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Designing 5-fluorouracil-loaded lipid nanoparticles using double emulsion and solvent evaporation for skin cancer therapy

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Abstract

Implementing the Quality by Design (QbD) approach enables pharmaceutical researchers to effectively reduce the number of experimental tests conducted and the associated time required. This aids in identifying important factors that substantially influence the quality of the goods, including vital material characteristics, formulation factors, and crucial procedure factors. This study aims to integrate the advantageous characteristics of Lipid Nanoparticles (LNPs) and the QbD approach to create an innovative Drug Delivery System (DDS) for treating skin cancers and actinic keratosis. LNPs, known for their high efficacy in the topical treatment of skin diseases, were formulated to address the skin layer's intricate structure and facilitate enhanced absorption. The present investigation employed the QbD methodology to fabricate LNP formulations to enhance the dermal permeation of 5-Fluorouracil (5-FU), a commonly utilized agent for treating non-melanoma skin cancer. The Lipid Nanoparticles containing 5-Fluorouracil (5-FLLNP) were synthesized using the Double Emulsion - Solvent Evaporation (DE-SE) technique. The DE-SE involves a dual-stage procedure encompassing two distinct emulsification steps, thereby introducing a heightened level of complexity to the overall process. The stability of nanoparticles produced through a DE-SE method is uncertain due to the various formulations and process attributes involved. Using a DE-SE technique in producing 5-FLLNP is a viable method for DDS, offering a means to achieve a high-quality product through implementing the QbD approach. Artificial Neural Networks (ANN) have successfully developed an optimal LNP formulation that ensures quality and falls within the designated Design Space (DS). 5-FLLNP formulation exhibited a greater cell viability of 73% compared to the pure 5-FU formulation, which had a cell viability of 68% for A431 cells.

Key Words: quality by design, LNPs, skin cancers, ANN, 5-Fluorouracil, double emulsion, solvent evaporation

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INTRODUCTION

QbD is a systematic approach that entails the deliberate design and expansion of work processes to guarantee a predetermined level of quality for the end product. Implementing these concepts gives rise to the paramount significance of "quality" in pharmaceutical engineering. The objective of QbD in pharmaceutics is to enhance comprehension of formulation and process variables and optimize the development of drug products that align most effectively with their intended objectives [1]. The QbD approach enables the mathematical expression of abstract concepts that emerge during the formulation development phase. QbD enables the comprehension and regulation of pharmaceutical quality and the variables associated with drug formulation and production. The individual operates within a design domain where identifying and optimizing key formulation and process parameters obviate the need for subsequent verification of the final product's quality. Applying QbD principles during product development leads to a more comprehensive understanding of the processes [2].

The utilization of contemporary technologies in manufacturing offers several advantages in quality enhancement, risk mitigation, and information management. These benefits are associated with implementing QbD as a strategic approach in business operations [3]. The QbD approach, formulated through a collaborative international endeavor, was formally outlined in the Q8 Pharmaceutical Development guideline of the International Council for Harmonization (ICH). Furthermore, the QbD methodology has gained significant significance in nano-sized DDS. Recent research has been conducted on various nano-sized DDS using the QbD approach. These systems include self-nano emulsified DDS, liposomes, LNPs, Nanostructured Lipid Carriers (NLC), phospholipid NPs, polymeric NPs, and nasal nano-sized compositions [4].

The traditional product development approach, which relied on experimentation,

has been superseded by a modeling-based approach known as QbD. ANNs are a class of computer systems that draw inspiration from the structure and functioning of the human brain. ANNs consist of a multitude of processing units, commonly referred to as artificial neurons [5]. Unlike other statistical assessments, neural networks necessitate less formal statistical training. ANNs do not necessitate the implementation of experimental design protocols characterized by stringent rules. Moreover, ANNs can draw conclusions based on historical or incomplete datasets. Due to this rationale, ANNs are regarded as rigorously defined mathematical techniques capable of addressing intricate nonlinear problems involving numerous variables and multiple solutions [6]. Therefore, neural networks have recently been applied in various pharmaceutical domains.

Non-melanoma and melanoma skin cancers are prevalent forms of cancer, exhibiting a rising occurrence attributed to factors such as ozone depletion, heightened tanning practices, and the advancing mean age of individuals. Caucasians worldwide experience non-melanoma skin cancer at the highest frequency compared to other types of cancer [7]. The main approach for managing non-melanoma skin cancer is surgical therapy, although the feasibility of this treatment may be limited by factors such as the lesion's dimensions and position. Surgical interventions may present challenges for patients, particularly when addressing aesthetic concerns related to anatomical regions. Topical chemotherapy represents a viable substitute for surgical intervention. 5-FU, a compound with two ionizable hydrogen atoms, is a widely utilized active component in managing skin malignancies, specifically in the therapeutic approach to actinic keratosis and basal cell carcinomas [8].

LNPs, which belong to the category of colloidal DDS, were created as a substitute for liposomes. When LNPs are applied topically, they create a cohesive film layer on the skin's surface. Film development is utilized to widen the spaces between corneocytes, improve skin hydration, and degrade the defensive ability of the stratum corneum [9]. Furthermore, the particular exteriors and morphology of the NPs contribute to achieving zero-degree kinetics and prolonged dynamic component release. Consequently, the LNPs can efficiently infiltrate the dermal cells briefly.

This paper presents the synthesis of 5-FLLNP using DE-SE to improve skin cancer treatment. Due to its increased prevalence and need for targeted therapies, skin cancer, including basal cell carcinoma, squamous cell carcinoma, and melanoma, is a major healthcare concern. 5-FU, a strong chemotherapeutic drug, can decrease skin cancer cell multiplication. However, solubility and stability concerns limit its practical use. LNPs improve 5-FU encapsulation, controlled release, and targeted skin distribution. Researchers use DE-SE to incorporate 5-FU into LNPs to increase medication stability, bioavailability, and systemic exposure, reducing side effects. This novel skin cancer treatment method may improve patient outcomes and target and manage this common and sometimes fatal illness.

RELATED WORKS

Skin cancer, including basal, squamous, and melanoma, is a global health issue due to its rising prevalence and rapid progression. Skin cancer therapy requires new medication delivery systems to target damaged regions while limiting systemic exposure. 5-FU is a strong chemotherapeutic medication that slows skin cancer cell proliferation. Due to its low solubility and quick breakdown under physiological circumstances, 5-FU requires specific drug delivery methods to be successfully used. In recent years, LNPs, notably W/O/W DE systems, have shown promise for regulated and localized 5-FU administration. This literature review covers W/O/W DE and SE methods for designing 5-FLLNP for skin cancer therapy, including key methodologies, recent developments, and potential benefits and drawbacks.

Singh et al. (2021) examined nanoparticle-delivered skin cancer therapy—the suggested technique required a complete literature analysis to appraise current research on this subject [10]. The review found that this method enhanced skin cancer treatment delivery and minimized systemic adverse effects. Nanoparticle stability and skin permeability were drawbacks.

In their 2022 work, Pandya, Chatterjee, and Ganti examined oral anticancer drug delivery methods using self-emulsifiers [11].

Their approach examined restrictions and current breakthroughs in this field. Implementation involved a literature review and self-emulsifying medication delivery system advances. The results showed better bioavailability and drug solubility in the gastrointestinal system, although formulation problems and patient factor variability were drawbacks.

Kazi et al. (2019) created folate-conjugated, 5-FU-loaded peptide-linked nanoparticles for tumor cell delivery [12]. Nanoparticle production and characterization were the proposed techniques. The implementation included in vitro and in vivo effectiveness tests. Selective tumor cell targeting and lower systemic toxicity were shown, although nanoparticle stability and clinical scale-up were problems. LNPs for hyperproliferative skin disorders were studied by Souto et al. (2021). The procedure involved developing and characterizing lipid nanocarriers [13]. Implementation included in vitro and in vivo performance studies. The results showed better medication penetration into the skin and therapeutic benefits. Stability issues were a drawback.

Panigrahi et al. (2021) used the double emulsion approach for PLGA-loaded nanoparticles to study pharmaceutical QbD. The proposed formulation optimization approach used QbD principles [14]. The implementation comprised experimental design and analysis. QbD's complexity and regulatory restrictions may be drawbacks. However, the outcome showed consistent and regulated nanoparticle manufacturing. According to Qushawy and Nasr (2020), solid lipid nanoparticles (SLNs) can deliver nano drugs. The procedure comprised SLN preparation and characterization. For application evaluation, in vitro and in vivo investigations were conducted. The findings showed enhanced medication stability and sustained release, although large-scale manufacturing and long-term stability were issues [15].

In cancer therapy, Ergin et al. (2023) suggested that 6-mercaptopurine-loaded solid LNP helps in reducing cytotoxicity [16]. The procedure comprised synthesizing, characterizing, and testing these nanoparticles in vitro and in vivo. The outcome showed enhanced medication distribution to hepatic cancer locations, perhaps lowering systemic adverse effects. However, nanoparticle manufacturing was complicated. QbD-guided early pharmaceutical development study by Amasya et al. (2019) produced lipid nanoparticles by high-pressure homogenization for skin cancer therapy [17]. The proposed approach optimized lipid nanoparticle manufacturing using QbD—implementation involved experimental design and analysis. The outcome revealed advantages in a well-defined and regulated production process but possible issues in implementing QbD principles for unique formulations.

Design and development of 5-FU-loaded lipid nanoparticles employing W/O/W DE and SE for skin cancer therapy is a vibrant field that offers intriguing alternatives to traditional therapeutic problems. This literature study gave insights into current research on this DDS's methods, benefits, and drawbacks. Encapsulating 5-FLLNP improves its stability, bioavailability, and skin cancer cell targeting. Many preclinical studies have shown promising findings, but translating them to clinical practice involves resolving scale-up issues, long-term stability, and comprehensive safety and effectiveness evaluations. However, these findings have great potential to improve skin cancer management, and more research and innovation are needed to develop effective 5-FLLNP to fight this common and often deadly disease.

DESIGNING 5-FLLNP USING W/O/W DE-SE

DE are called the "emulsions of emulsion" system, and their complicated heterodisperse nature characterizes them. There are two distinct emulsions: water in oil in water (W/O/W) emulsion and Oil in Water in Oil (O/W/O) emulsion. An emulsion can generally be described as a dispersed phase inside another continuous phase. However, the DE process consists of three distinct phases using two distinct emulsifiers or surfactants. Typically, the interior and exterior layers of the emulsion are composed of identical materials. William Seifriz first introduced the notion of DE-SE in 1925 and researched the influence of oil phase density on the characteristics of the resulting emulsion [18].

Nevertheless, the amount of water in the oil droplets would lead to their quick destabilization, resulting in a singular emulsion. Hence, these particular emulsions are called metastable or transitional

emulsions, exhibiting characteristics between water-in-oil (W/O) and oil-in-water (O/W) emulsions. The phenomenon of water mobility between the internal aqueous and continuous aqueous phases is attributed to the transient instability of DE-SE. The Osmotic Pressure (OP) is re-equilibrated by the solute difference between the dispersed water and the subsequent aqueous phases. The durability of DE mostly relies on the solute content or OP of the constituent phases. The elevated OP in the external phase induces the expansion of internal droplets within the emulsion, whereas reduced OP leads to the constriction of internal droplets.

In addition to OP arising from the disparity in solute concentration between the aqueous phases, Laplace pressure, influenced by droplet size and surface tension, similarly induces elevated pressure within internal droplets. This heightened pressure might potentially lead to premature formation and breakage of the DE-SE. DE-SE can be generated by either a one-step procedure or a two-step procedure. The procedure of emulsification, which occurs in a single step, leads to the spontaneous development of DE. This characteristic has garnered attention due to the procedure's inherent simplicity. Nevertheless, the possible restriction of this method lies in the inadequate trapping efficiency of medicines. The two-step emulsification procedure of DE-SE has been shown in Fig. 1.

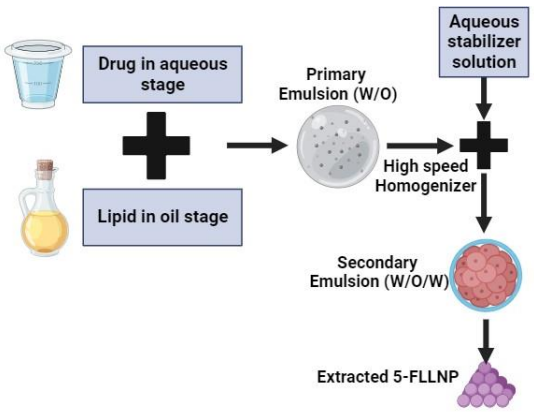

Fig.1. Designing 5-FLLNP using W/O/W DE-SE.

The initial phase involves the preparation of a W/O emulsion, commonly referred to as the main emulsion. The second stage involves the creation of a secondary emulsion, specifically a W/O/W emulsion, utilizing the W/O emulsion created in the initial step as the oil phase. Subsequently, it has been established that the two-step emulsification method has emerged as the prevailing approach. Numerous researchers have employed this technique to generate durable DE-SE. The process of preparing primary and secondary emulsions may be outlined as follows:

Step 1: Preparation of the main W/O emulsion

In the first step, the quantified quantity of a drug ingredient soluble in water is dissolved in an aqueous phase. On the other hand, pharmaceuticals that are soluble in lipids are dispersed in an appropriate organic solvent. Subsequently, the aqueous phase containing the medicine is introduced into the polymeric phase, composed of organic materials, while undergoing intense agitation, resulting in a W/O emulsion. The primary emulsion is typically formulated using high shear settings and a hydrophobic surfactant to generate stable and finely dispersed droplets of W/O emulsion.

Step 2: Preparation of Secondary Emulsification (W/O/W)

The initial W/O emulsion is introduced into a subsequent aqueous phase containing an appropriate emulsifier, such as Poly Vinyl Alcohol (PVA). This process is accompanied by constant stirring or homogenization, after which the organic solvent is eliminated by evaporation. In the second stage, a hydrophilic surfactant is employed unconventionally to enhance the stabilization of the exterior interface of the oil globules inside an O/W emulsion. The DE has the potential to undergo adsorption onto an appropriate adsorbent. Alternatively, 5-FLLNP can be separated using high-speed centrifugation or ultracentrifugation, followed by thorough washing. The second emulsification process is often conducted with little shear to prevent the disruption of primary or internal emulsion droplets.

Experiments

During the initial phase of QbD, it is essential to establish the Quality Target Product Profile (QTPP) by leveraging existing knowledge. Subsequently, it is imperative to identify the important quality features and process factors. The subsequent phase entails the development of the procedure and the delineation of the design

domain. The QbD stages outlined below were adhered to throughout the study.

Defining QTPP

As per the stipulations outlined in ICH Q8 (R2), the QTPP may be defined as a forward-looking synopsis of the quality attributes of a pharmaceutical product that is ideally to be attained to guarantee the intended quality while duly considering the safety and effectiveness of such product. The definition of qualitative targets encompasses aspects such as the expected therapeutic efficacy, quality, dosage form, stability, administration method, and appearance of the pharmacological product. The objective of this study was to produce a topical solution using 5-FLLNP.

To attain the QTPP, an assessment was conducted on literature research and data obtained from pre-formulation experiments. This evaluation facilitated the establishment of the Knowledge Space (KS) about the formulation components. The LNP formulations of 16 SLN and 16 NLC consist of components that fall inside the KS associated with inputs designed for the W/O/W DE-SE approach [17]. Within the realm of knowledge, various lipids, namely tripalmitin, and tristearin, were employed alongside lecithin as the co-lipid. Additionally, different ratios of liquid lipids were utilized to form NLCs, specifically Transcutol, at levels of 25% and 35%. Furthermore, different quantities of 5-FU were included, with amounts of 35 mg and 45 mg being employed. Lastly, distinct concentrations of PVA in the outer phase were employed, specifically 0.5% and 1%.

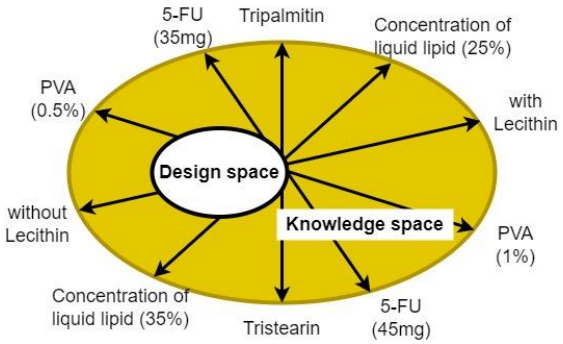

Fig.2. Designing 5-FLLNP using W/O/W DE-SE.

Fig. 2 depicts a schematic representation of the KS and DS. The top and bottom level boundaries of the KS for a given constraint and the lipids employed are defined by the two orientations of the arrows. A total of 32 formulae were generated within the domain of expertise.

Defining Critical Quality Features (CQF)

As per the definition provided by the ICH, a CQF refers to a feature or characteristic of a product that is of a physiological, biochemical, biotic, or bacteriological nature. This attribute needs to fall within a suitable limit, range, or distribution to guarantee the product's intended quality. The CQFs identified within the KS were the particle dimension, distribution of particle dimensions (polydispersity score), encapsulation effectiveness, and the quantity of 5-FLLNP released after 7 hours in in-vitro trials.

Assessment and the formation of the DS

The DS refers to the multidimensional amalgamation and interplay of input factors, such as material qualities and progression constraints, empirically shown to provide quality following the ICH Q8 (R2) guidelines. The DS encompasses the complex interrelationships between the QTPP and CQFs in a multivariate functional manner. The LNP formulations were evaluated in this work using INform V5.01. This software was used to analyze the data, establish the DS, and optimize formulation. The software mentioned above package is an ANN established by Intelligensys Ltd. The software in question possesses the ability to acquire knowledge from provided data. Upon implementation, it generates a DS and then facilitates the optimization of formulations by adjusting the quantities of components or altering process conditions within a specified range. During the program's deployment, the data about the LNP formulations created within the designated parameter range were sent to the ANN.

The back-propagation learning process was executed, including 5 inputs and outputs, as depicted in Table 1. One of the primary objectives of model development is to establish a tool that may be utilized for making predictions. Nevertheless, it is important to acknowledge the potential danger that the model may exhibit overfitting, where it becomes too focused on the training data and fails to generalize well to new, unseen data. This phenomenon might be likened to the model "memorizing" the training data rather than effectively

predicting outcomes at other periods. Validation is a crucial step in implementing neural network models, as it helps mitigate potential issues. To assess the model's validity, some data is deliberately excluded from the training phase and reserved as Test Data.

Tab.1. Parameters for ANN (Back-propagation).

Parameter	Value
Number of inputs, outputs	5, 5
Number of hidden layers	1
Number of nodes	1
Transfer function	Sigmoid
Output transfer function	Linear
Learning rate	0.75
Target Epochs	500

Subsequently, the provided inputs can be utilized for Test Data, enabling the prediction of corresponding attributes. By comparing the anticipated and empirically measured qualities, one may evaluate the level of predictability exhibited by the model. The training parameters for the model are provided in Table 1. Once the data had been transmitted to the program and the variables had been established, the training phase commenced, leading to the development of the neural network models. To optimize the training process and achieve the most effective neural network model, appropriate parameters were identified and implemented to facilitate the self-training of the program. The optimal model is defined as the one exhibiting the maximum level of predictability.

RESULTS AND DISCUSSION

The investigation of the morphological characteristics of 5-FLLNP formulations that are considered best was conducted using a Transmission Electron Microscope (TEM). 5-FLLNP have been suspended in distilled water and subsequently deposited onto copper grids. These grids were then inserted into the microscope and subjected to scanning at a voltage of 120 kV.

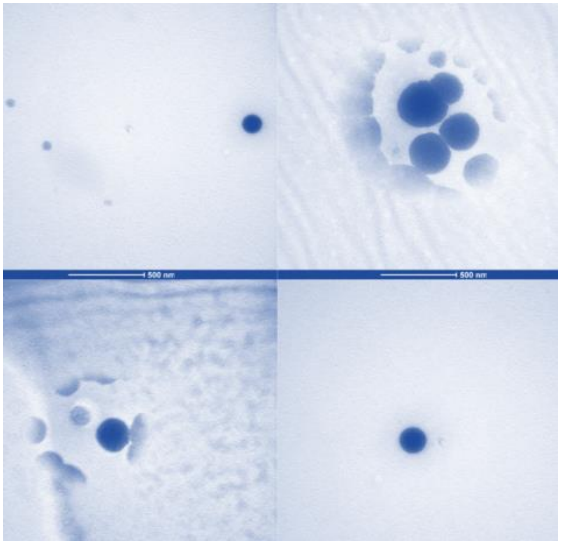

Fig.3. TEM image of 5-FLLNP.

Fig. 3 depicts the TEM image of 5-FLLNP. The 5-FLLNP exhibited a spherical morphology. The diameters of the LNPs seen by TEM are comparable to the sizes measured using the light-scattering analyzer.

The cytotoxic effects of an optimized 5-FLLNP were evaluated in vitro using A431 human epidermoid carcinoma cells. 5-FU, a potent anticancer drug, is a pyrimidine analog that functions as a chemotherapy medication and is categorized as an antimetabolite. Antimetabolites function as competing inhibitors of natural substances involved in the biosynthesis of cellular constituents. The principal method by which 5-FU exerts its effects is by suppressing DNA synthesis, specifically targeting the S phase of the cell cycle during mitotic division. The primary objective of cancer chemotherapy is to induce the death of tumor cells while minimizing harm to normal cells. To ascertain the appropriate dosages for administration, HaCaT cell lines were subjected to incubation with escalating concentrations (10-2 M, 10-3 M, 10-4 M) of pure 5-FU.

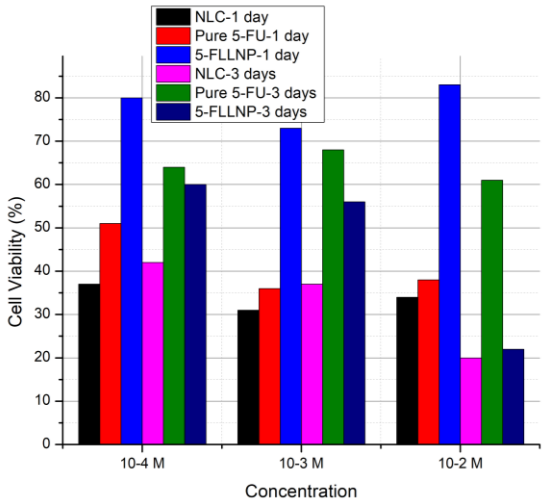

Fig.4. Cell viability (%) of A431 cells for varying concentrations of drugs.

Fig. 4 displays the percentages of cell viability in A431 cells after being subjected to different medication concentrations for diverse durations. At a concentration of 10-4 M, it was observed that NLC and 5-FLLNP exhibited significantly higher cell viability (37% and 80%, respectively) in comparison to pure 5-FU (51%) following a 24-hour exposure period. This suggests that these drug carriers possess improved cytocompatibility. Nevertheless, when the concentration was increased to 10-3 M, the vitality of the cells dropped across all formulations. Notably, the 5-FLLNP formulation exhibited a greater cell viability of 73% compared to the pure 5-FU formulation, which had a cell viability of 68%. Notably, at a concentration of 10-2 M, the cell viability saw a considerable decrease for all formulations within a 24-hour period. Among these formulations, 5-FLLNP exhibited the highest level of cytotoxicity, with a recorded cell viability of 22%. During the three-day duration of treatment, both NLC and 5-FLLNP demonstrated constant cell viability values, but the pure 5-FU indicated a more significant decline in cell viability. The findings of this study indicate that NLC and 5-FLLNP can increase drug delivery and maintain cytotoxic effects over an extended period. This suggests these formulations might be utilized for controlled drug release and improved therapeutic outcomes.

CONCLUSION

This research aims to combine the beneficial attributes of LNPs with the QbD methodology to develop a novel DDS for managing skin malignancies and actinic keratosis. LNPs, which have demonstrated notable effectiveness in the topical management of skin disorders, were developed to target the complex architecture of the skin and promote improved absorption into the skin. The current study applied the QbD approach to developing LNP formulations to improve the transdermal absorption of 5-FU, a frequently employed therapeutic drug for non-melanoma skin cancer. The 5-FLLNP were fabricated utilizing the DE-SE methodology. The DE-SE method comprises two separate emulsification phases, adding a greater degree of intricacy to the entire technique. The stability of nanoparticles generated via a DE-SE approach remains unknown due to the diverse formulations and process variables implicated. Currently, using a DE-SE technology in synthesizing 5-FLLNP presents itself as a feasible strategy for DDS, providing a pathway to get a product of superior quality by employing the QbD methodology. ANN has created an ideal LNP formulation that guarantees high quality and adheres to the specified design parameters. 5-FLLNP formulation revealed a better cell vitality of 73% than the pure 5-FU formulation, which had a cell viability of 68% for A431 cells.

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