0.50) and mixing speed (400 rpm and 600 rpm)¹⁴. The experimental runs (R) are performed in a random order to minimize any drift effect. Two final validation runs (V) are performed at central values (1.5 % PVA aqueous solution, 0.415 FOS and 500 rpm mixing speed). To prepare the organic phase, a freshly synthesized Far-ATBP2 in methanol batch is diluted with acetone to reach a concentration of 1 mg/mL. The organic phase is added dropwise to PVA aqueous solution. The final solution is left under mild stirring overnight, at room temperature, to allow the evaporation of the organic solvents.

To remove unutilized/excess PVA from NP suspension, purification is done using Amicon Ultra-4 centrifugal filter (100 kDa MWCO) at x5000 g, for three cycles of 10 min each, at 20 °C and in 0.001 % TEA (to preserve imine bond).

3.9 Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS)

Using Zetasizer Ultra (Malvern Panalytical, Malvern, UK), z-average hydrodynamic diameter (D_H) and polydispersity index (PDI) of NPs are obtained by DLS measurements, and for zeta potential of NPs ELS is measured. For simultaneous DLS and ELS measurements, samples are diluted with Milli-Q water by 1:7 ratio, placed in a folded cuvette and analyzed in three technical replicates. A disposable micro-cuvette is used instead of the folded cuvette when DLS measurements alone are needed.

3.10 Scanning Electron Microscopy (SEM)

The morphology of NPs is visualized using SEM (Zeiss EVO HD15 - Carl Zeiss) at 5 kV and 5,000x to 20,000x magnification under high vacuum. NP suspension is placed on a silica wafer, air-dried, and sputter-coated with a 10 nm gold layer, using Quorum Q150R ES sputter coater (Quorum Technologies Ltd) before visualization. The size of NPs is calculated by ImageJ software.

3.11 pH-responsive NP Dissociation

For investigation of NPs dissociation under different environmental pH conditions, the NP suspensions are incubated for 24 h at 37 °C in 8 mM buffer solutions of pH 7.4 and pH 4.5. Using Zetasizer Ultra (Malvern Panalytical, Malvern, UK), D_H , PDI and scattering intensity (kcps) is obtained, by taking DLS measurements in disposable micro-cuvettes at 0 h and 24 h.

3.12 Freeze-Drying NP Stability

NP suspensions are subjected to overnight freeze-drying at -110 °C and 0.200 mBar. The dried powder is resuspended in Milli-Q water and then analyzed using Zetasizer Ultra (Malvern Panalytical, Malvern, UK) to take DLS measurements for obtaining D_H and PDI.

3.13 Cell Viability & Biocompatibility

The assay was performed by a trained person in the lab, i.e., Camilla Passi. For the assay, cells are seeded (2×10^4 cells/well) in a 96-well plate and grown for 48 h. The samples are diluted from their respective stock solutions with the culture medium to reach the final concentrations (0, 7.8, 15.6, 31.3, 62.5, and 125 $\mu\text{g/mL}$) and incubated for 24 h. For the experiment, the medium is supplemented with 0.1% DMSO to allow Far dilution without altering cell viability. As a positive control, 2 % Triton-X was applied. After 24 h , cells are washed with PBS and incubated with 10 % MTT reagent. After 4 h incubation with MTT reagent, the medium is aspirated, and DMSO is added, followed by incubation for 20 min. The absorbance of formed formazan is measured by Tecan plate reader (Infinite M200, Tecan, Männedorf, Switzerland) at 550 nm.

4. RESULTS AND DISCUSSION

4.1 Synthesis of Far-PL30

The chemical principle behind Far-PL30 synthesis is the Schiff base reaction, which is a condensation reaction between an aldehyde or ketone and a primary amine. The hydrophobicity of the aldehydic Far decreases by conjugation with hydrophilic PL polymer and the resultant Far-PL (*Figure 1*) has an amphiphilic nature, which helps in NPs formation. The reaction is reversible and the resultant conjugate degrades under acidic pH. Far-PL30 formation has been well characterized in the previous study⁹ and is also visible by change in color of the reaction mix (from colorless to yellow).

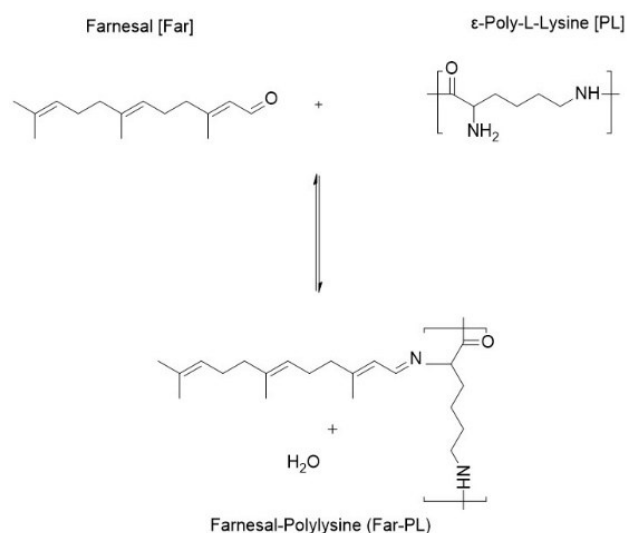


Figure 1. Synthesis scheme of Far-PL.

4.2 Preparation of Far-PL30 NPs

As shown in *Figure 2A*, NPs of varied sizes were obtained based on the preparation method. Nanoprecipitation yielded NPs (Far-PL30-NA) with smallest size, i.e., $D_H \approx 118.5$ nm, with monodispersed size distribution ($PDI \approx 0.08$). On other hand, Nanoemulsification yielded NPs (Far-PL30-EM) with largest size, i.e., $D_H \approx 197.9$ nm, also with monodispersed size distribution ($PDI \approx 0.10$). NPs formed by self-assembly (Far-PL30-SA) were monodispersed ($PDI \approx 0.08$) and had moderate sizes ($D_H \approx 163.6$ nm). The colloidal NP suspensions were also characterized in terms of surface charges by measuring their zeta potential (*Figure 2B*) and all preparations methods yield positive zeta potential, which can be attributed to excess of unreacted NH_2 groups in PL. Far-PL30-SA had the highest zeta potential ($\approx +55.5$ mV), followed by Far-PL30-NA ($\approx +49.2$ mV) and then Far-PL30-EM ($\approx +42.9$ mV).

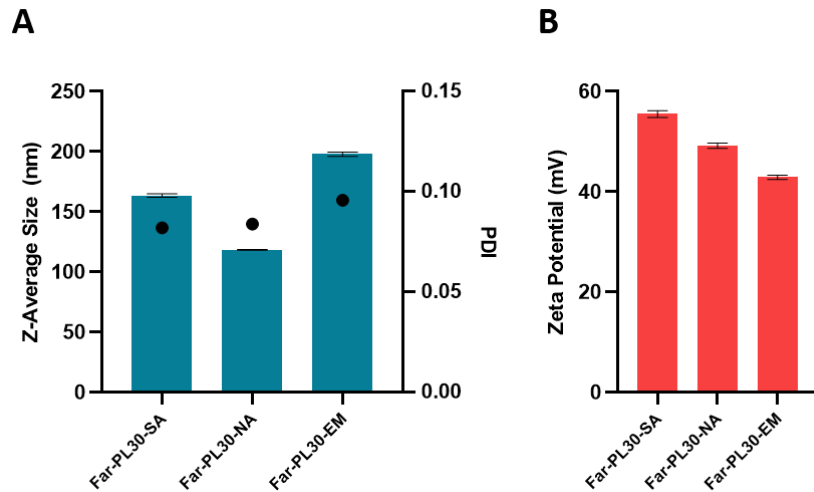


Figure 2. A) D_H (nm) and PDI of Far-PL30 NPs B) Zeta potential (mV) of Far-PL30 NPs, obtained from different preparation methods [$n=3$ and $N=1$].

After freeze-drying NPs prepared by all three methods at $-110\text{ }^{\circ}\text{C}$ and 0.200 mBar, the D_H was evaluated (Figure 3). NPs prepared by SA showed decrease in size (82.1 nm) and increase in PDI (0.48) after freeze-drying, indicating they were unstable and formed aggregates, which can be attributed to lack of stabilizer (PVA) in the formulation. NPs prepared by NA and EM (containing PVA as a stabilizer in formulation) remained stable with similar D_H before and after freeze-drying with PDIs less than 0.20 (monodispersed). While both NA and EM provide stable and monodispersed NPs, NE is a high-energy method that could be detrimental to AMPs, which is not the case with NA.

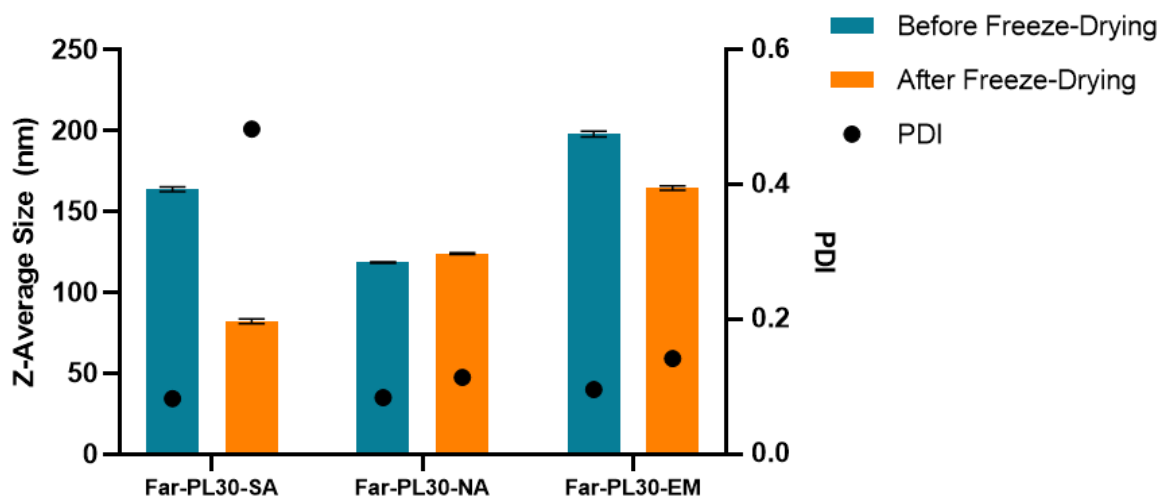


Figure 3. D_H (nm) and PDI of Far-PL30 NPs before and after freeze-drying [$n=3$ and $N=1$].

These findings suggest that NA may serve as a preferred method for formulating AMP-based NPs, as it is a low-energy process that minimizes thermal degradation of AMPs while enabling the formation of stable NP structures.

4.3 Synthesis of Farnesal-AMP Conjugates

4.3.1 Far-ATBP1

The chemical principle behind Far-ATBP1's synthesis is the Schiff base reaction (like in Far-PL synthesis). The reaction is a nucleophilic addition between the aldehyde group of Far and the primary amino groups of ATBP1 (*Figure 4*).

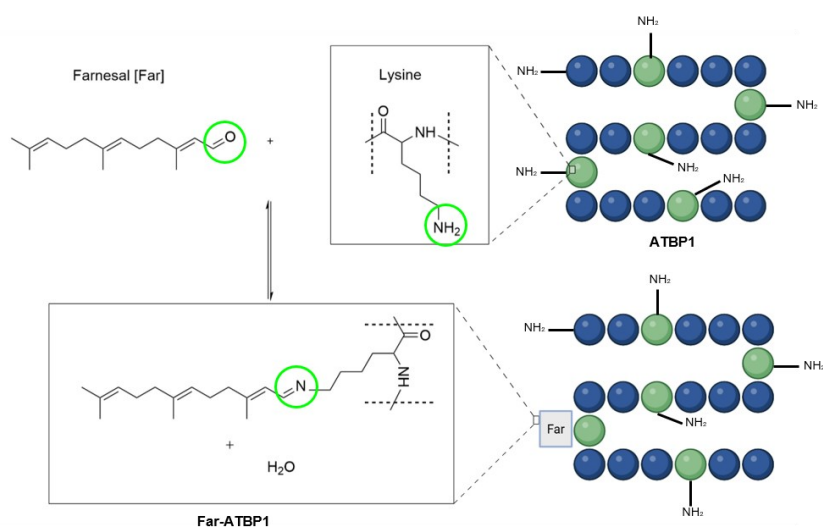


Figure 4. Synthesis scheme of Far-ATBP1.

To investigate successful conjugation, three different reaction mixtures were evaluated (Far-ATBP1-40, Far-ATBP1-66, and Far-ATBP1-200) by changing the molar ratio between the available NH_2 groups of ATBP1 and Far (6:2.4, 6:4, and 6:12, respectively) in the reaction. The synthesis was monitored by performing ^1H -NMR analysis at 0 h and 24 h (*Figure 5*) and by observing visible color change (colorless to yellow). The aldehyde proton peak ($\delta \approx 10$ ppm) from Far in the reaction mixture should decrease in the event of a successful conjugation reaction, along with color change. Far-ATBP1-40 and Far-ATBP1-66 showed decrease in aldehyde proton peak but were not accompanied by color change. This suggests that the change in aldehyde peak might not be because of conjugation, but rather due to hydrolysis, evaporation, or other means of material loss.

Far-ATBP1-200 reaction mixture showed decrease in aldehyde proton peak over time accompanied by color change (colorless to yellow) indicating a successful conjugation. This

was aided by using acetic acid as catalyst for the reaction¹⁵, adding Far in excess to drive the forward reaction (favoring conjugation) and applying higher temperature to improve molecular motion and reaction probability of ATBP1¹⁶.

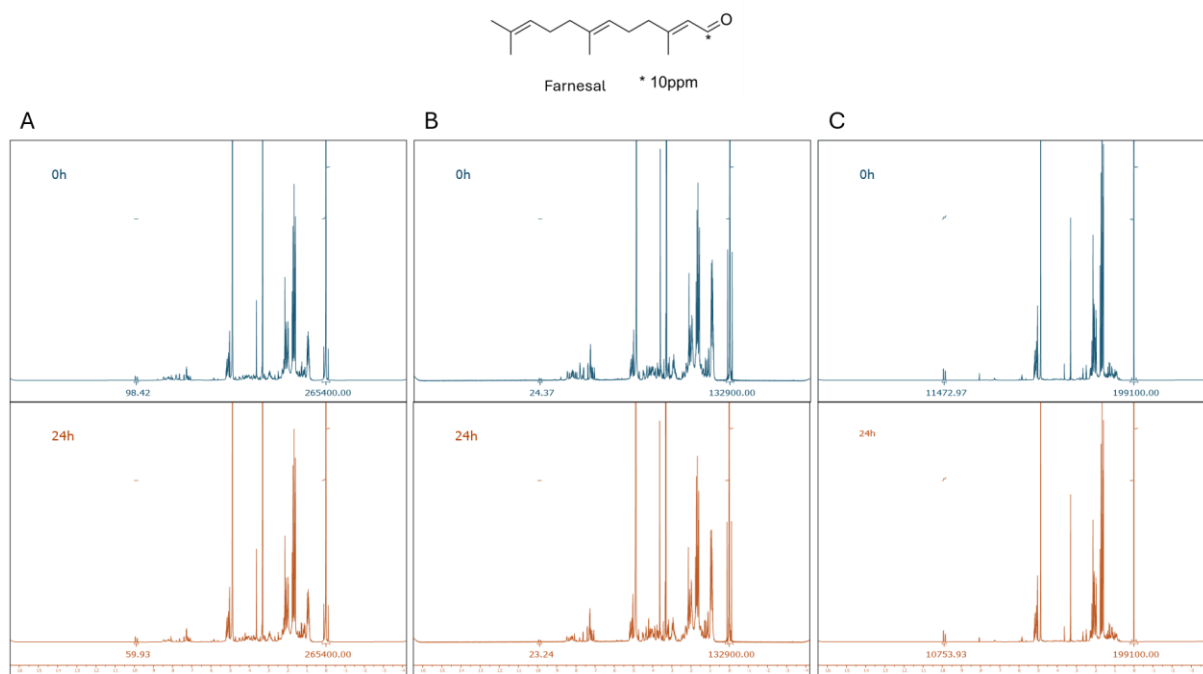


Figure 5. ¹H-NMRs with TMS as internal standard ($\times 10^{15}$ H) of A) Far-ATBP1-40 at 0 h and 24 h B) Far-ATBP1-66 at 0 h and 24 h C) Far-ATBP1-200 at 0 h and 24 h.

4.3.2 Far-ATBP2

Like Far-ATBP1 and Far-PL, the chemical principle behind Far-ATBP2 synthesis is also the Schiff base reaction, being a nucleophilic addition between the aldehyde group of Far and the primary amino groups of ATBP2 (see Figure 6).

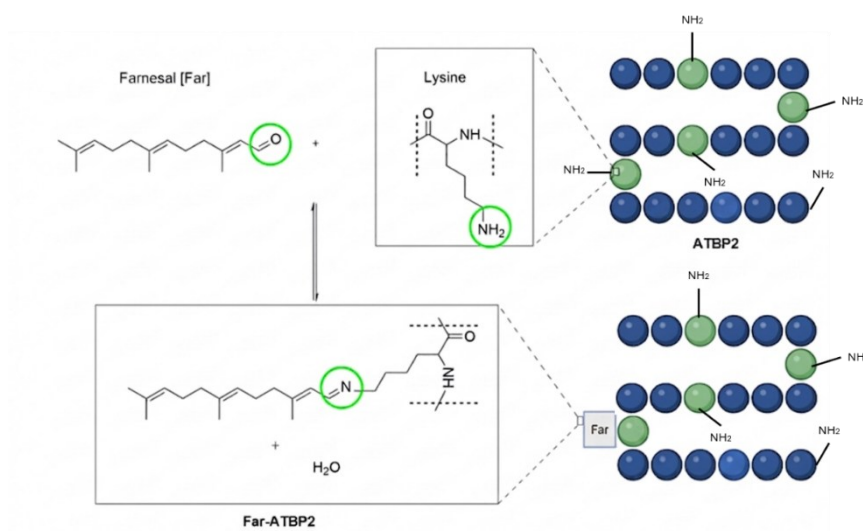


Figure 6. Synthesis scheme of Far-ATBP2.

Investigation of successful conjugation was done for the reaction mixture “Far-ATBP2-200” with the molar ratio between the available NH₂ groups of ATBP2 and Far of 5:10 in the reaction. The synthesis was monitored by performing ¹H-NMR analysis and observing visible color change. As shown in *Figure 7*, Far-ATBP2-200 reaction mixture showed slight decrease in aldehyde proton peak ($\delta \approx 10\text{ppm}$) from Far after 24 h, accompanied by color change (colorless to yellow) indicating conjugation occurred between Far and ATBP2.

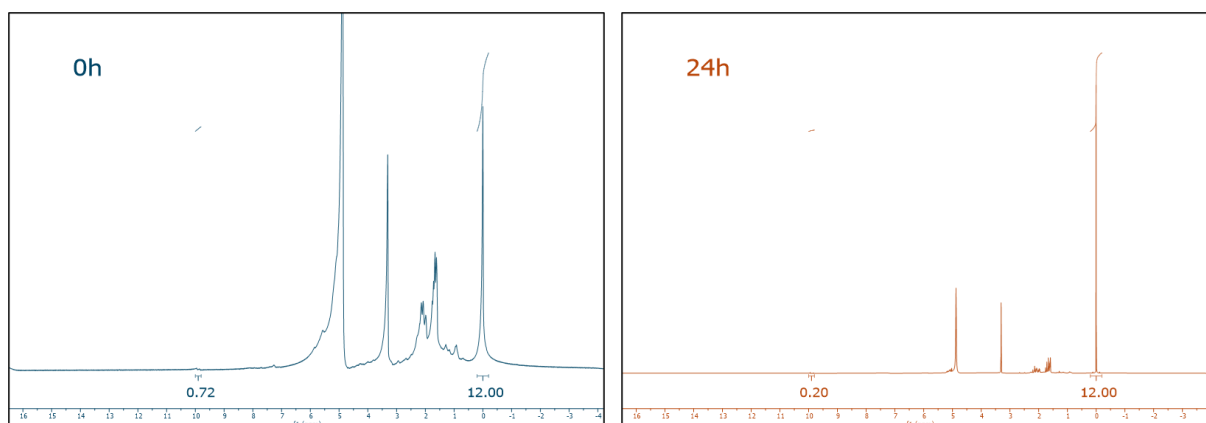


Figure 7. ¹H-NMRs of Far-ATBP2-200 at 0 h and 24 h with TMS as internal standard.

4.3.3 Purification and Characterization

Far-ATBP1

For further analysis, Far-ATBP1 conjugates required purification from unutilized Far and acetic acid in the reaction mixture. For this purpose, washing was done by performing dialysis using membrane with MWCO of 1 kDa that would only allow Far-ATBP1 to be retained as residue and leave rest of the substances to be filtered out into dialysate. The dialyzed Far-ATBP1 was freeze-dried to obtain solid powder.

To confirm the presence of imine bond, FT-IR analysis of Far, ATBP1 and Far-ATBP1 was performed. Transmittance between wavenumbers of 1500 cm⁻¹ and 1800 cm⁻¹ was focused (region for imine bond¹⁷). As shown in *Figure 8*, Far-ATBP1 and ATBP1 showed similar signals compared to Far. Since ATBP1 has bonds in the region of interest inherently, imine bond in Far-ATBP1 could not be observed, and the FT-IR measurements were inconclusive.

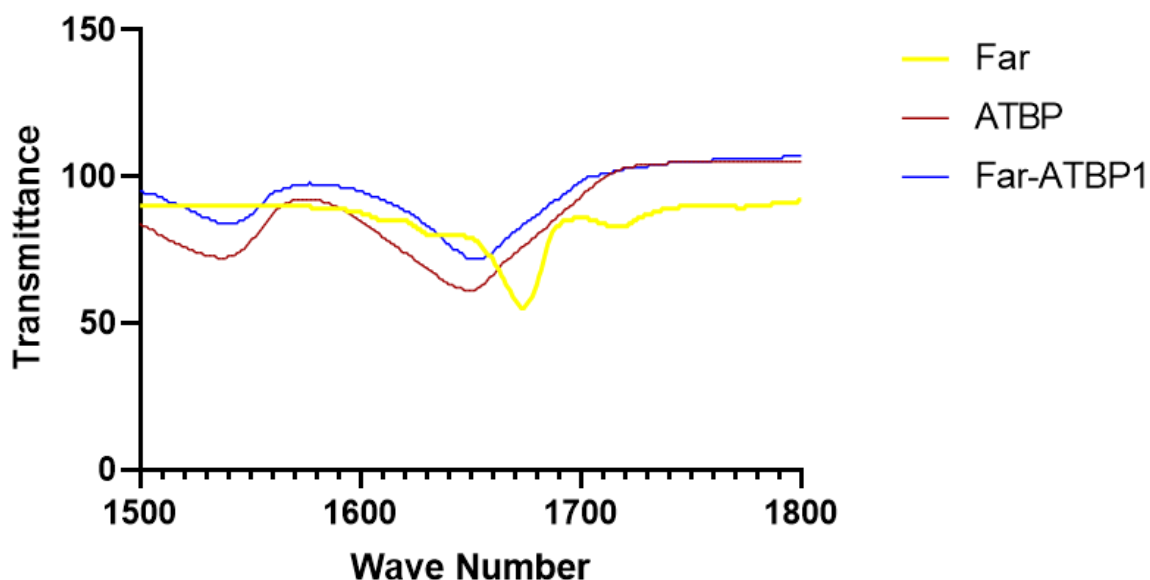
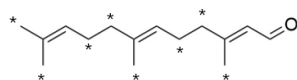


Figure 8. FT-IR Spectra of Far, ATBP1 and Far-ATBP1 focused on wavenumber range from 1500 cm^{-1} to 1800 cm^{-1} .

To confirm and calculate the conjugation rate of Far per ATBP1 peptide in Far-ATBP1, ^1H -NMR measurement comparison of washed FA1 conjugates and ATBP1 was done. To improve the yield of recovered Far-ATBP1, ionic strength of the dialysate was increased by adding 20 mM NH_4Cl . The said ionic strength would provide counterions, that can shield Far-ATBP1 from sticking electrostatically to the dialysis membrane wall, therefore improving its recovery in the residue¹³. The residue was freeze-dried and then analyzed by ^1H -NMR spectroscopy (see Figure 9). The region of interest (R.O.I) was from chemical shifts of 0.7 ppm and 2.5 ppm, as this region contains signals from methyl groups (12H) and methylene groups (8H) of Far, ATBP1 and would exclude signals from NH_4Cl ($\delta \approx 6.0\text{ ppm} - 7.5\text{ ppm}$).



Farnesal

R.O.I: 0.7 – 2.5 ppm

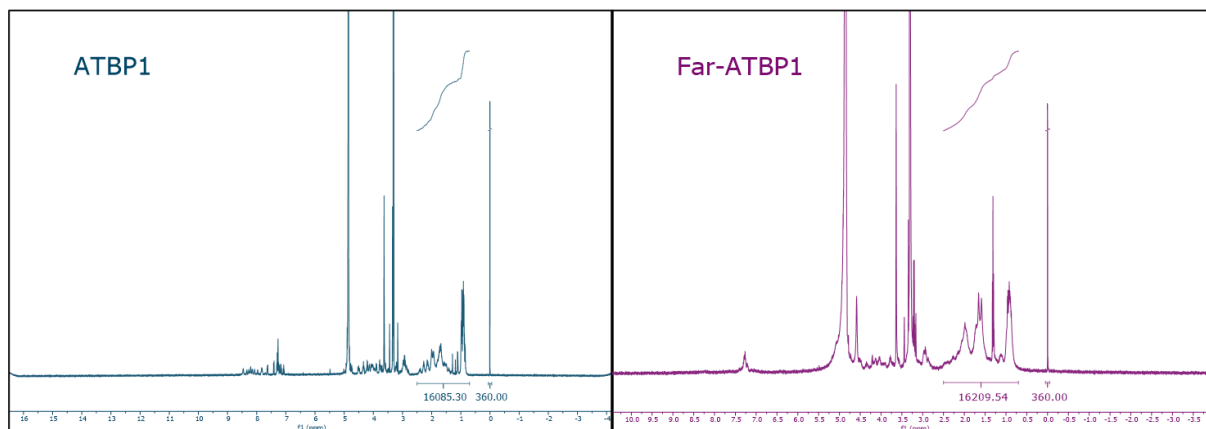


Figure 9. ^1H -NMRs of dialyzed ATBP1 (~ 0.5 mg) and Far-ATBP1 (~ 0.5 mg) with TMS as internal standard ($\times 10^{15}\text{H}$) depicting a 5% conjugation rate of Far per ATBP1.

The results showed that ~ 0.5 mg of ATBP1 contained 1.608×10^{19} hydrogens in R.O.I ($H_{\text{pep}} = 3.216 \times 10^{19}$ hydrogens per mg) and ~ 0.5 mg of Far-ATBP1 contained 1.62×10^{19} hydrogens in R.O.I ($H_{\text{conj}} = 3.24 \times 10^{19}$ hydrogens per mg). The number of hydrogens in Far-ATBP1 contributed by Far in the R.O.I (ΔH) can be calculated by:

$$\Delta H = H_{\text{conj}} - H_{\text{pep}} = 3.24 \times 10^{19} - 3.216 \times 10^{19} = 2.4 \times 10^{17} \text{ H per mg}$$

Each Far has 20 hydrogens in R.O.I, so the moles of Far molecules per mg are:

$$n_{\text{far}} = \frac{\Delta H}{20 \times N_A} = \frac{2.4 \times 10^{17}}{20 \times 6.022 \times 10^{23}} = 1.99 \times 10^{-8} \text{ mol}$$

For calculating conjugation rate of Far per ATBP1:

$$\frac{n_{\text{far}}}{n_{\text{ATBP1 per mg}}} = \frac{1.99 \times 10^{-8}}{4.06 \times 10^{-7}} \times 100 \approx 5\%$$

So, the conjugation rate of Far in Far-ATBP1 came out to be 5%, which can also be interpreted as twenty molecules of ATBP1 conjugated with one molecule of Far. The low conjugation rate might be due to steric hindrance from neighboring amino acids (e.g., bulky side chains) or the 3D structure of ATBP1, which does not allow all primary amino groups in the structure to be available for conjugation.

Far-ATBP2

Like Far-ATBP1, Far-ATBP2 conjugates were washed by dialysis and the residue was freeze-dried. Calculation of conjugation rate of Far per ATBP2 was done by comparing ^1H -NMR measurements of dialyzed Far-ATBP2 and ATBP2 with TMS as internal standard. The region of interest (R.O.I) was again from chemical shifts of 0.7 ppm and 2.5 ppm for the same reasons mentioned earlier.

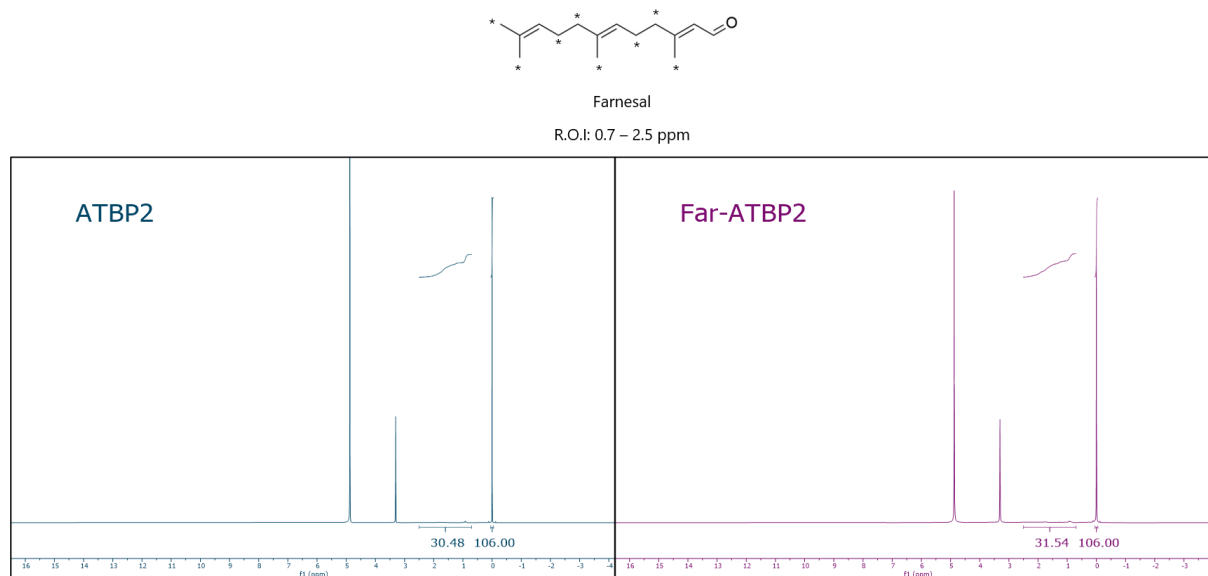


Figure 10. ^1H -NMRs of dialyzed ATBP2 (~ 0.45 mg) and Far-ATBP2 (~ 0.4 mg) with TMS as internal standard ($\times 10^{18}\text{H}$) depicting 213.5% conjugation rate of Far per ATBP2.

As shown in Figure 10, 0.45 mg of ATBP2 contains 30.48×10^{18} hydrogens in R.O.I ($H_{\text{pep}} = 68.19 \times 10^{17}$ hydrogens per mg) and 0.4 mg of Far-ATBP2 contains 31.54×10^{18} hydrogens in R.O.I ($H_{\text{conj}} = 78.85 \times 10^{18}$ hydrogens per mg). The number of hydrogens in Far-ATBP2 contributed by Far in the R.O.I (ΔH) can be calculated by:

$$\Delta H = H_{\text{conj}} - H_{\text{pep}} = 78.85 \times 10^{18} - 68.18 \times 10^{18} = 10.67 \times 10^{18} \text{ H per mg}$$

Each Far has 20 hydrogens in R.O.I, so the moles of Far molecules per mg are:

$$n_{\text{far}} = \frac{\Delta H}{20 \times N_A} = \frac{10.67 \times 10^{18}}{20 \times 6.022 \times 10^{23}} = 8.86 \times 10^{-7} \text{ mol}$$

For calculating conjugation rate of Far per ATBP2:

$$\frac{n_{\text{far}}}{n_{\text{ATBP2 per mg}}} = \frac{8.86 \times 10^{-7}}{4.15 \times 10^{-7}} \times 100 \approx 213.5\%$$

So, the conjugation rate of Far in Far-ATBP2 came out to be 213.5%, which can also be interpreted as 1 molecule of ATBP2 conjugated with ~2 molecules of Far. The higher conjugation rate (compared to 5% of Far-ATBP1) can be attributed to the primary and 3D structure of ATBP2, that is more open in conformation and allows more primary amino groups to be available for conjugation.

4.4 NP Formation of Farnesal-AMP Conjugates

4.4.1 Self-Assembled (SA) NPs

The Far-ATBP1 and Far-ATBP2 self-assembled NPs (SA NPs) were formed during the dialysis-based washing step, where gradual solvent exchange promoted spontaneous assembly of the amphiphilic conjugates. In both systems, covalent linkage of hydrophobic Far to the hydrophilic peptides ATBP1 or ATBP2 generated amphiphilic hybrids capable of organizing into nanoscale structures upon exposure to aqueous medium. DLS/ELS measurements confirmed NP formation for both conjugates: Far-ATBP1-SA NPs exhibited a D_H of 393 nm, PDI 0.13, and zeta potential +35.72 mV, while Far-ATBP2-SA NPs showed a slightly larger D_H of 403.2 nm, a broader PDI of 0.48, and a zeta potential of +25.06 mV. SEM imaging (*Figure 11*) supported these findings, revealing predominantly spherical Far-ATBP1-SA NPs with an average size of 380.7 nm, and a mixture of spherical and irregularly shaped Far-ATBP2-SA NPs averaging 422.4 nm.

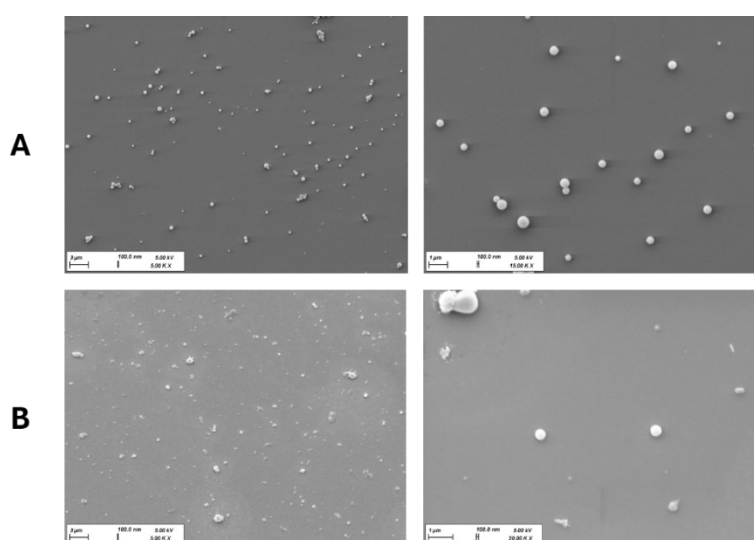


Figure 11. SEM images A) Far-ATBP1-SA NPs B) Far-ATBP2-SA NPs

While dialysis-induced self-assembly is simple and gentle—making it attractive for peptide-based nanocarriers—it also presents notable limitations. The gradual solvent exchange can lead to heterogeneous nucleation, resulting in broader size distributions, as

seen in the higher PDI of Far-ATBP2-SA NPs. Self-assembly during dialysis is also highly sensitive to concentration, diffusion rate, and batch-to-batch variability, making precise control over NP size and morphology challenging.

4.4.2 Far-ATBP1 Nanoprecipitated (NA) NPs

Nanoprecipitation of the Far-ATBP1 conjugate yielded well-defined NPs, reflecting the improved control over nucleation and growth offered by rapid solvent displacement. Unlike the dialysis-based self-assembly process—which relies on slow solvent exchange and often produces larger, more heterogeneous structures—the nanoprecipitation workflow enables instantaneous supersaturation of the amphiphilic conjugate, driving uniform nucleation events. The inclusion of poly(vinyl alcohol) (PVA) further contributed to particle stabilization by providing steric hindrance during the precipitation step, thereby minimizing aggregation and promoting formation of discrete nanostructures.

Zetasizer analysis confirmed that nanoprecipitation successfully produced Far-ATBP1-NA NPs with a markedly reduced D_H of 156.8 nm, a low PDI of 0.09, and a zeta potential of +23.6 mV. These values demonstrate a substantial improvement in size control and monodispersity compared to the self-assembled Far-ATBP1-SA NPs (393 nm, PDI 0.13). SEM imaging (*Figure 12*) corroborated these findings, revealing predominantly spherical NPs with an average diameter of 181 nm, consistent with DLS data. The moderately positive surface charge is expected to enhance colloidal stability through electrostatic repulsion, reducing the likelihood of agglomeration and supporting long-term dispersion¹⁸. Collectively, these results demonstrate that nanoprecipitation effectively mitigates the size heterogeneity and diffusion-driven variability inherent to the self-assembly method.

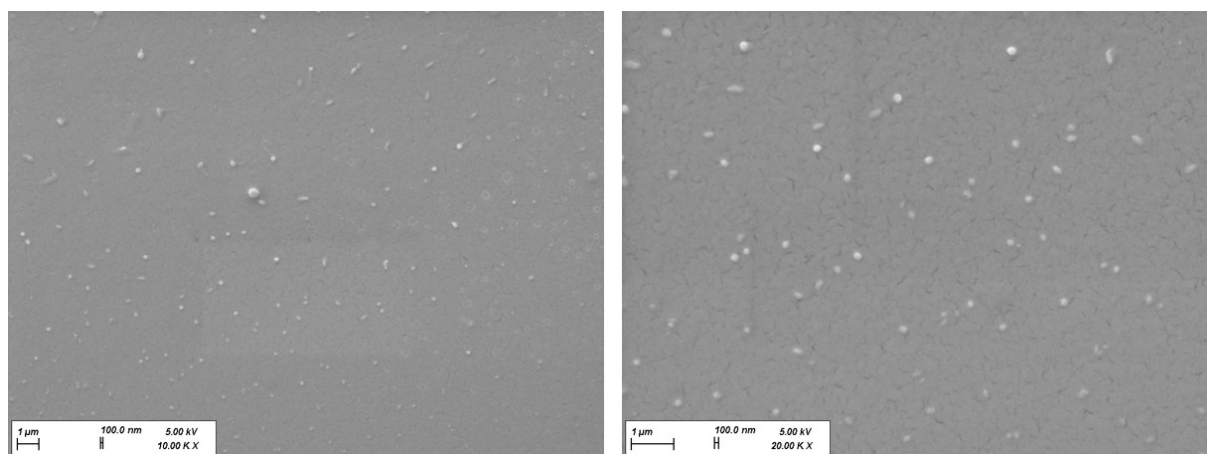


Figure 12. SEM images of Far-ATBP1-NA NPs.

4.4.3 Far-ATBP2 Nanoprecipitated (NA) NPs

Application of the nanoprecipitation technique to the Far-ATBP2 conjugate similarly produced NPs with improved size uniformity and stability relative to their self-assembled counterparts. The rapid solvent displacement characteristic of nanoprecipitation promotes synchronized nucleation events, preventing the broad size distribution and irregular morphologies observed during dialysis-induced assembly. As with Far-ATBP1, PVA served as an effective stabilizer, limiting particle–particle interactions during formation and supporting the development of spherical, well-dispersed nanostructures.

Zetasizer analysis confirmed that Far-ATBP2-NA NPs possessed a substantially reduced D_H of 183.9 nm, a PDI of 0.06, and a zeta potential of +15.2 mV. These values indicate tight size control and excellent monodispersity, representing a major improvement over the self-assembled Far-ATBP2-SA NPs, which were considerably larger (403.2 nm) and exhibited a broad size distribution (PDI 0.48). SEM imaging (*Figure 13*) further validated these results, showing spherical NPs with an average diameter of 240.8 nm, consistent with DLS measurements. The positive surface charge is expected to promote electrostatic repulsion between particles, reducing agglomeration and enhancing dispersion stability¹⁸. Overall, nanoprecipitation effectively resolved the heterogeneity and morphological variability associated with self-assembly, yielding Far-ATBP2 NPs with properties more suitable for reproducible biological performance.

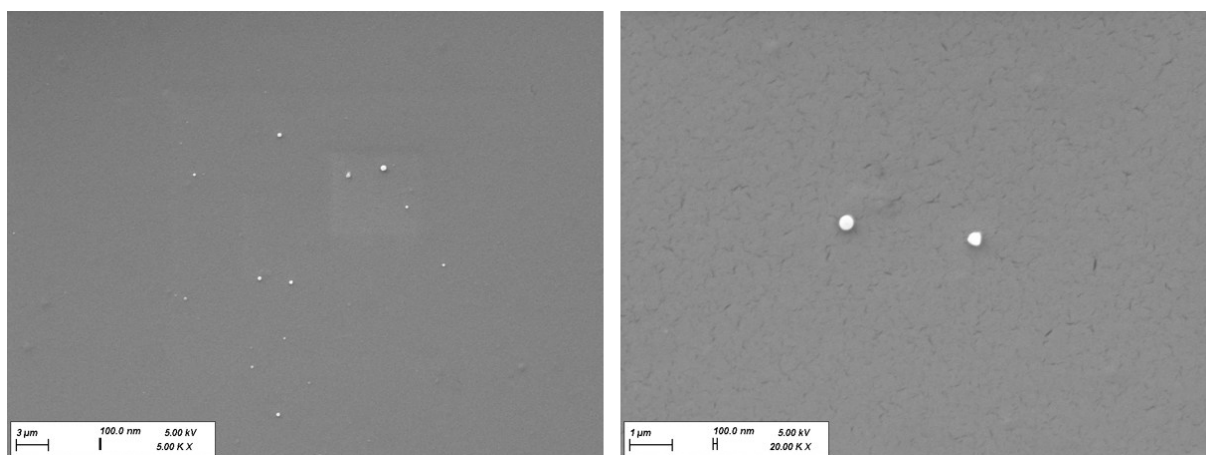


Figure 13. SEM images of Far-ATBP2-NA NPs.

4.4.4 DOE-Based Optimization

Far-ATBP2 exhibited a higher degree of Far conjugation relative to Far-ATBP1, suggesting greater hydrophobic loading and, consequently, a stronger propensity for forming stable

nanoprecipitated structures. Given this enhanced conjugation efficiency and the superior performance potential of Far-ATBP2-based formulations, an optimization of its nanoprecipitation process was done. To evaluate how key formulation parameters influence NP formation, a screening fractional factorial design (2^{3-1}) was employed. This DOE approach enables identification of dominant factors affecting NP size while minimizing experimental burden.

Three process variables¹⁴ were selected based on their established roles in governing nucleation and growth during nanoprecipitation: PVA % in the aqueous phase, FOS, and mixing speed (rpm). Each parameter was assessed at two levels to capture its directional influence on the response variable—NP size. The fractional factorial design required four experimental runs, each representing a unique combination of factor levels, as summarized in *Table 1*.

Experiment Run #	Factor 1 (X1)	Factor 2 (X2)	Factor 3 (X3)
1	-1	-1	+1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	+1
PVA % (X1) : 1% (-1) & 2% (+1)			
FOS (X2) : 0.33(-1) & 0.5 (+1)			
Mixing speed (X3) : 400 rpm (-1) & 600 rpm (+1)			

Table 1. Design of Experiment for Far-ATBP2-NA NPs.

Based on the said design 4 batches of Far-ATBP2-NA NPs were made (1 for each run) for obtaining the model equation. The D_H & PDI of prepared batches were tested by DLS measurements and output can be seen in *Figure 14*. The results indicated that PVA % and FOS correlate with particle size of NPs positively (i.e., increase in D_H) while mixing speed correlates negatively (i.e., decrease in D_H). The magnitude of correlation from FOS was highest (16.225) followed by PVA % (4.225) and mixing speed had lowest correlation (-1.125). In combination, only PVA % and FOS interaction may lead to increase in NP D_H . These results can be easily explained, as increasing the amount PVA used in aqueous phase would result in more PVA

absorbing to NP surface and thus increasing its size. Similarly increasing the organic phase fraction would increase the amount of Far-ATBP2 in each NP composition and thus increase its size.

To validate the model equation, 2 batches of NPs were made at 0 levels of all factors i.e., center points (PVA % = 1.5, FOS = 0.415 and Mixing Speed = 500 rpm). DLS measurements showed that Far-ATBP2-NA-V1 had a D_H of 183.9 nm and Far-ATBP2-NA-V2 had a D_H of 165.9 nm. The average D_H of validation batches is 174.9 nm, which is within the 95% confidence interval of model prediction 170.8 nm (89.9nm and 250nm) and thus the model equation is validated, however the none of the process parameters bear a p-value less than 0.05 and were not significant, suggesting that more than 2 validation batches need to be prepared for better idea of the actual variation of the manufacturing process. All 6 batches of Far-ATBP2-NA NPs were monodispersed in size distribution with PDIs less than 0.20.

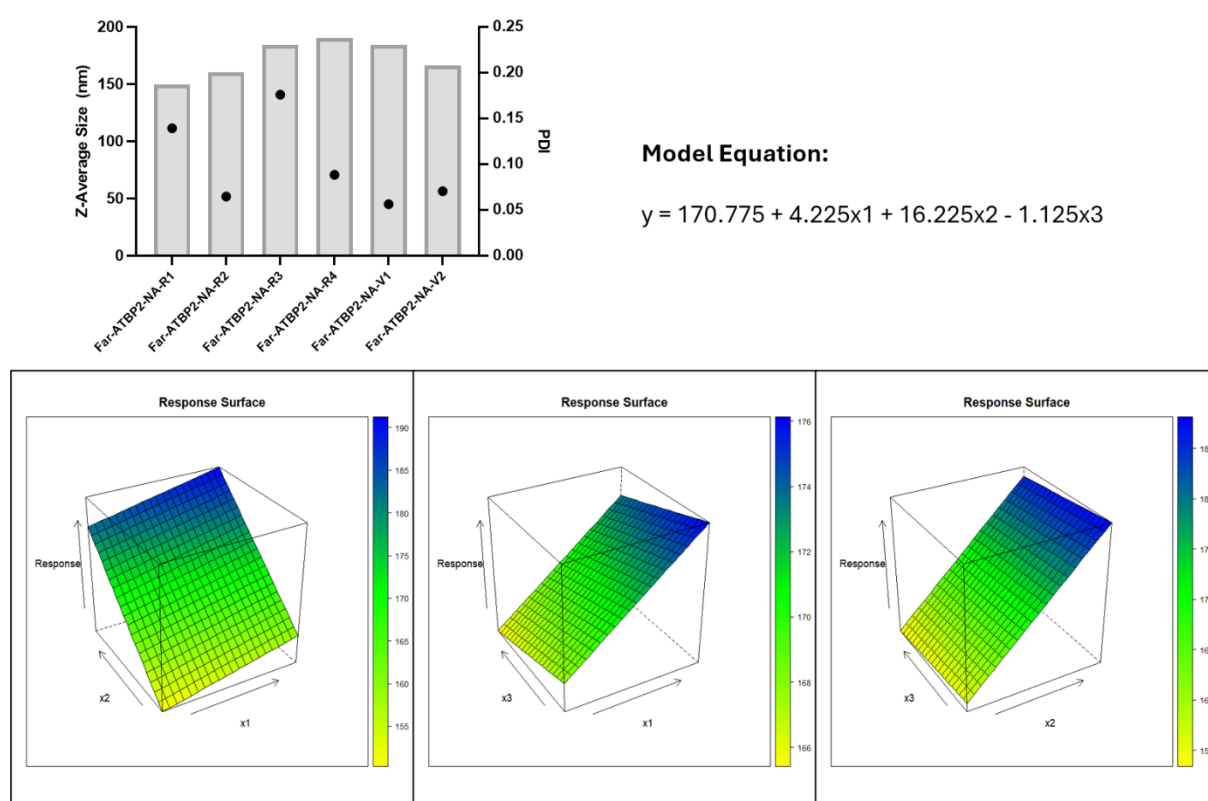


Figure 14. Results after DOE: D_H & PDI of prepared batches, Model Equation and Response surface graphs for combination of factors.

4.5 pH-Responsive NP Dissociation

Evaluating the pH-responsive dissociation of Far-ATBP1-NA and Far-ATBP2-NA NPs is essential for establishing their intended “smart” behavior, namely the selective release of

AMPs under acidic conditions characteristic of intracellular compartments such as endosomes. To probe this, NPs were incubated in pH 7.4 and pH 4.5 buffers at 37 °C for 24 h, followed by DLS analysis to monitor changes in D_H , PDI, and scattering intensity.

For Far-ATBP1-NA, the data (Figure 15) showed an increase in PDI and a decrease in scattering intensity in both pH environments after 24 h. This pattern suggests destabilization of the NP population, likely driven by aggregation or partial disassembly. The instability observed may be attributed to the lower degree of Far conjugation in Far-ATBP1, which reduces hydrophobic core integrity, as well as the intrinsic sensitivity of the imine linkage, which is susceptible to hydrolysis. However, meaningful interpretation of this trend requires further investigation. Additional studies examining stability across a broader range of pH values, shorter incubation intervals, and multiple independent batches will be necessary to determine whether the observed dissociation is reproducible and statistically significant.

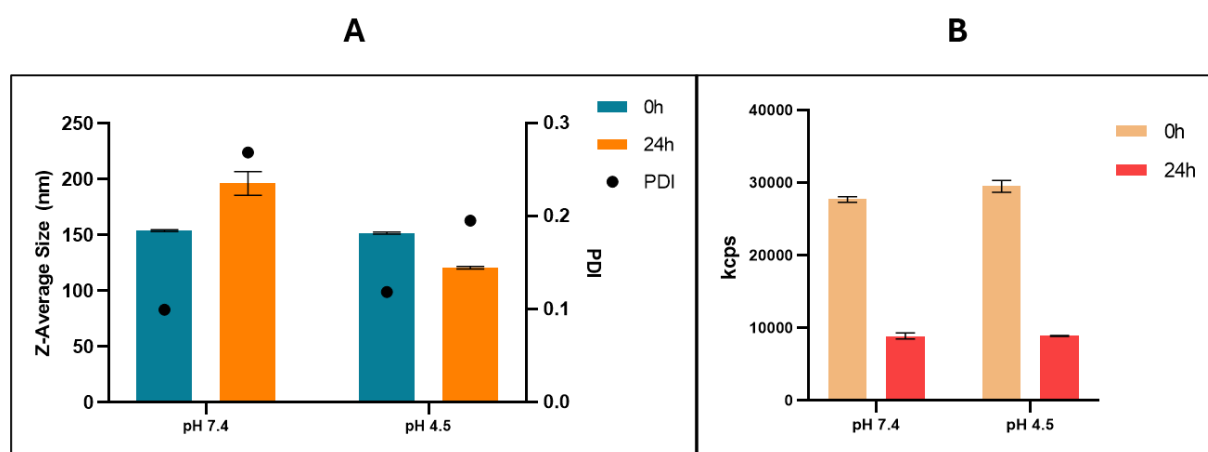


Figure 15. Far-ATBP1-NA NPs DLS results after incubating in buffers pH 7.4 and pH 4.5 A) D_H (nm) and PDI B) Scattering Intensity (kcps)[$n=3$ and $N=1$].

For Far-ATBP2-NA however, the data (Figure 16) showed that after 24 h, the PDI and scattering intensity remained largely unchanged at pH 7.4, indicating that the NPs maintained their size distribution and colloidal stability under neutral conditions. At pH 4.5, however, a slight increase in PDI and a decrease in scattering intensity were observed, suggesting partial dissociation or structural loosening in acidic environments. Mechanistically, this behavior aligns with the expected pH-responsiveness of the imine bond, which undergoes accelerated hydrolysis under acidic conditions, leading to controlled destabilization of the NPs.

These results collectively indicate that Far-ATBP2-NA NPs exhibit selective dissociation at acidic pH, consistent with the intended design of a pH-triggered release system. Nonetheless,

as with Far-ATBP1-NA, further examining stability across a broader range of pH values, shorter incubation intervals, and multiple independent batches will be necessary to determine whether the observed dissociation is reproducible and statistically significant.

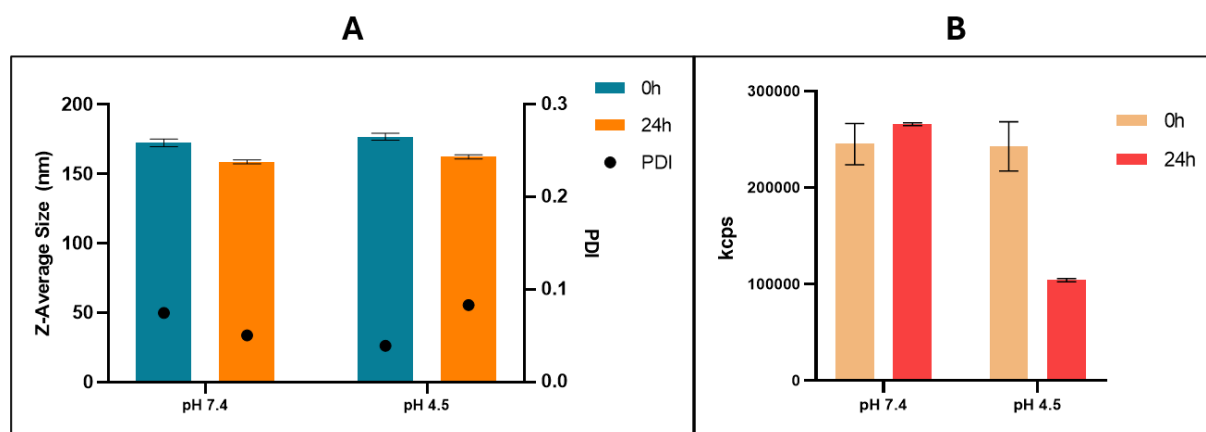


Figure 16. Far-ATBP2-NA NPs DLS measurement results after incubating in buffers of pH 7.4 and pH 4.5 A) D_H (nm) and PDI B) Scattering Intensity (kcps)[$n=3$ and $N=1$].

4.6 Freeze-Drying Stability

The stability of Far-ATBP2-NA NPs following lyophilization was evaluated to determine whether the formulation could withstand drying and reconstitution—an essential requirement for long-term storage and translational use. After freeze-drying, the resulting powder was re-dispersed in water and analyzed by DLS to assess changes in D_H and PDI (Figure 17). Upon reconstitution, the NPs maintained a D_H below 200 nm, indicating that the overall size of the nanostructures was largely preserved. However, the PDI increased to 0.28, suggesting a broader size distribution and partial loss of colloidal uniformity.

This increase in PDI indicates that Far-ATBP2-NA NPs experience structural destabilization during the freeze-drying process, likely due to stresses associated with ice crystal formation and dehydration. Incorporating a cryoprotectant such as sucrose or trehalose could mitigate this issue by stabilizing the NP interface, preserving hydration shells, and preventing aggregation during freezing and sublimation. Further optimization studies are therefore needed to establish a robust freeze-drying process. In addition to stability assessment, the theoretical yield of the nanoprecipitation process was determined by weighing the freeze-dried Far-ATBP2-NA material. The yield was calculated to be 40%, providing a baseline for future optimization efforts aimed at improving process efficiency and maximizing recovery.

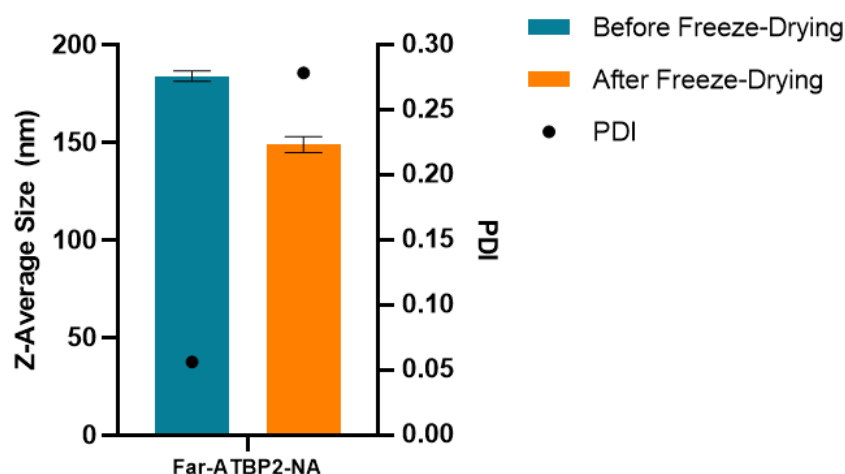


Figure 17. D_H (nm) and PDI of Far-ATBP2-NA NPs before and after freeze-drying [$n=3$ and $N=1$].

4.7 Cell viability and Biocompatibility

To quantify the biocompatibility and cytotoxicity profile of the prepared conjugates, cell viability assay was performed. Using the MTT assay, metabolic activity of A549 cells was tested after incubating them for 24 h with Far, ATBP1, Far-ATBP1, Far-ATBP2 and ATBP2. As shown in Figure 18, the results indicate that at lower concentrations (7.8 – 62.5 $\mu\text{g/mL}$) none of the tested materials relatively impact viability of A549 cells i.e., cell viability >90 % across Far, ATBP1, ATBP2, Far-ATBP1 and Far-ATBP2. At 125 $\mu\text{g/mL}$ however, it can be noted that ATBP1 (42 % viability) was more cytotoxic than Far-ATBP1 (80 % viability), indicating that conjugation has reduced cytotoxicity of ATBP1. Cell viability in Far-ATBP2 (117 %) was higher than ATBP2 (96 %) indicating both were not cytotoxic at 125 $\mu\text{g/mL}$. Both Far-ATBP1 and Far-ATBP2 were less cytotoxic than Far (29 % viability). These results suggest that both conjugates have better biocompatibility than their respective AMPs. The effect can be explained by decrease in the number of positively charged $-\text{NH}_2$ groups in both peptides after imine formation, which can otherwise be cytotoxic¹⁹. The samples that present >100 % cell viability across the concentration range of 7.8 $\mu\text{g/mL}$ – 125 $\mu\text{g/mL}$ may be doing so due to multiple factors (including stress induced metabolic hyperactivation). This is a limitation of the MTT Assay and is a well-documented phenomenon²⁰. For the meaningful interpretation of this trend, further investigation is needed by performing MTT assay on higher concentrations (e.g., 250 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$) and repeating the experiment multiple times with different batches for evaluating statistical significance.

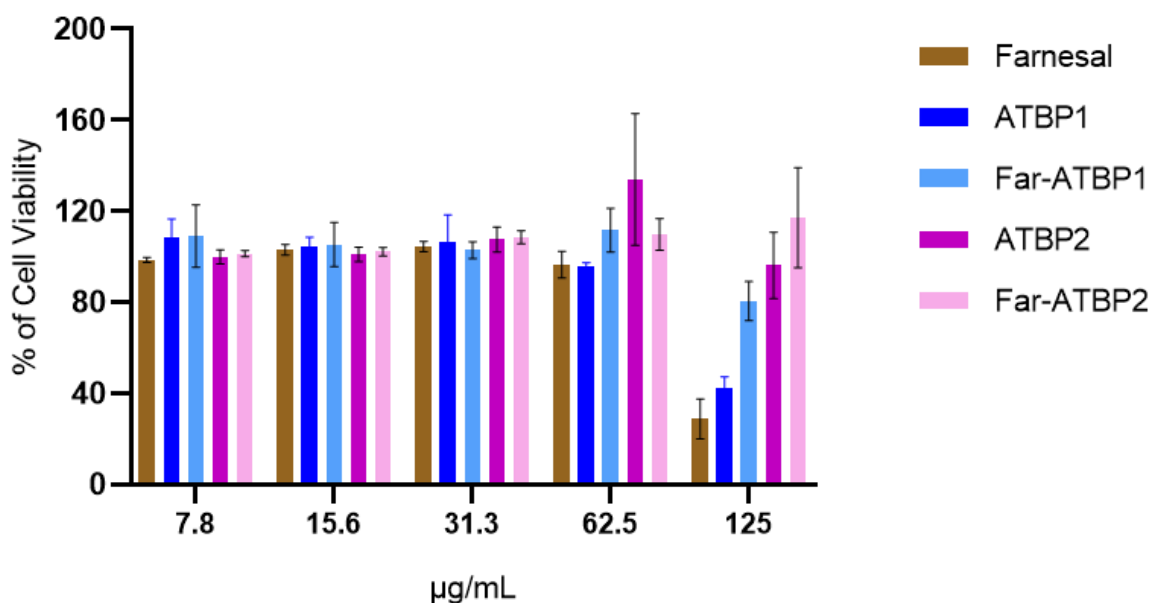


Figure 18. Cell viability of A549 cells analyzed by MTT assay after 24h incubation with Farnesal, ATBP1, Far-ATBP1, ATBP2 and Far-ATBP2 at different concentrations (7.8 – 125 µg/mL) [n=3 and N=1].

5. CONCLUSION & FUTURE PERSPECTIVES

The present work successfully synthesized the amphiphilic conjugates Far-ATBP1 and Far-ATBP2 by linking the hydrophobic isoprenoid Far to two linear 20-mer antituberculosis peptides through acid-sensitive Schiff base chemistry. ¹H-NMR analysis confirmed imine formation, although the data suggest that steric accessibility within the peptide structures may have influenced the extent of conjugation. To strengthen these observations, future studies should incorporate optimized chromatographic methods (such as HPLC or SEC) to more accurately quantify conjugation efficiency.

Cell viability studies using the A549 lung epithelial cell line demonstrated that both conjugates exhibited improved biocompatibility compared to their native free peptides. This enhancement is likely due to the reduction in free amine groups following imine formation, which decreases the overall cationic charge associated with AMP-induced cytotoxicity. Although these initial results are promising, repeated MTT assays will be required to confirm reproducibility and establish statistical significance.

Formulation of the conjugates into NPs via nanoprecipitation was achieved successfully. Far-ATBP1-NA NPs were obtained but showed instability under both physiological (pH 7.4)

and acidic (pH 4.5) conditions within 24 h, indicating susceptibility to hydrolysis. In contrast, Far-ATBP2-NA NPs remained stable at pH 7.4 and degraded selectively at pH 4.5, demonstrating desirable pH-responsive behavior. These findings suggest that differences in conjugation degree and intrinsic imine sensitivity may govern NP stability. A more detailed investigation of dissociation kinetics and direct measurement of AMP release using chromatographic techniques will be essential to validate these conclusions.

Further optimization of Far-ATBP2-NA NPs using a fractional factorial DOE identified PVA concentration, organic phase fraction, and mixing speed as influential parameters affecting particle size. Although the DOE model provided useful insights, additional evaluation of nanoprecipitation variability is warranted. Moreover, lyophilized Far-ATBP2-NA NPs exhibited increased polydispersity, indicating physical instability that must be addressed in future formulation work.

Overall, the results of this study establish a foundation for developing Far-AMP conjugates and their NP formulations as potential pH-responsive anti-TB systems. Future research should assess cellular uptake in alveolar macrophages and evaluate antimicrobial activity against *Mtb* to determine whether Far conjugation enhances therapeutic efficacy. Strategies to improve NP stability, including the use of cryoprotectants during freeze-drying, should be explored during scale-up. Finally, translating these NPs into microparticle-based dry-powder inhalation systems may offer a promising route toward clinical application in pulmonary TB therapy.

6. REFERENCES

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