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STRATEGIC DEVELOPMENT AND EVALUATION OF NANOSUSPENSION FORMULATIONS FOR IMPROVING ORAL BIOAVAILABILITY OF LOW-SOLUBILITY DRUGS

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Abstract: Diabetic wounds remain difficult to manage because persistent hyperglycaemia, oxidative stress, chronic inflammation, microbial burden and impaired angiogenesis act simultaneously to delay tissue repair. The present study developed a topical nanostructured lipid carrier (NLC) containing hesperetin (HES) and ferulic acid (FA), followed by incorporation into a pH- and temperature-responsive hydrogel. Excipients were selected on the basis of drug–lipid solubility and emulsification screening, and the NLC was optimized using a three-factor central composite design with lipid concentration, surfactant concentration and sonication time as independent variables. The optimized system used a beeswax:ethyl oleate lipid mixture, Tween 80:poloxamer 188 as the surfactant blend and 150 s sonication. The observed particle size was 271 nm with a PDI of 0.459, zeta potential of −37.1 mV, and entrapment efficiencies of 91.53 ± 0.32% for HES and 90.12 ± 0.56% for FA. Thermal, diffraction and spectroscopic studies supported incorporation of the actives into the lipid matrix without evidence of new chemical interactions. The FA:HES 8:2 combination produced synergistic DPPH scavenging with a combination index of 0.605 and an IC50 of 13.49 µg. NLC loading increased 24-h release to 86.49% for HES and 76.49% for FA compared with 32.53% and 20.63%, respectively, from drug suspension. The NLC showed approximately 3.5-fold higher skin permeation than the comparator, low hemolysis (2 ± 0.365%) and a low HET-CAM irritation score (0.51 ± 0.01). The dual-responsive hydrogel extended release to 48 h, and the formulation demonstrated antibacterial activity, HaCaT compatibility and 92.4 ± 4.9% scratch-wound contraction at 48 h. In the diabetic rat wound model, the optimized hydrogel produced 93.2 ± 3.4% wound closure by day 15 and was associated with increased VEGF and reduced ICAM-1 levels. Overall, the findings support the formulation as a promising multifunctional topical platform for diabetic wound management, although additional long-term safety, mechanistic and translational studies are required before clinical application.

Keywords - Diabetic wound healing, Hesperetin, Ferulic acid, Nanostructured lipid carrier, Dual-Responsive Hydrogel, Topical drug delivery.

I. INTRODUCTION

Diabetic foot ulceration is a chronic complication in which normal wound repair is disrupted by the combined effects of hyperglycaemia, peripheral neuropathy, vascular insufficiency, oxidative stress, persistent inflammation and susceptibility to microbial infection. Unlike an uncomplicated acute wound, the diabetic wound may remain in an inflammatory state, with impaired angiogenesis and extracellular-matrix remodelling. Recent work continues to identify oxidative stress, ferroptosis, impaired vascular responses and inflammatory dysregulation as important therapeutic targets. Hesperetin has recently been

shown to promote diabetic wound healing through a SIRT3-associated anti-ferroptotic mechanism, supporting the biological rationale for its inclusion in a multifunctional wound formulation [1].

Ferulic acid is another phenolic compound with a well-described antioxidant and wound-repair profile. Experimental work in streptozotocin-induced diabetic rats demonstrated faster epithelialisation together with improved hydroxyproline and hexosamine content and enhancement of endogenous antioxidant status after ferulic-acid treatment [2]. More recently, ferulic acid has also been incorporated into NLC systems for wound-healing applications, where lipid-based delivery improved the formulation properties and supported cell migration [3]. These observations suggest that combining two mechanistically complementary polyphenols may be more useful than relying on a single antioxidant.

Despite their biological promise, HES and FA have formulation limitations. Their limited aqueous solubility can restrict the amount available at the wound surface and may reduce reproducibility of topical dosing. NLCs are attractive in this context because they combine a solid lipid with a liquid lipid, allowing disruption of the highly ordered lipid matrix, increased loading capacity and controlled release. Current literature indicates that NLC-based topical systems can improve local retention, skin hydration, drug stability and sustained release, while NLC–gel systems can further increase residence time at the wound site [4–6].

The present work therefore used a two-stage delivery strategy: first, a HES–FA NLC was optimized by quality-by-design principles; second, the optimized NLC was incorporated into a pH- and temperature-responsive hydrogel composed of carboxymethyl chitosan and poloxamer 407. The study evaluated analytical performance, formulation optimization, physicochemical characteristics, drug release, antioxidant activity, skin permeation, preliminary biocompatibility, antibacterial activity, cellular wound contraction and wound healing in streptozotocin-induced diabetic rats. The study was designed to address several barriers of diabetic wound treatment within one topical system rather than treating oxidative stress, microbial burden and impaired tissue repair as isolated problems.

II. MATERIALS AND METHODS

MATERIALS

Hesperetin and ferulic acid were the active compounds. White beeswax and ethyl oleate were investigated as the solid and liquid lipid components, respectively. Tween 80 and poloxamer 188 were evaluated as surfactants. Carboxymethyl chitosan, poloxamer 407 and genipin were used in development of the responsive hydrogel. HPLC-grade methanol and other analytical-grade materials were used for assay and characterization. The work employed UV–visible spectroscopy, HPLC, particle-size analysis, FTIR, DSC, PXRD, TEM, SEM, CLSM, ELISA and related laboratory instrumentation.

PREFORMULATION AND ANALYTICAL METHODS

Organoleptic properties, aqueous and methanolic solubility, melting behaviour and partition coefficient were assessed before formulation. HES and FA showed poor aqueous solubility, with measured water solubilities of 0.463 ± 0.12 and 0.12 ± 0.14 mg/mL, respectively. The measured logP values were 2.60 for HES and 1.63 for FA. DSC confirmed melting events at approximately 253.99 °C for HES and 175.05 °C for FA. UV spectra showed analytical maxima at 289 nm for HES and 312 nm for FA.

Separate UV methods were validated in methanol and phosphate-buffered saline (pH 7.4). Linearity, precision, accuracy, robustness, limit of detection and limit of quantification were assessed. A Vierordt simultaneous-equation method was also established at 289 and 312 nm. For chromatographic confirmation and quantitative assay, an autosampling Shimadzu Prominence-i LC-2030C plus HPLC with a C18 column (150 × 4.6 mm, 5 µm) and PDA detection was used. Isocratic methanol:water (30:70, v/v), 1 mL/min flow, 25 °C and 295 nm detection were employed over a 10-min run. HES and FA eluted at approximately 3.7 and 4.75 min, respectively. The method showed R^2 values of 0.9971 and 0.9984, recoveries of 99.6% and 99.22%, and precision values of 1.19% and 1.28% RSD for HES and FA. The validation approach was consistent with the analytical-validation principles described in ICH Q2(R1) [7].

EXCIPIENTS SCREENING AND NLC PREPARATION

Liquid and solid lipids were screened according to their ability to solubilize HES and FA. Ethyl oleate provided the highest measured solubility among the liquid lipids tested, 14.4 ± 0.37 mg/mL for HES and 11.5 ± 0.24 mg/mL for FA. White beeswax provided the highest solid-lipid solubilization, 9.7 ± 0.53 mg/g for HES and 7.1 ± 0.61 mg/g for FA, and was miscible with ethyl oleate. Surfactant screening identified Tween 80 plus poloxamer 188 at a 1:1 ratio as the most effective emulsifier combination, with 92.16 ± 1.8% transmittance in the screening experiment.

The NLCs were prepared by melt emulsification followed by probe ultrasonication. The lipid phase contained a beeswax:ethyl oleate combination and the actives were incorporated while the lipid phase was maintained at 80 °C. The aqueous surfactant phase containing Tween 80 and poloxamer 188 was also heated to 80 °C and added under high-speed stirring. The resulting pre-emulsion was sonicated using a probe sonicator at 80% amplitude for three cycles. The optimization design varied lipid concentration, surfactant concentration and sonication time while maintaining the HES and FA loading and surfactant ratio constant.

DESIGN OF EXPERIMENTS AND OPTIMIZATION

A three-factor central composite design generated 20 experimental runs comprising factorial, axial and centre points. Lipid concentration (X1), surfactant concentration (X2) and sonication time (X3) were the independent variables. Particle size (Y1), PDI (Y2), HES entrapment efficiency (Y3) and FA entrapment efficiency (Y4) were the responses. The formulation target was particle size below 300 nm, minimum PDI and maximum entrapment efficiencies. Quadratic models were fitted and assessed using ANOVA, lack-of-fit testing, coefficient of determination, adjusted and predicted R^2 values, and adequate precision.

NLC CHARACTERIZATION

The optimized NLC was characterized for particle size, PDI, zeta potential, morphology, entrapment efficiency, thermal behaviour, crystallinity and chemical compatibility. Drug release was assessed in PBS (pH 7.4) and compared with a drug suspension. Release data were fitted to zero-order, first-order, Higuchi and Korsmeyer–Peppas models. DPPH radical-scavenging activity was used to evaluate the antioxidant effect of individual compounds, combinations and the NLC. The combination index was interpreted to assess synergy.

DUAL-RESPONSIVE HYDROGEL

A pH- and temperature-responsive hydrogel was prepared using carboxymethyl chitosan and poloxamer 407 and loaded with the optimized HES–FA NLC using a swelling-loading approach. The hydrogel was examined by SEM, FTIR and PXRD. Swelling behaviour was investigated at different pH and temperature conditions, with particular attention to pH 7.4 and 37 °C. In vitro release from the hydrogel was followed for 48 h. Antibacterial activity, HaCaT cell compatibility, scratch-wound contraction, skin permeation, hemolysis, HET-CAM irritation and wound-healing efficacy were evaluated as reported in the thesis.

IN VIVO WOUND-HEALING EVALUATION

The thesis reports evaluation in streptozotocin-induced diabetic rats using a wound-healing model. Wound closure, histopathology and VEGF and ICAM-1 responses were evaluated. Histological findings were compared among untreated diabetic wounds and formulation-treated groups. Because the supplied thesis text does not provide a complete, publication-ready description of animal number, allocation, randomisation, wound dimensions, dosing frequency and animal-ethics approval number, these details have deliberately not been invented in this manuscript. They should be inserted from the original laboratory/animal-house records before submission to a journal.

III. RESULTS AND DISCUSSION

PREFORMULATION AND ANALYTICAL PERFORMANCE

The preformulation findings supported selection of a lipid-based carrier. HES and FA were poorly soluble in water and showed moderate lipophilicity, consistent with the need for a formulation capable of maintaining both compounds at the wound surface. Thermal and diffraction studies confirmed that the starting materials were crystalline, providing a useful baseline for subsequent assessment of drug incorporation into the NLC matrix.

The analytical methods showed acceptable performance across the studied ranges. In methanol, the UV methods produced R^2 values of 0.9999 for HES and 0.9992 for FA, with mean recoveries of 99.68% and 99.59%, respectively. Intraday and interday precision remained below 2% RSD. The simultaneous Vierordt method produced $R^2 = 0.9995$ for both compounds and mean recoveries of 98.77% for HES and 98.09% for FA. These results support the suitability of the analytical procedures for formulation testing. The HPLC method provided additional selectivity and confirmed that both analytes could be quantified in the presence of the NLC matrix.

Table 1. Summary of analytical performance for HES and FA.

Parameter	HES	FA
UV linearity range in methanol	1–10 µg/mL	1–10 µg/mL
R^2 (methanol)	0.9999	0.9992
Mean recovery	99.68%	99.59%
LOD	0.90 µg/mL	1.03 µg/mL
LOQ	2.90 µg/mL	3.20 µg/mL
HPLC R^2	0.9971	0.9984
HPLC recovery	99.60%	99.22%
HPLC precision	1.19% RSD	1.28% RSD

EXCIPIENTS AND OPTIMIZATION

The excipient-screening stage provided a rational basis for the NLC composition. Ethyl oleate was selected as the liquid lipid because it showed the highest measured solubilization of both actives, whereas beeswax provided the greatest solid-lipid solubilization. The Tween 80–poloxamer 188 combination produced substantially higher emulsification performance than the individual surfactants. This selection is consistent with the general formulation rationale for NLCs, where the combination of solid and liquid lipids can create a less ordered matrix and accommodate lipophilic compounds [4,5].

Across the 20 CCD runs, particle size ranged from 242 to 385 nm, PDI from 0.287 to 0.500, HES entrapment from 78.12% to 92.71% and FA entrapment from 79.16% to 91.12%. The quadratic models were significant for all four responses ($p < 0.0001$), while lack-of-fit was not significant. The model R^2 values were 0.9982 for particle size, 0.9777 for PDI, 0.9613 for HES entrapment and 0.9887 for FA entrapment. The predicted R^2 values were also acceptable, indicating that the models were useful for navigating the experimental design space.

Numerical optimization targeted low particle size and PDI together with high entrapment. The predicted optimum comprised 3.84 mg lipid mixture, 5 mg surfactant blend and 150 s sonication. The model predicted 268.8 nm particle size, 0.442 PDI, 89.9% HES entrapment and 89.4% FA entrapment. Experimental confirmation gave 271 nm, 0.459, 91.53% and 90.12%, respectively. The close agreement between predicted and observed responses, with prediction errors below 5%, provides a strong formulation-development argument. The desirability value of 0.90 further indicates that the selected composition satisfied the predefined response targets.

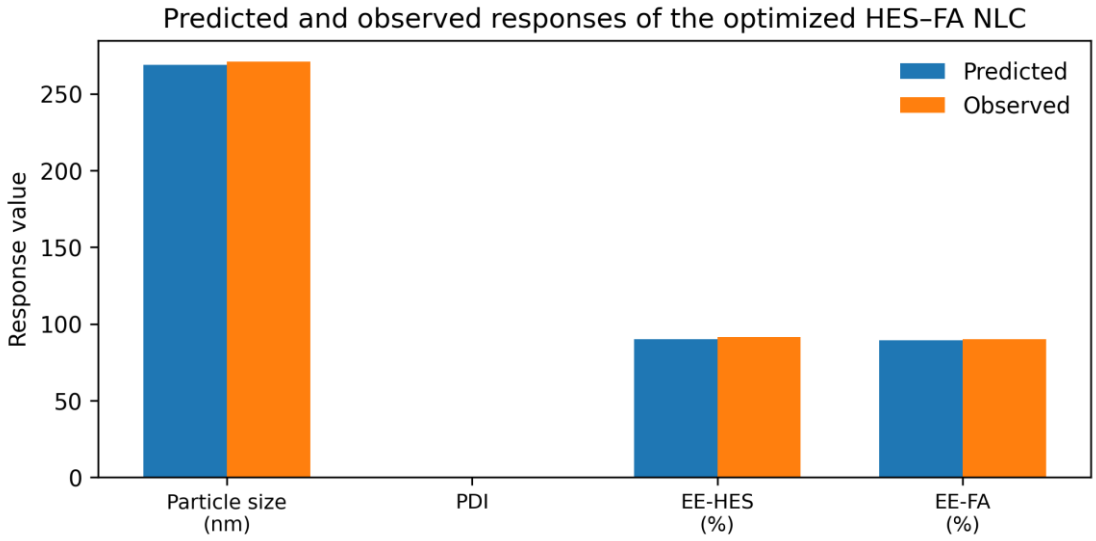

Figure 1. Predicted and observed responses for the optimized HES–FA NLC.

Table 2. Regression performance and confirmation of the optimized NLC.

Response	Model R^2	Adjusted R^2	Predicted R^2	Observed optimum
Particle size	0.9982	0.9984	0.9948	271 nm
PDI	0.9777	0.9577	0.8397	0.459
HES EE	0.9613	0.9265	0.8179	91.53%
FA EE	0.9887	0.9786	0.9420	90.12%

PHYSICOCHEMICAL CHARACTERISTICS OF THE OPTIMIZED NLC

The optimized NLC had a mean hydrodynamic diameter of 271 nm and a zeta potential of −37.1 mV. TEM and SEM showed nearly spherical, smooth and well-separated particles, with TEM giving an individual particle size of approximately 275 nm. The close agreement between dynamic light scattering and microscopy supports the reliability of the nanoscale size measurement. The relatively high PDI of 0.459, however, indicates a broader distribution than would normally be preferred for a highly uniform colloidal system. The dissertation itself recognises this limitation and suggests that incorporation into the hydrogel may improve practical stability.

Entrapment efficiencies were high, reaching 91.53 ± 0.32% for HES and 90.12 ± 0.56% for FA. The high loading is consistent with the measured solubility of both compounds in the selected lipid mixture. DSC showed disappearance of the characteristic drug and beeswax thermal events after NLC formation, while PXRD demonstrated a marked reduction in sharp crystalline reflections. FTIR spectra retained the characteristic drug-associated bands without new peaks or major shifts. Together, these observations support physical incorporation and partial amorphization of HES and FA within the lipid matrix rather than formation of a new chemical entity.

Table 3. Physicochemical profile of the optimized HES–FA NLC.

Characteristic	Observed result	Interpretation
Particle size	271 nm	Nanoscale carrier suitable for topical delivery
PDI	0.459	Broad distribution; requires attention during scale-up
Zeta potential	−37.1 mV	Supports colloidal electrostatic stability
HES entrapment	91.53 ± 0.32%	High drug incorporation
FA entrapment	90.12 ± 0.56%	High drug incorporation
TEM size	≈275 nm	Consistent with particle-size analysis
Morphology	Smooth, near-spherical	Favourable colloidal morphology

DRUG RELEASE AND RELEASE KINETICS

The NLC produced a biphasic release profile. More than 45% of both compounds was released during the first four hours, followed by a slower phase through 24 h. At 24 h, cumulative release reached 86.49% for HES and 76.49% for FA, compared with 32.53% and 20.63% from the corresponding drug suspension. Thus, the NLC increased the 24-h release approximately 2.66-fold for HES and 3.71-fold for FA relative to suspension. The initial phase can reasonably be attributed to surface-associated or more readily accessible drug, while the later phase reflects diffusion from the lipid matrix.

Among the kinetic models, the Higuchi and Korsmeyer–Peppas models provided the strongest fits. For NLC release, Korsmeyer–Peppas R^2 values were 0.961 for HES and 0.959 for FA, with reported release exponents of 0.350 and 0.380. These values are consistent with a diffusion-dominant mechanism under the model assumptions. The result is important because it indicates that the NLC can provide a rapid initial availability followed by continued release rather than an abrupt loss of the active compounds.

ANTIOXIDANT SYNERGY

The antioxidant experiments provided one of the clearest formulation-selection findings. HES alone showed an IC50 of 1209 µg, whereas FA showed an IC50 of 22.27 µg. The FA:HES 8:2 combination reduced the IC50 to 13.49 µg and produced a combination index of 0.605, indicating synergism under the applied DPPH model. At 200 µg/mL, the 8:2 solution and HES–FA NLC produced 88.3% and 90.1% DPPH inhibition, respectively. Importantly, the NLC did not significantly diminish the intrinsic antioxidant response, supporting the choice of the combined actives for subsequent formulation.

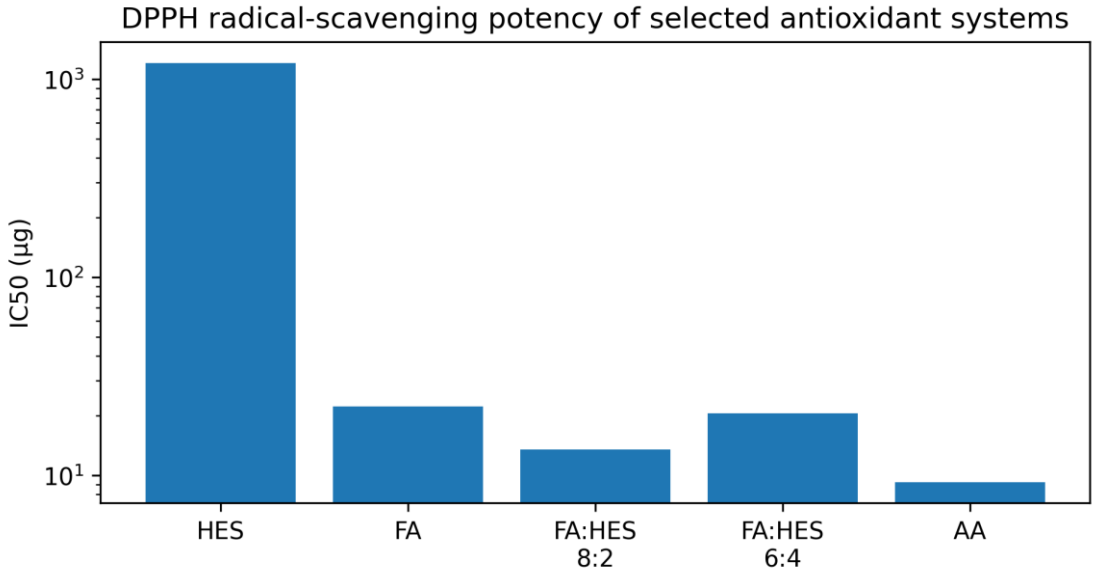

Figure 2. DPPH IC50 values of selected HES–FA combinations and ascorbic acid.

F. DUAL-RESPONSIVE HYDROGEL BEHAVIOUR

The hydrogel displayed the intended stimulus-responsive behaviour. Swelling increased at pH 7.4 because ionisation of carboxyl groups in carboxymethyl chitosan increased electrostatic repulsion and water uptake. Temperature also influenced the system, with poloxamer 407 undergoing micellar organisation and gelation around physiological temperature. This behaviour is particularly relevant to topical application because a formulation can be handled during administration and then form a more structured depot under wound conditions. Recent work independently supports the utility of poloxamer 407/chitosan thermosensitive systems for diabetic wound dressings [8].

Loading the NLC into the hydrogel changed the release profile. During 48 h, the hydrogel released 72.25% of HES and 66.31% of FA. Although the percentage released was lower than from the NLC at 24 h, the time extension is a desirable formulation feature for wound care because the three-dimensional polymer network acts as an additional diffusion barrier. The reported kinetic analysis identified Higuchi and Korsmeyer–Peppas behaviour as the best-fitting descriptions, with diffusion as the dominant contribution and possible effects from polymer relaxation, swelling and limited erosion.

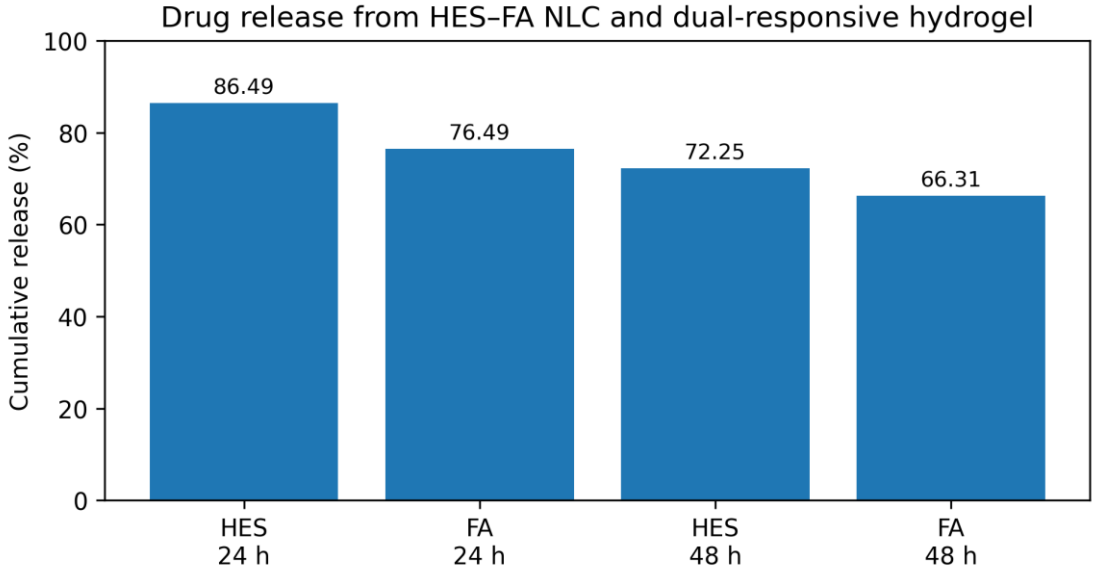

Figure 3. Endpoint cumulative release reported for HES–FA NLC and the dual-responsive hydrogel.

Table 4. Comparative release performance of the developed systems.

System	HES release	FA release	Observation
Drug suspension, 24 h	32.53%	20.63%	Low release
HES–FA NLC, 24 h	86.49%	76.49%	Higher and biphasic release
HES–FA NLC hydrogel, 48 h	72.25%	66.31%	Extended, controlled release

G. Permeation and preliminary biocompatibility

The dissertation reports approximately 3.5-fold greater skin permeation for the NLC system than the comparator in the CLSM experiment. This finding is mechanistically plausible because nanosized lipid carriers can improve contact with the skin and may facilitate local partitioning of lipophilic compounds. Nevertheless, the result should be interpreted as a skin-permeation finding rather than as proof of systemic bioavailability. The formulation also showed low hemolysis (2 ± 0.365%) and an HET-CAM irritation score of 0.51 ± 0.01, which were interpreted in the thesis as evidence of preliminary biocompatibility and low irritation.

Antibacterial and cellular wound-healing response

The HES–FA NLC hydrogel inhibited Bacillus subtilis and Escherichia coli more strongly than the NLC and control groups, with reported statistical significance ($p < 0.05$). This multifunctional behaviour is relevant to diabetic wounds, where bacterial colonisation can maintain inflammatory signalling and interfere with tissue repair. HES and FA both possess reported antioxidant and antimicrobial activities, while the chitosan-derived hydrogel matrix may additionally contribute to antimicrobial performance [2,3,9].

In HaCaT cell experiments, the hydrogel was reported to be compatible with the cell model and produced 92.4 ± 4.9% wound contraction after 48 h in the scratch assay. This result provides a useful bridge between physicochemical performance and biological response: the formulation not only retained the active compounds but also supported a cellular environment favourable to closure of a model wound. The finding is consistent with the broader literature showing that polyphenol-loaded lipid systems can promote cell migration and proliferation [3].

In vivo wound healing and histopathology

The optimized HES–FA NLC hydrogel produced 93.2 ± 3.4% wound closure by day 15 in the streptozotocin-induced diabetic rat model, with a reported $p < 0.001$ association with increased VEGF and reduced ICAM-1. Histologically, untreated diabetic wounds showed persistent inflammation, oedema, disorganised extracellular matrix and poor fibroblast proliferation. Formulation-treated wounds showed greater epithelialisation, more organised fibroblasts and collagen, increased angiogenesis and a more mature epidermal architecture. The hydrogel-treated group was described as approaching normal skin architecture, with keratinised epidermis, organised collagen bundles, hair follicles and minimal inflammatory-cell infiltration.

Taken together, these findings suggest that the formulation acts through several complementary mechanisms: antioxidant protection, local antimicrobial activity, sustained topical exposure, enhanced tissue penetration, support of cell migration and a pro-angiogenic response. This multi-target approach is consistent with current understanding of diabetic wound pathophysiology and with recent reviews emphasising that nano-delivery systems can address oxidative stress, inflammation, infection and impaired angiogenesis simultaneously [4–6,10]. The present study is therefore stronger as a multifunctional topical platform than as a single-drug wound-healing product.

Critical appraisal of the thesis data

Several strengths increase the manuscript's publication potential. First, the study follows a rational formulation-development sequence from preformulation to excipient screening, statistical optimization, physicochemical characterization and biological evaluation. Second, the CCD models are statistically strong, with high R^2 values and non-significant lack-of-fit. Third, the study compares the NLC with a drug suspension and subsequently incorporates the optimized NLC into a responsive hydrogel, allowing the contribution of each delivery stage to be discussed. Fourth, the biological package includes antioxidant, permeation, irritation, antibacterial, cellular and in vivo end points rather than relying on a single assay.

At the same time, several points should be corrected before submission to a high-selectivity journal. The dissertation cover title refers to nanosuspension formulations and oral bioavailability, whereas the experimental chapters actually describe HES–FA NLCs and a topical dual-responsive hydrogel for diabetic wounds. The manuscript title has therefore been aligned with the actual experimental work. The reported PDI of 0.459 is relatively broad and should be presented as a limitation rather than described simply as satisfactory. The term 'bioavailability' should be avoided unless a pharmacokinetic study demonstrates systemic exposure; the present evidence supports local topical delivery and skin permeation. Finally, animal-study reporting must be completed with the original ethical approval, animal numbers, randomisation, wound dimensions, dosing schedule and statistical details before journal submission.

IV. CONCLUSION

A HES–FA nanostructured lipid carrier was successfully developed using a quality-by-design approach and subsequently incorporated into a pH- and temperature-responsive hydrogel. The optimized NLC had a particle size of 271 nm, PDI of 0.459, zeta potential of −37.1 mV and high entrapment efficiencies for both HES and FA. The formulation converted the poorly water-soluble actives into a nanoscale lipid system with improved release and skin permeation characteristics. The FA:HES 8:2 combination showed synergistic antioxidant behaviour, while the hydrogel provided prolonged release and stimulus-responsive swelling. Preliminary biocompatibility, antibacterial, cellular and in vivo results further supported the formulation's multifunctional wound-healing potential. The 93.2 ± 3.4% wound closure reported at day 15, together with increased VEGF, reduced ICAM-1 and improved histological organisation, provides a coherent preclinical signal. However, the formulation should be considered a promising research-stage topical system rather than a clinically established treatment. Further work should address long-term stability, scale-up, detailed toxicology, complete animal-study reporting, mechanistic biomarkers and translational efficacy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL AND REPORTING NOTE

The supplied dissertation reports an in vivo streptozotocin-induced diabetic rat study but does not provide an animal-ethics approval identifier in the accessible manuscript text. The final journal version should include the applicable Institutional Animal Ethics Committee approval number and the complete animal-reporting information before submission. This information has not been fabricated.

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