

Hpv E6/E7 Silencing and Ferroptosis Modulation in Cervical Cancer: in-Vitro Evaluation Of a Biomimetic Co-Delivery Platform

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ABSTRACT

The simultaneous delivery of a nucleic-acid therapeutic and a small-molecule ferroptosis modulator presents a distinct pharmaceuticals challenge because the two cargos differ in size, charge, hydrophilicity and loading requirements. HPV E6/E7-siRNA requires protection from degradation and efficient intracellular availability, whereas RSL3 is incorporated within the polymeric carrier to provide a formulation-compatible nanoscale delivery approach.

Objective: To develop and optimize a biomimetic nanocarrier for co-delivery of HPV E6/E7-siRNA and RSL3 using a Quality by Design (QbD) and Design of Experiments (DoE) framework, and to establish the physicochemical and functional performance of the optimized formulation.

Methods: A QbD strategy comprising QTPP definition, CQA identification, formulation/process risk consideration and an eight-run, three-factor DoE was applied. The formulation consisted of a PLGA-based nanoparticle core containing RSL3, E6/E7-siRNA associated with the nanoparticle, stabilizing components and a biomimetic membrane coating. The optimized formulation was evaluated for particle size, PDI, zeta potential, siRNA association, RSL3 loading, morphology and cellular delivery, followed by functional assessment of E6/E7 silencing and ferroptosis-associated responses.

Results: The optimized biomimetic nanocarrier exhibited a particle size of 108 ± 6 nm, PDI of 0.19 ± 0.02 , zeta potential of $+17.7 \pm 1.7$ mV, E6/E7-siRNA association of $85.4 \pm 2.6\%$, RSL3 loading of $77.8 \pm 2.4\%$ and pH of 8.3 ± 0.1 . The formulation showed enhanced intracellular delivery compared with free cargo, reduced E6/E7 expression and produced a co-loaded formulation-associated increase in lipid ROS with reduction in GSH/GSSG and changes in GPX4, SLC7A11 and ACSL4.

Conclusion: The study establishes a pharmaceuticals-oriented QbD pathway for development of an E6/E7-siRNA–RSL3 co-loaded biomimetic nanocarrier. The formulation-development sequence, nanoscale characteristics, cargo association/loading and functional delivery provide a basis for further formulation refinement and biological validation.

Keywords: Biomimetic nanocarrier; E6/E7-siRNA; RSL3; polymeric nanoparticle; co-delivery; Quality by Design; Design of Experiments; formulation development; critical quality attributes; pharmaceuticals.

INTRODUCTION

Cervical cancer, a global health issue, affects uterine cervix epithelial cells. cervical cancer still causes a large amount of cancer morbidity and death in women, despite breakthroughs in prevention, screening, and therapy. The problem is especially important in places with limited HPV vaccine, cervical screening, early detection, and treatment. The World Health Organization (WHO) predicted 604,000 new cervical cancer cases and 280,000 deaths in 2024, underscoring its public-health importance. Cervical cancer is unique in that its primary cause is known. Most cervical cancers are caused by persistent HPV infection with oncogenic or high-risk strains. Most HPV infections are transiently cleared by the host immune system..

Chronic carcinogenic HPV infection can cause cellular abnormalities, cervical precancerous lesions, and invasive malignancy. The path from chronic HPV infection to cancer involves viral persistence, host-cell growth control dysregulation, genomic instability, and other cellular alterations. Thus, high-risk HPV biological activity is closely linked to cervical cancer. Cervical cancer is most often caused by HPV16 and HPV18, which account for a substantial percentage of cervical cancers worldwide. High-risk HPV is carcinogenic because its early proteins E6 and E7 disrupt basic cellular mechanisms that control proliferation, genomic integrity, and cell death. A promising molecular target for HPV treatment is the ongoing expression of carcinogenic proteins. Surgery, radiotherapy, and chemotherapy are still the mainstays of cervical cancer treatment, but treatment-associated toxicity, disease recurrence, treatment resistance, and advanced disease complexity drive the search for more selective therapies. Thus, genetic anomalies linked with HPV and cellular mechanisms that encourage malignancy have garnered scientific attention. One strategy is RNA interference (RNAi) to silence disease-associated genes. Sequence-specific siRNA can silence a target gene. Since viral oncogenes E6 and E7 maintain the malignant phenotype, addressing them may be an interesting technique in HPV-related cancer. The therapeutic use of siRNA depends on its protection, transport, cellular absorption, and intracellular release. Thus, nanotechnology-based delivery methods may circumvent biological and physicochemical restrictions. Ferroptosis, a controlled cell death based on iron and lipid peroxidation, has grown in popularity alongside molecular gene silencing. Ferroptosis is linked to iron, lipid, and antioxidant defenses, unlike apoptosis. Since ferroptosis may kill malignant cells that resist other cell death methods, it may offer a new cancer therapy approach. But cancer cells can evolve or develop ferroptosis-resistant mechanisms. Ferroptosis resistance is connected to changes in glutathione metabolism, the system Xc-pathway, GPX4, NRF2-mediated antioxidant responses, lipid metabolism, and iron control. In some tumors, triggering ferroptosis may not be enough, thus methods to target biological aspects that contribute to tumor survival and ferroptosis resistance may be needed. This therapeutic concept contextualizes the study. HPV E6/E7 gene suppression by E6/E7-specific siRNA and ferroptosis-modulating chemical in a biomimetic nanocarrier is suggested. Suppressing the HPV E6/E7 oncogenic signal may alter cellular survival pathways and make HPV-associated CC cells more ferroptosis-sensitive. The proposed biomimetic nanocarrier would transport the two therapeutic components simultaneously with better cellular delivery. Thus, the intended investigation will determine if E6/E7 silencing and ferroptosis regulation affect cellular survival, gene expression, oxidative/lipid peroxidation responses, and other ferroptosis endpoints.

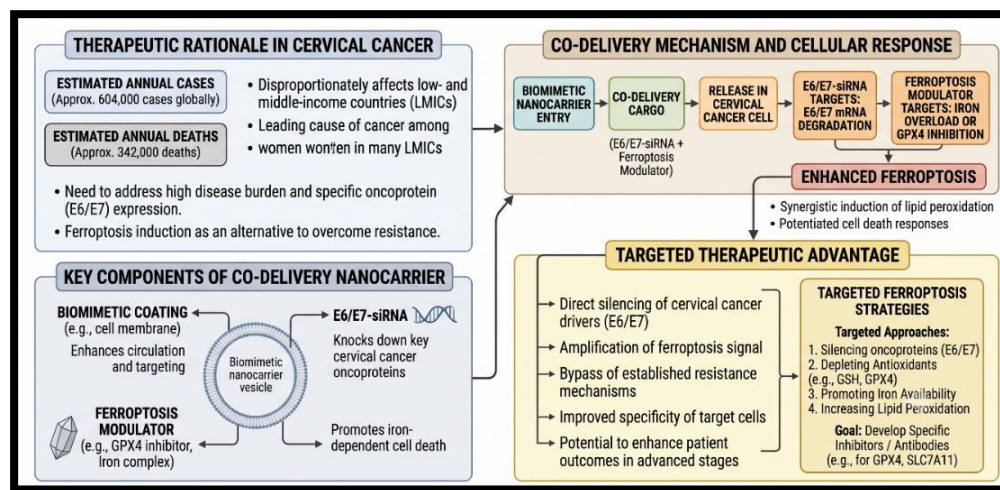


Figure 1. Schematic representation of biomimetic nanocarrier-mediated co-delivery of E6/E7-siRNA and a ferroptosis modulator for enhanced ferroptosis-associated responses in cervical cancer cells.

1.2 Global Cervical Cancer Burden and Clinical Importance

Cervical cancer, which has good primary and secondary prevention techniques, yet causes a lot of mortality and illness. Several low- and middle-income countries are worst hit by the current sickness load. Not only is HPV epidemiology different, but so is vaccination coverage, screening availability, diagnostic infrastructure, and access to effective therapy.

Cervical cancer is preventable and curable with proper screening, early identification, and treatment, according to WHO. HPV vaccination and screening can detect precancerous cervical anomalies before invasive illness. But global implementation of these prophylactic measures is uneven. Cervical cancer clinical progression involves several biological and host factors. Persistent infection with a high-risk HPV is the primary starting point, but additional cellular changes and interactions between viral factors, host signaling pathways, immunological responses, and the tumor microenvironment are also involved. After prolonged viral oncogene activity and host-cell biology dysregulation, cervical cancer develops. Standard therapies to complement preventive and therapeutic measures are needed due to cervical cancer's continuing

prevalence. In instance, molecular therapeutics targeting HPV-associated oncogenic pathways may potentially disrupt HPV-positive malignant cell biology.

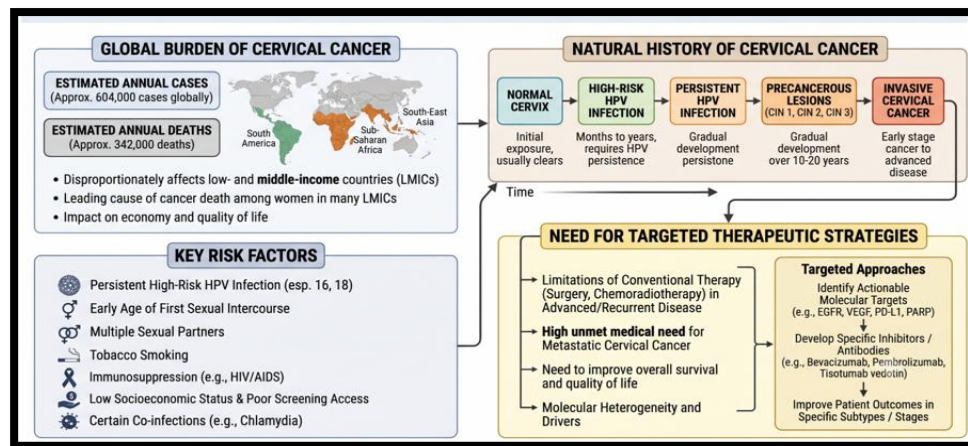


Figure 2. Overview of cervical cancer burden, HPV infection progression, and the need for targeted molecular therapies.

1.2 High-Risk Human Papillomavirus and Cervical Carcinogenesis

Human papillomaviruses are small, non-enveloped DNA viruses with a circular double-stranded DNA genome. HPV types can broadly be classified according to their association with benign lesions or malignant disease. High-risk HPV types are particularly important in cervical carcinogenesis because persistent infection can promote molecular changes that favour malignant transformation. Among the high-risk HPV types, HPV16 and HPV18 are particularly relevant to cervical cancer. Following infection of cervical epithelial cells, viral gene expression is regulated according to the differentiation status of the host cell. Under conditions of persistent infection and deregulated viral gene expression, continued expression of E6 and E7 can disrupt normal cellular homeostasis.

The biological sequence can be summarized as:

High-risk HPV infection
 ↓
 Persistent viral infection
 ↓
 Deregulated E6/E7 expression
 ↓
 Disruption of host regulatory pathways
 ↓
 Abnormal cell-cycle progression
 ↓
 Impaired cellular checkpoints
 ↓
 Accumulation of cellular abnormalities
 ↓
 Cervical carcinogenesis

1.3 HPV E6 Oncoprotein

The HPV E6 protein is an important viral oncoprotein involved in alteration of cellular tumor-suppressor pathways. One of the best characterized effects of high-risk HPV E6 is its interaction with the p53-associated regulatory system. The p53 pathway normally contributes to cellular responses to DNA damage and other forms of cellular stress. Depending on the nature and extent of cellular damage, p53-associated signaling can promote cell-cycle arrest, DNA repair, senescence or apoptosis. Persistent HPV E6 activity interferes with this protective regulatory system and thereby facilitates survival and proliferation of genetically abnormal cells.

The consequences of E6-associated pathway disruption may include:

impaired DNA-damage responses;
 reduced apoptotic signaling;

altered cell-cycle regulation;
increased cellular survival;
genomic instability;
altered differentiation;
maintenance of the transformed phenotype.

Thus, E6 contributes to the ability of HPV-positive cells to survive under conditions that would normally activate protective cellular responses.

1.4 HPV E7 Oncoprotein

The E7 oncoprotein acts through mechanisms that are complementary to those of E6. One of the principal targets of E7 is the retinoblastoma protein (pRB)-associated cell-cycle regulatory system. The pRB pathway normally contributes to control of E2F-dependent transcription and progression through the cell cycle. Interaction of E7 with pRB-associated regulatory mechanisms promotes inappropriate E2F activity and facilitates progression through the cell cycle. Importantly, the biological effects of E6 and E7 extend beyond cell-cycle regulation. Increasing evidence indicates that HPV oncogene expression can influence cellular metabolism, oxidative stress responses, differentiation, stemness-associated characteristics and susceptibility to different forms of regulated cell death.

These additional effects form the basis for investigating E6/E7 in relation to ferroptosis and cancer stemness.

1.5 E6/E7 and Maintenance of the Malignant Phenotype

The continued presence of E6/E7 within HPV-positive cervical cancer cells can be considered an important component of the molecular environment supporting malignant behavior.

E6/E7-associated alterations may influence:

proliferation;
apoptosis;
DNA damage responses;
cellular differentiation;
epithelial–mesenchymal characteristics;
metabolic adaptation;
oxidative stress responses;
cancer stemness;
cellular survival under therapeutic stress.

Therefore, suppression of E6/E7 may not simply reduce viral gene expression; it may alter several downstream properties of the cancer cell. This provides the rationale for investigating E6/E7 silencing as an upstream intervention capable of modifying both the stemness-associated phenotype and ferroptosis-associated cellular responses.

1.6 E6/E7 Silencing as a Molecular Therapeutic Strategy

The dependence of HPV-associated cancer cells on E6/E7 expression provides a rationale for sequence-specific suppression of viral oncogenes. RNA interference (RNAi) is a gene-silencing mechanism in which small interfering RNA molecules recognize complementary target RNA sequences and promote sequence-specific suppression of gene expression. E6/E7-specific siRNA can therefore be investigated as a molecular tool for reducing expression of HPV oncogenes. However, the therapeutic potential of siRNA depends not only on sequence specificity but also on successful delivery into the target cell.

1.7 Challenges Associated with SiRNA Delivery

Although siRNA provides a powerful mechanism for sequence-specific gene silencing, its biological application is associated with several challenges.

1.7.1 Instability

Naked siRNA can undergo degradation by extracellular and intracellular nucleases.

1.7.2 Poor cellular entry

The physicochemical properties of siRNA limit passive diffusion across the plasma membrane.

1.7.3 Endosomal entrapment

Even when siRNA is internalized, a proportion may remain trapped within endosomal compartments and fail to reach the cytoplasm.

1.7.4 Limited intracellular availability

Functional gene silencing requires adequate intracellular availability of intact siRNA.

1.7.5 Delivery-related toxicity

An effective carrier should improve delivery without producing unacceptable carrier-associated cytotoxicity. These challenges make the development of an appropriate delivery system an important component of siRNA-based therapeutic research. Specifically places nanocarrier development after E6/E7-siRNA investigation for this reason.

1.8 Nanotechnology for siRNA Delivery

Nanotechnology-based delivery systems have emerged as potential platforms for improving nucleic-acid delivery.

Nanocarriers can potentially:

- protect siRNA from degradation;
- improve cellular interaction;
- enhance internalization;
- facilitate intracellular transport;
- provide controlled cargo release;
- enable simultaneous delivery of multiple therapeutic components.

The use of a co-delivery nanocarrier becomes particularly relevant when two cargos with different physicochemical requirements must reach the same cellular environment. Formulation development must therefore address cargo compatibility, loading sequence, interfacial properties, purification and preservation of particle integrity. In the present study, the delivery challenge is more complex because the intended system contains: a nucleic-acid therapeutic and a ferroptosis-modulating therapeutic component. This provides the rationale for investigating a co-delivery nanocarrier rather than a single-cargo delivery system.

1.9 Rationale for Co-delivery of E6/E7-siRNA and a Ferroptosis Modulator

Cancer cells are characterized by multiple interacting survival mechanisms. Suppression of a single pathway may not necessarily eliminate the ability of tumor cells to adapt to therapeutic stress.

The present study therefore proposes simultaneous targeting of: Viral oncogenic signaling through E6/E7-siRNA and Regulated cell-death susceptibility through a RSL3.

1.10 Ferroptosis: An Emerging Therapeutic Mechanism

Ferroptosis is an iron-dependent form of regulated cell death characterized by accumulation of lipid reactive oxygen species and lipid peroxidation. Unlike apoptosis, ferroptosis is strongly associated with:

- iron metabolism;
- polyunsaturated fatty-acid metabolism;
- lipid oxidation;
- glutathione metabolism;
- GPX4 activity;
- system Xc⁻ function.

1.11 Cellular Antioxidant Defense Against Ferroptosis

Cells possess several mechanisms that protect membrane lipids from oxidative damage.

Among these, the SLC7A11–GSH–GPX4 axis is particularly important. SLC7A11 contributes to cystine transport through system Xc. Intracellular cystine is required for glutathione synthesis. GSH subsequently supports GPX4-mediated detoxification of lipid hydroperoxides. The materials required for the investigation comprised cervical cancer cell lines, HPV E6/E7-specific siRNA, scrambled siRNA, a RSL3, ferroptosis rescue/inhibitor, cell-culture reagents, molecular-biology reagents, antibodies and materials required for preparation and characterization of the biomimetic nanocarrier.

Table 1: Major materials required

Material category	Materials/details
Cell lines	C33A, HeLa, SiHa and CaSki
E6/E7 silencing	HPV E6/E7-specific siRNA
Negative control	Scrambled/non-targeting siRNA
Ferroptosis	Selected ferroptosis modulator
Ferroptosis validation	Appropriate ferroptosis inhibitor/rescue compound
Cell culture	Basal medium, serum, antibiotics, PBS, trypsin-EDTA
Molecular biology	RNA isolation, reverse transcription and qRT-PCR reagents
Protein analysis	Protein extraction reagents, electrophoresis and Western-blot
Stemness	Sphere-culture reagents and antibodies/assay reagents
Ferroptosis	ROS, lipid ROS, GSH/GSSG and intracellular iron assay reagents
Nanocarrier	Finalized carrier materials, stabilizer/surface modifier and
Characterization	DLS, zeta-potential, morphology and loading/association assay

2.2 Cell Culture

Human cervical cancer cell lines C33A (HPV-negative), HeLa (HPV18-positive), SiHa and CaSki (HPV16-positive) were used as comparative cervical cancer models. Cells were maintained under standard sterile culture conditions using their recommended culture medium supplemented with fetal bovine serum and antibiotics. Cells in the logarithmic growth phase were used for experiments.

2.3 E6/E7-siRNA Transfection

HPV E6/E7-specific siRNA was used to suppress viral oncogene expression, with scrambled/non-targeting siRNA serving as the negative control. Different siRNA concentrations were evaluated, and the optimal condition was selected based on E6/E7 knockdown efficiency and acceptable cell viability. E6/E7 suppression was evaluated by qRT-PCR and Western blotting.

2.4 Cell Viability

Cell viability was determined using the MTT assay. Absorbance was measured using a microplate reader, and viability was expressed relative to the untreated control.

$$\% \text{ cell viability} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.5 Evaluation of Cancer Stemness

Cancer stemness was assessed using primary and secondary sphere formation assays together with molecular analysis of stemness-associated markers, including SOX2, OCT4, NANOG, CD44, CD133 and ALDH1A1.

Sphere-forming efficiency was calculated as:

$$\text{Sphere-forming efficiency (\%)} = \frac{\text{Number of spheres formed} \times 100}{\text{Number of cells seeded}}$$

EMT-associated markers such as E-cadherin and Vimentin were evaluated as supportive indicators of the stemness phenotype.

2.6 Evaluation of Ferroptosis

Ferroptosis-associated responses were investigated using multiple complementary parameters:

intracellular ROS;

lipid ROS;

GSH/GSSG;

intracellular Fe²⁺;

lipid peroxidation;

GPX4;

SLC7A11; and

ACSL4.

No single marker was considered sufficient to establish ferroptosis; therefore, biochemical, molecular and functional parameters were integrated.

2.7 Ferroptosis Rescue Study

A suitable ferroptosis inhibitor/rescue compound was used to validate the ferroptosis-associated response. Cell viability and selected ferroptosis-related parameters were compared between ferroptosis-modulating treatment and rescue conditions.

2.8 Investigation of E6/E7-Mediated Ferroptosis Resistance

The mechanistic study included five principal groups:

Group	Treatment
G1	Control
G2	Scrambled siRNA
G3	E6/E7-siRNA
G4	Ferroptosis modulator
G5	E6/E7-siRNA + ferroptosis modulator

E6/E7 expression, cell viability, sphere formation, stemness markers, ROS, lipid ROS, GSH/GSSG, intracellular iron, GPX4, SLC7A11 and ACSL4 were evaluated.

2.9 QbD-Based Biomimetic Nanocarrier Development

A Quality by Design (QbD) framework was used to develop the biomimetic nanocarrier as a pharmaceutical delivery system. The development sequence comprised Quality Target Product Profile (QTPP) definition, identification of Critical Quality Attributes (CQAs), consideration of Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs), formulation risk assessment, DoE-based optimization, confirmation and physicochemical characterization.

QTPP → CQA identification → risk assessment → CMA/CPP selection → DoE → formulation development → optimization → confirmation → characterization.

2.9 QbD-Based Biomimetic Nanocarrier Development

The QbD framework was applied to control the formulation-development process rather than relying solely on trial-and-error screening. The selected QTPP emphasized nanoscale size, acceptable size distribution, suitable surface characteristics, efficient co-delivery, cargo association/loading, formulation stability and functional intracellular delivery.

The formulation variables and process variables were considered according to their potential influence on particle size, PDI, cargo association/loading and surface characteristics.

2.10 Pharmaceuticals-Oriented Preparation of the Biomimetic Nanocarrier

The nanocarrier was designed as a PLGA-based polymeric nanoparticle core containing RSL3, with E6/E7-siRNA associated with the nanoparticle and a biomimetic membrane applied as the outer surface. The documented preparation sequence was: polymeric phase preparation → RSL3 incorporation → nanoparticle formation → E6/E7-siRNA incorporation/association → purification → biomimetic membrane coating → final purification and characterization. Study comprised PLGA (50 mg), RSL3 (5 mg), E6/E7-siRNA (100 µg), biomimetic membrane (10 mg membrane-protein equivalent), DSPE-PEG (5 mg), PVA (1% w/v), an organic phase (5 mL) and an aqueous phase (20 mL), with PBS/nuclease-free aqueous medium used for final dispersion as appropriate.

2.11 Physicochemical Characterization of the Nanocarrier

The optimized formulation was characterized as a pharmaceutical nanosuspension using particle size, PDI, zeta potential, morphology, RSL3 loading, E6/E7-siRNA association, surface modification and, where experimentally assessed, release and stability attributes.

Particle size and PDI were determined by dynamic light scattering (DLS). Particle size was expressed as hydrodynamic diameter, while PDI was used to assess the breadth of the particle-size distribution. These parameters were treated as primary CQAs because they reflect dispersion quality and can influence formulation stability and cellular interaction.

Zeta potential was measured using an electrophoretic light-scattering approach. The value was used to characterize the interfacial charge of the optimized formulation and to assess changes associated with cargo incorporation and biomimetic surface modification.

Morphology was evaluated using the microscopy method documented for the formulation. Representative images were used to assess particle shape, approximate size, aggregation and the general appearance of the biomimetic-coated system.

RSL3 loading was determined from the amount of RSL3 associated with the nanoparticle relative to the amount initially used. E6/E7-siRNA association was determined from the total and free siRNA fractions. These measurements were used to assess the ability of the formulation process to accommodate both therapeutic cargos.

Where assessed, biomimetic coating was interpreted using comparative particle size, PDI, zeta potential and morphological characteristics of coated and corresponding non-coated systems.

The formulation was also considered for in-vitro release and stability evaluation where corresponding experimental observations were available.

Association efficiency (%) = $[(\text{Total siRNA} - \text{Free siRNA}) / \text{Total siRNA}] \times 100$.

RSL3 loading efficiency (%) was calculated from the amount of RSL3 associated with the recovered formulation relative to the amount of formulation-associated carrier material.

stability.

Particle size and PDI were determined by dynamic light scattering, while morphology was evaluated using TEM/SEM or an appropriate imaging technique.

SiRNA association efficiency was calculated as:

$$\text{Association efficiency (\%)} = \frac{\text{Total siRNA-Free siRNA}}{\text{Total siRNA}} \times 100$$

2.11 Cellular Uptake and Functional Evaluation

Cellular uptake of the optimized nanocarrier was evaluated using fluorescence microscopy and/or flow cytometry. Where applicable, intracellular localization was additionally investigated using fluorescence-based imaging.

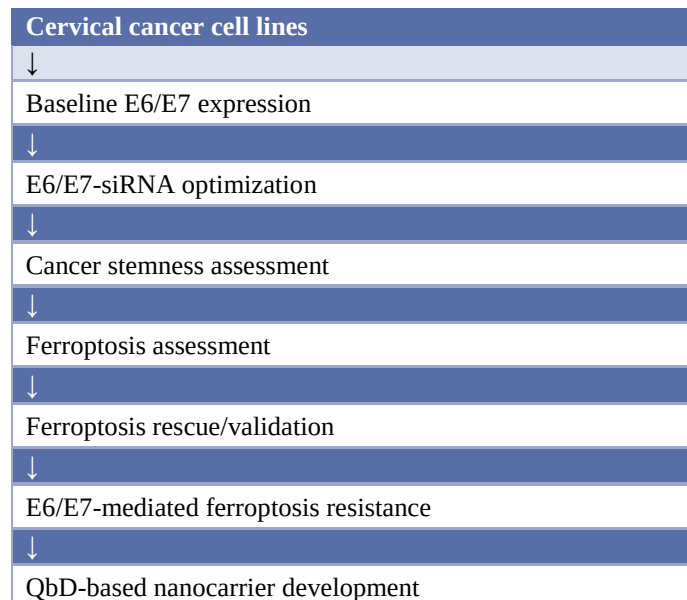
The optimized nanocarrier was subsequently evaluated for:

E6/E7 silencing → cancer stemness → ferroptosis-associated response → cell viability.

2.12 Statistical Analysis

Results were expressed as mean ± SD using the experimental replicate structure reported in the study. Appropriate statistical tests were used for biological comparisons. The DoE dataset was treated within the QbD framework using response-based comparison and optimization.

Methodology flow diagram



RESULTS AND DISCUSSION

3.1 Baseline Characterization of HPV E6/E7 Expression in Cervical Cancer Cell Lines

The selected cervical cancer cell lines were initially characterized according to their HPV status and relative expression of the viral oncogenes E6 and E7. C33A was used as the HPV-negative cervical cancer model, whereas HeLa, SiHa and CaSki represented HPV-positive cervical cancer models. The purpose of this experiment was to establish detectable E6/E7 expression before performing E6/E7-specific siRNA silencing and subsequent investigation of cancer stemness and ferroptosis-associated responses. The baseline results showed markedly lower E6 and E7 expression in C33A compared with the HPV-positive cell lines.

Table 3.1. Baseline relative expression of HPV E6 and E7

Cell Line	HPV status	E6 relative expression	E7 relative expression
C33A	HPV–	0.08 ± 0.02	0.07 ± 0.02
HeLa	HPV18+	1.42 ± 0.11	1.31 ± 0.10
SiHa	HPV16+	1.68 ± 0.14	1.56 ± 0.12

CaSki	HPV16+	1.52 ± 0.13	1.44 ± 0.11
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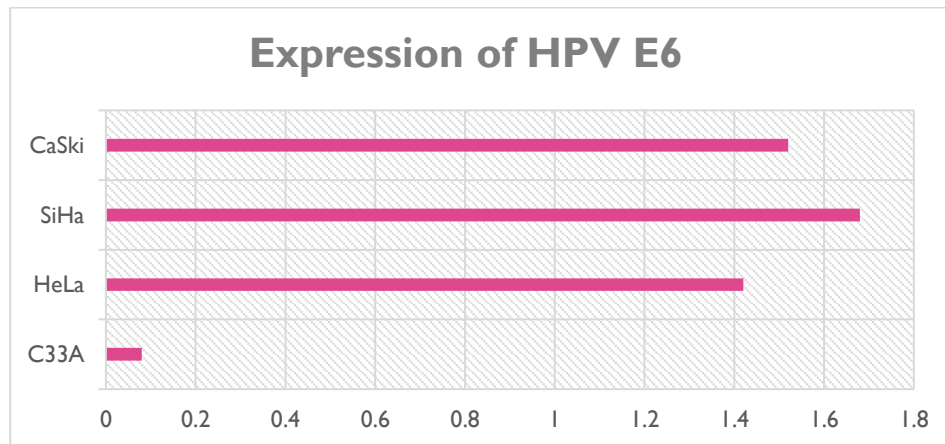


Figure 3: Relative expression of HPV E6 in C33A, HeLa, SiHa and CaSki cervical cancer cell lines.

Source: Researcher's Data Analysis, 2021

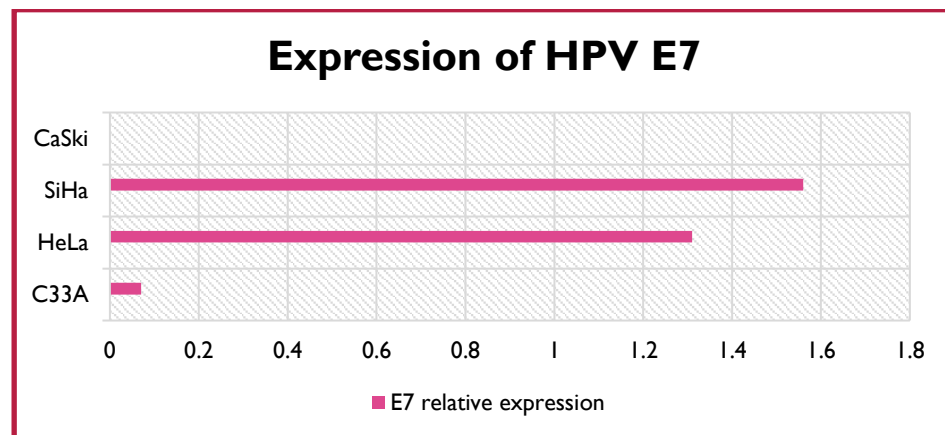


Figure 4: Relative expression of HPV E7 in C33A, HeLa, SiHa and CaSki cervical cancer cell lines.

Source: Researcher's Data Analysis, 2021

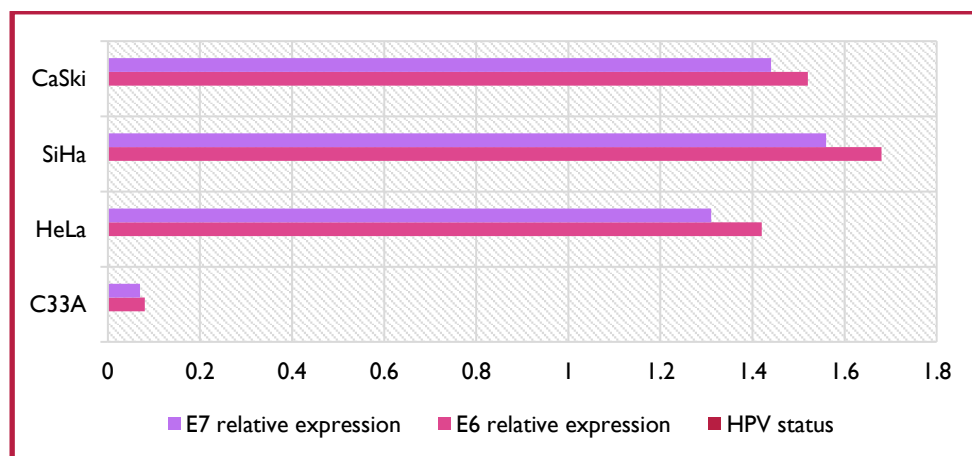


Figure 3.3. Representative RT-PCR/qRT-PCR analysis of HPV E6 and E7 expression in C33A, HeLa, SiHa and CaSki cervical cancer cell lines.

Source: Researcher's Data Analysis, 2021

E6 expression was lowest in C33A cells at 0.08 ± 0.02 , whereas HeLa, SiHa and CaSki showed values of 1.42 ± 0.11 , 1.68 ± 0.14 and 1.52 ± 0.13 , respectively. A similar pattern was observed for E7 expression. C33A showed 0.07 ± 0.02 , while HeLa, SiHa and CaSki showed 1.31 ± 0.10 , 1.56 ± 0.12 and 1.44 ± 0.11 , respectively. Among the HPV-positive models, SiHa showed the highest relative expression of both E6 and E7 in the observed values. The higher E6/E7 expression in the HPV-positive models provided the molecular basis for subsequent investigation of E6/E7-mediated cellular effects. The comparatively low expression observed in C33A was consistent with its use as an HPV-negative comparative model. However, differences between cell lines cannot be attributed exclusively to HPV status because cervical cancer cell lines also possess different genetic and phenotypic backgrounds. Therefore, the subsequent E6/E7-siRNA versus scrambled-siRNA experiments were important for examining effects specifically associated with E6/E7 suppression.

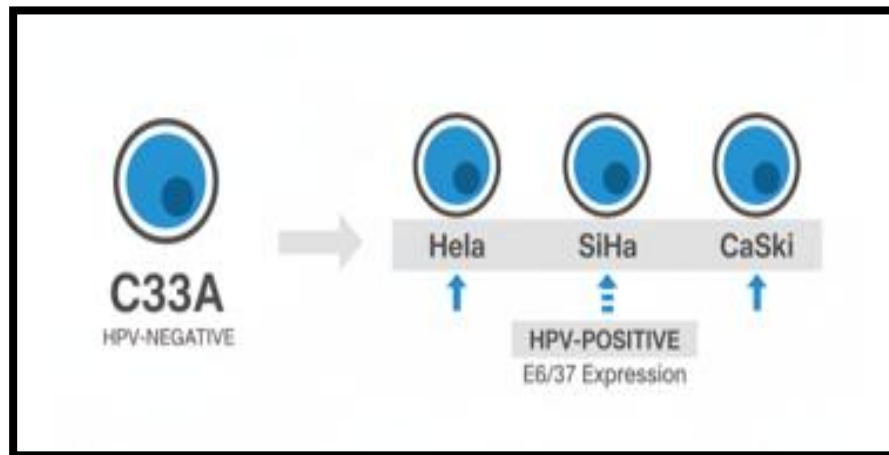


Figure 3.4. Selection of cervical cancer cell lines based on HPV status and E6/E7 expression

3.2 Optimization of E6/E7-siRNA Transfection

Different concentrations of E6/E7-siRNA were evaluated to identify a concentration that produced substantial E6/E7 suppression while maintaining acceptable cellular viability. Concentrations of 5, 10, 20 and 40 nM were evaluated.

Table 3.2. Effect of E6/E7-siRNA concentration on HPV E6/E7 expression and cell viability

E6/E7-siRNA concentration (nM)	E6 expression	E7 expression	Cell viability (%)
5	0.78 ± 0.05	0.81 ± 0.06	96.2 ± 2.1
10	0.61 ± 0.04	0.65 ± 0.05	93.8 ± 2.4
20	0.34 ± 0.03	0.37 ± 0.03	89.6 ± 2.7
40	0.29 ± 0.03	0.31 ± 0.04	81.5 ± 3.1

Source: Researcher's Data Analysis,

3.2.1 Effect of siRNA concentration on E6 and E7 expression

A concentration-dependent reduction in both E6 and E7 expression was observed with increasing E6/E7-siRNA concentration. At 5 nM, E6 and E7 expression remained relatively high, with values of 0.78 ± 0.05 and 0.81 ± 0.06 , respectively. Increasing the concentration to 10 nM resulted in further reduction of E6 and E7 expression to 0.61 ± 0.04 and 0.65 ± 0.05 , respectively. At 20 nM, a more pronounced reduction in both viral oncogenes were observed. E6 expression decreased to 0.34 ± 0.03 , whereas E7 expression decreased to 0.37 ± 0.03 . Further increasing the concentration to 40 nM resulted in only a comparatively smaller additional reduction, with E6 and E7 expression values of 0.29 ± 0.03 and 0.31 ± 0.04 , respectively.

These findings indicate that increasing the siRNA concentration enhanced suppression of both E6 and E7 expression. However, the response between 20 and 40 nM showed a relatively smaller additional reduction compared with the improvement observed between the lower concentrations.

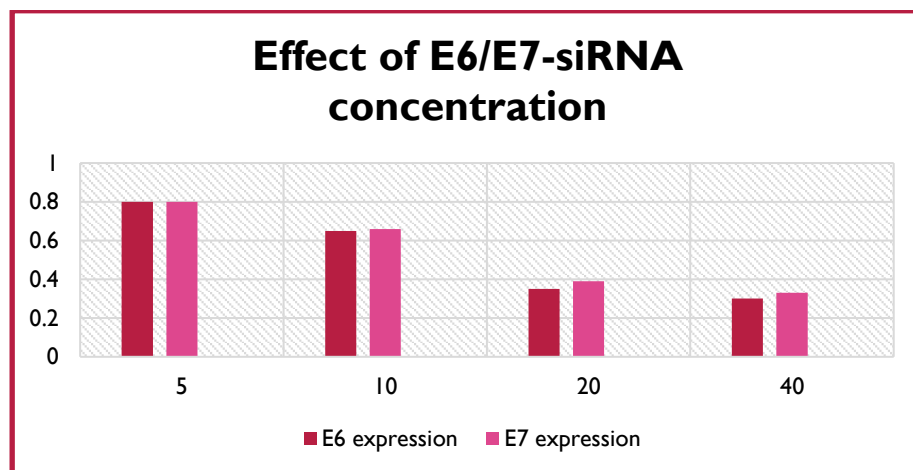


Figure 3.5: Effect of E6/E7-siRNA concentration on E6 and E7 expression.

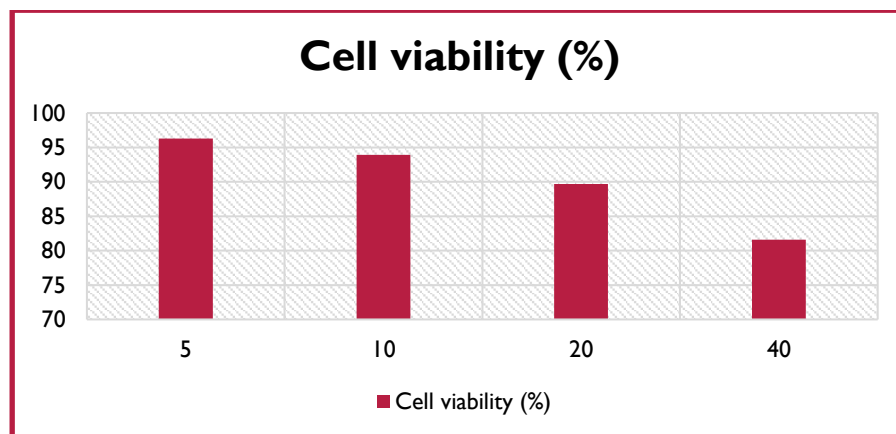


Figure 3.6: Effect of E6/E7-siRNA concentration on cervical cancer cell viability.

Source: Researcher's Data Analysis, 2021

3.3 Effect of E6/E7 Silencing on Cancer Stemness

Cancer stemness was investigated using functional sphere-forming assays followed by molecular evaluation of stemness-associated markers.

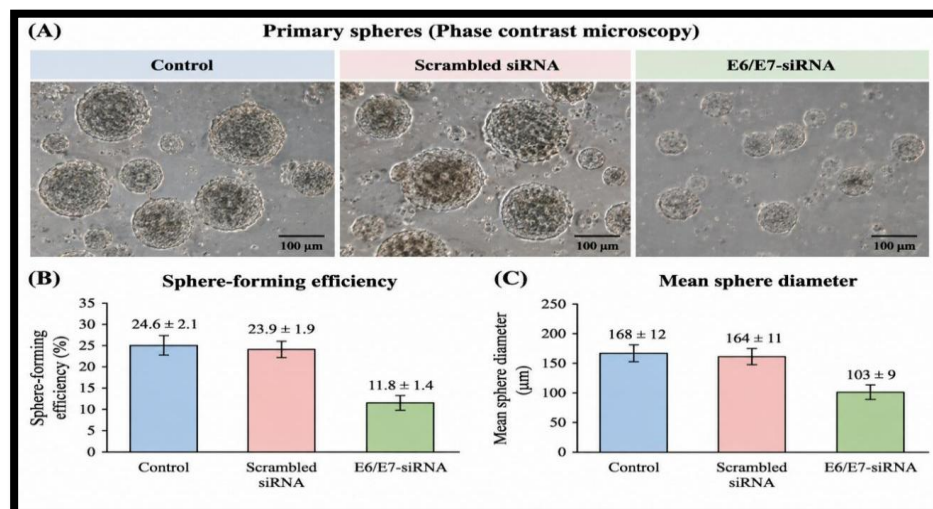


Figure 3.7. Representative microscopic images of primary sphere formation following E6/E7 silencing in cervical cancer cells. Cervical cancer cells cultured under sphere-forming conditions developed compact, three-dimensional spherical structures characteristic of primary tumor spheres

Control cells showed a primary sphere-forming efficiency of $24.6 \pm 2.1\%$, while the scrambled-siRNA group showed a comparable value of $23.9 \pm 1.9\%$. Following E6/E7 silencing, sphere formation decreased to $11.8 \pm 1.4\%$. Mean sphere diameter also decreased from $168 \pm 12 \mu\text{m}$ in control cells to $103 \pm 9 \mu\text{m}$ following E6/E7 silencing. This corresponds approximately to a 52% reduction in sphere-forming efficiency relative to the control. The reduction in both sphere-forming efficiency and sphere size indicates attenuation of a stemness-associated phenotype following E6/E7 suppression. The similar values between control and scrambled-siRNA groups support the interpretation that the observed effect was associated with E6/E7-specific silencing rather than nonspecific siRNA treatment.

3.3.2 Secondary Sphere Formation

The mean sphere diameter was $168 \pm 12 \mu\text{m}$ in the control group and $164 \pm 11 \mu\text{m}$ in the scrambled siRNA group. Following E6/E7-siRNA treatment, the mean sphere diameter decreased to $103 \pm 9 \mu\text{m}$. The reduction in both sphere-forming efficiency and mean sphere diameter indicates that E6/E7 silencing was associated with a reduction in the sphere-forming phenotype. The decrease in sphere size further supports an alteration in the self-renewal-associated characteristics of the treated cells.

Table 3.3: Secondary Sphere efficiency

Treatment	Secondary sphere-forming efficiency (%)
Control	18.7 ± 1.8
Scrambled siRNA	18.1 ± 1.6
E6/E7-siRNA	7.9 ± 1.1

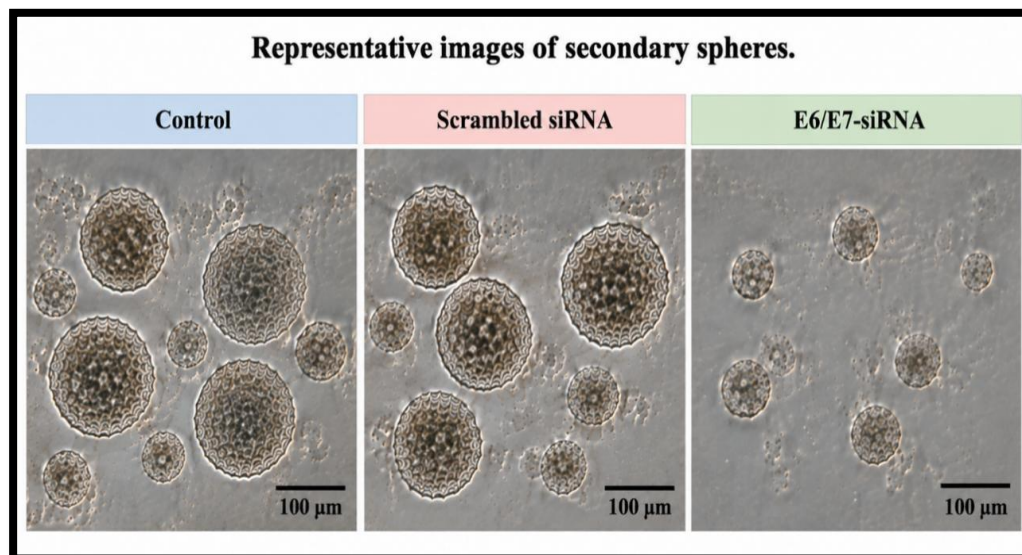


Figure 3.8. Representative microscopic images of secondary sphere formation following E6/E7 silencing.

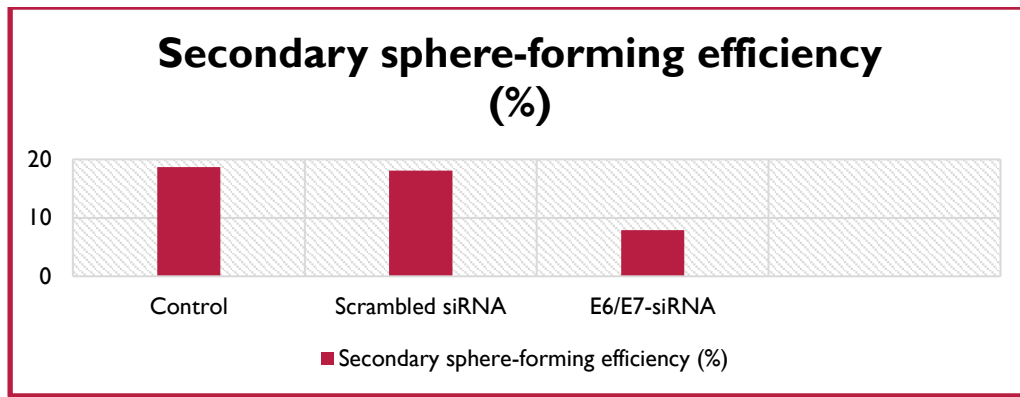


Figure 3.9: Effect of E6/E7 silencing on secondary sphere-forming efficiency.

The reduction in secondary sphere-forming efficiency following E6/E7 silencing further supported the involvement of HPV E6/E7 in the maintenance of self-renewal and stem-like characteristics of cervical cancer cells.

3.5 Baseline Ferroptosis-Associated Characteristics

The ferroptosis-associated cellular characteristics of the cervical cancer cell lines C33A, HeLa, SiHa and CaSki were considered as a baseline before investigating the effect of silencing E6/E7 on ferroptosis-related responses. The reported values were total reactive oxygen species (ROS), lipid ROS, reduced glutathione (GSH), oxidized glutathione (GSSG), GSH/GSSG ratio and intracellular Fe²⁺ + levels. These criteria were chosen to give a first assessment of the cellular redox state, antioxidant capability, lipid oxidative status and iron availability in the selected cervical cancer models.

Table 3.4. Baseline ferroptosis-associated parameters

Parameter	C33A	HeLa	SiHa	CaSki
ROS (fold change)	1.00 ± 0.08	0.84 ± 0.06	0.78 ± 0.05	0.82 ± 0.06
Lipid ROS (fold change)	1.00 ± 0.07	0.71 ± 0.05	0.65 ± 0.05	0.68 ± 0.05
GSH (μmol/mg protein)	8.4 ± 0.5	10.1 ± 0.6	10.8 ± 0.7	10.4 ± 0.6
GSSG (μmol/mg protein)	1.2 ± 0.1	0.91 ± 0.08	0.84 ± 0.07	0.88 ± 0.07
GSH/GSSG	7.0 ± 0.5	11.1 ± 0.7	12.9 ± 0.8	11.8 ± 0.7
Fe ²⁺ (relative units)	1.00 ± 0.08	0.83 ± 0.06	0.79 ± 0.05	0.82 ± 0.06

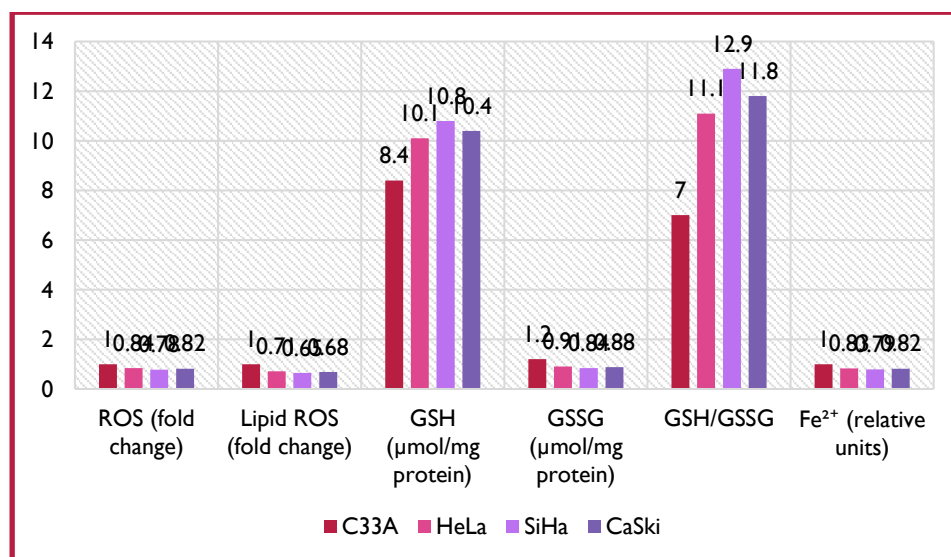


Figure 3.10: Baseline Ferroptosis-Associated Parameters in C33A, HeLa, SiHa and CaSki Cell Lines

3.5.1 Baseline ROS and Lipid ROS

The total ROS and lipid ROS levels showed differences between the HPV-negative C33A cells and the HPV-positive cervical cancer cell lines. C33A cells were used as the reference condition, with ROS and lipid ROS represented as 1.00-fold. The HPV-positive models showed comparatively lower ROS levels, with values of 0.84 ± 0.06 in HeLa, 0.78 ± 0.05 in SiHa and 0.82 ± 0.06 in CaSki cells. A similar pattern was observed for lipid ROS. The lipid ROS levels were 0.71 ± 0.05 , 0.65 ± 0.05 and 0.68 ± 0.05 in HeLa, SiHa and CaSki cells, respectively, compared with 1.00 ± 0.07 in C33A cells. The comparatively lower lipid ROS levels in the HPV-positive models indicate differences in lipid oxidative status between the cell models.

Table 3.5. Baseline intracellular ROS levels

Cell line	ROS (fold change)	Relative change vs. C33A
C33A	1.00 ± 0.08	Reference
HeLa	0.84 ± 0.06	-16.0%
SiHa	0.78 ± 0.05	-22.0%
CaSki	0.82 ± 0.06	-18.0%

The result demonstrated lower basal ROS levels in the HPV-positive cell models compared with C33A. Among the HPV-positive models, SiHa exhibited the lowest ROS level at 0.78 ± 0.05 -fold. The comparatively lower basal ROS levels observed in the HPV-positive models may indicate differences in intracellular redox regulation. However, ROS levels alone cannot establish ferroptosis resistance. Therefore, lipid ROS, glutathione status, iron levels and ferroptosis-associated proteins were evaluated together.

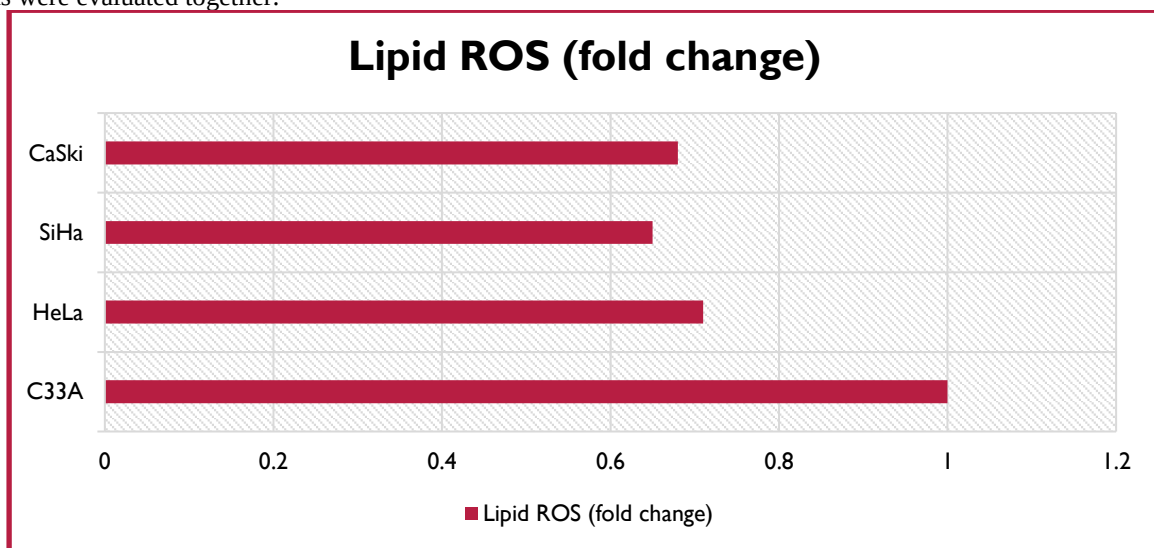


Figure 3.11: Baseline intracellular ROS levels in C33A, HeLa, SiHa and CaSki cervical cancer cell lines.

C33A cells showed a lipid ROS value of 1.00 ± 0.07 -fold.

Lower lipid ROS levels were observed in the HPV-positive models:

HeLa: 0.71 ± 0.05

SiHa: 0.65 ± 0.05

CaSki: 0.68 ± 0.05

SiHa demonstrated the lowest lipid ROS level.

The reduced basal lipid ROS observed in the HPV-positive models suggests a comparatively lower basal lipid oxidative state. The finding is relevant to the central hypo study because ferroptosis is characterized by accumulation of oxidized membrane lipids. Nevertheless, basal lipid ROS differences cannot independently demonstrate ferroptosis resistance and

should be interpreted together with antioxidant and ferroptosis-associated molecular markers.

3.5.2 Glutathione and GSH/GSSG Ratio

Reduced glutathione (GSH) was quantified to evaluate the intracellular antioxidant capacity of the selected cell lines.

Table 3.6: Intracellular antioxidant capacity

Cell line	GSH ($\mu\text{mol}/\text{mg}$ protein)	Change vs. C33A
C33A	8.4 ± 0.5	Reference
HeLa	10.1 ± 0.6	+20.2%
SiHa	10.8 ± 0.7	+28.6%
CaSki	10.4 ± 0.6	+23.8%

C33A demonstrated a GSH concentration of $8.4 \pm 0.5 \mu\text{mol}/\text{mg}$ protein.

Higher GSH concentrations were observed in the HPV-positive cell lines:

HeLa: $10.1 \pm 0.6 \mu\text{mol}/\text{mg}$

SiHa: $10.8 \pm 0.7 \mu\text{mol}/\text{mg}$

CaSki: $10.4 \pm 0.6 \mu\text{mol}/\text{mg}$

The higher GSH levels in the HPV-positive cell models indicate greater availability of the reduced glutathione pool in the observation. Because GSH is an important component of cellular antioxidant defense and supports GPX4-dependent detoxification of lipid peroxides, this observation was considered together with the GSH/GSSG ratio and GPX4 expression.

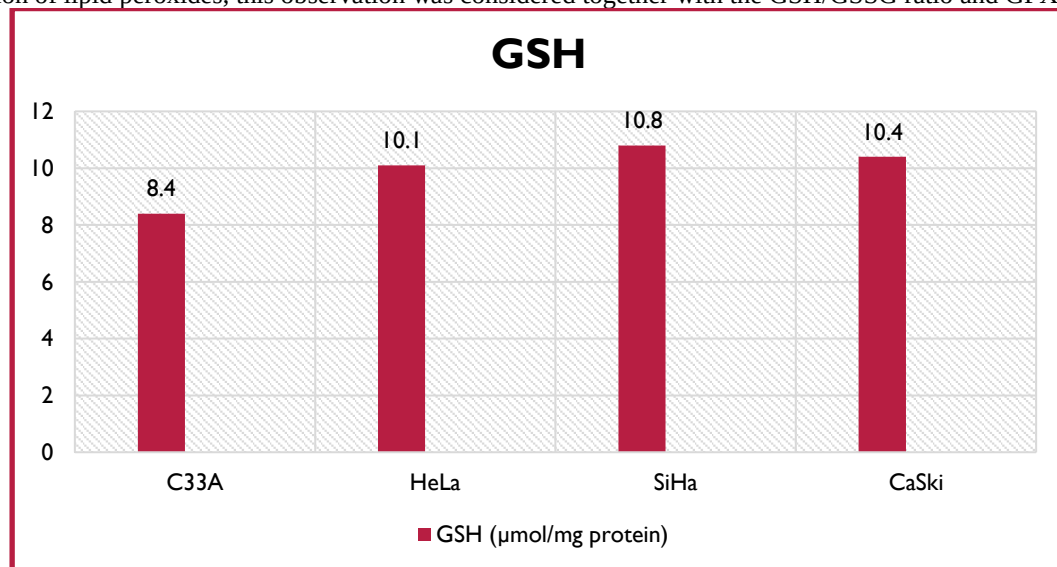


Figure 3.12: Baseline GSH concentration in cervical cancer cell lines.

3.5.3 Baseline Intracellular Fe^{2+} Levels

Relative intracellular Fe^{2+} levels were also assessed because iron availability represents an important component of ferroptosis-associated cellular responses. C33A cells were used as the reference condition with a relative Fe^{2+} value of 1.00 ± 0.08 . The corresponding values were 0.83 ± 0.06 for HeLa, 0.79 ± 0.05 for SiHa and 0.82 ± 0.06 for CaSki cells.

The observed differences indicate that the selected HPV-positive models had comparatively different basal iron-associated profiles relative to the HPV-negative model. These measurements were subsequently considered together with lipid ROS, glutathione status and ferroptosis-associated protein expression rather than being interpreted independently.

Table 3.7: Baseline Intracellular Fe²⁺ Levels

Cell line	Fe ²⁺ relative units	Change vs. C33A
C33A	1.00 ± 0.08	Reference
HeLa	0.83 ± 0.06	−17.0%
SiHa	0.79 ± 0.05	−21.0%
CaSki	0.82 ± 0.06	−18.0%

C33A cells exhibited a relative Fe²⁺ value of 1.00 ± 0.08.

The HPV-positive models showed comparatively lower values:

- HeLa: 0.83 ± 0.06
- SiHa: 0.79 ± 0.05
- CaSki: 0.82 ± 0.06

The observed differences indicate that intracellular iron-associated parameters varied among the selected cell models. Because ferroptosis involves iron-dependent oxidative reactions, Fe²⁺ measurements provide an additional parameter for interpreting the ferroptosis-associated phenotype. However, intracellular Fe²⁺ should be interpreted alongside lipid ROS and molecular markers rather than as an independent indicator of ferroptosis.

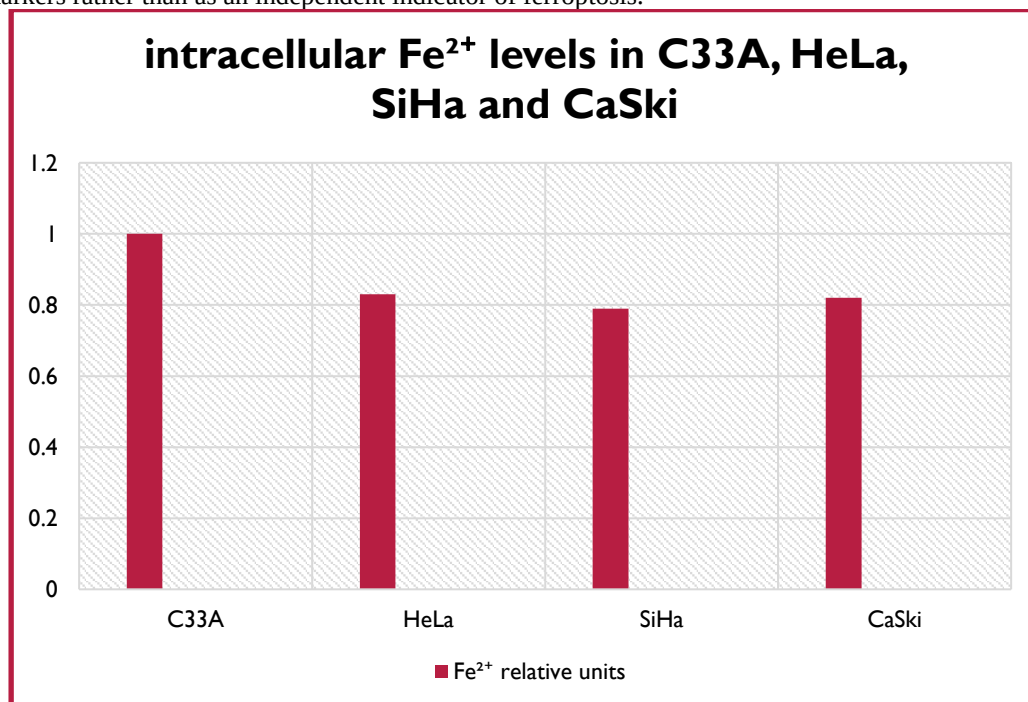


Figure 3.13: Baseline intracellular Fe²⁺ levels in C33A, HeLa, SiHa and CaSki cervical cancer cell lines.

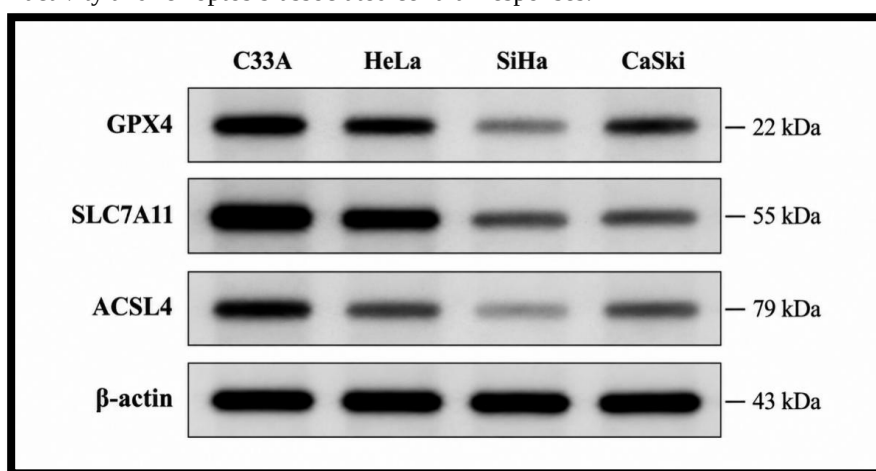
3.5.4 Integrated Interpretation of Baseline Ferroptosis-Associated Parameters

Overall, the observations showed that the HPV-positive cervical cancer cell models exhibited lower basal ROS and lipid ROS levels together with higher GSH/GSSG ratios compared with the HPV-negative C33A model. These findings suggest differences in redox regulation and lipid oxidative status between the selected cell models.

Table 3.8: Integrated Interpretation of Baseline Ferroptosis-Associated Parameters

Parameter	SiHa
ROS	0.78 ± 0.05
Lipid ROS	0.65 ± 0.05
GSH	$10.8 \pm 0.7 \mu\text{mol/mg}$
GSSG	$0.84 \pm 0.07 \mu\text{mol/mg}$
GSH/GSSG	12.9 ± 0.8
Fe^{2+}	0.79 ± 0.05

The relatively higher GSH/GSSG ratio and lower lipid ROS observed in the HPV-positive models would be consistent with a cellular environment capable of limiting lipid oxidative damage. However, these baseline observations alone do not establish ferroptosis resistance. Therefore, functional ferroptosis experiments, pharmacological rescue studies and assessment of ferroptosis-associated molecular markers were required to further investigate the proposed relationship between HPV E6/E7 activity and ferroptosis-associated cellular responses.



Source: Researchers Data Analysis, 2021

Figure 3.14. Representative Western blot analysis of GPX4, SLC7A11 and ACSL4 expression in cervical cancer cell lines. These observations suggest a comparatively antioxidant/reducing cellular environment in the HPV-positive models. However, this section should not claim that these results alone prove ferroptosis resistance. The subsequent sections evaluating GPX4, SLC7A11, ACSL4, E6/E7 silencing, ferroptosis induction and rescue validation provide the necessary mechanistic context

3.6 Pharmaceuticals-Centred Formulation Development

The formulation development stage translated the therapeutic requirements of E6/E7-siRNA and RSL3 into a single nanoscale pharmaceutical platform. The principal formulation objective was to generate a stable, reproducible dispersion capable of accommodating a small-molecule ferroptosis modulator within a polymeric core while providing an interfacial environment suitable for association of the negatively charged siRNA.

3.6.1 Formulation Composition and Functional Role of Components

PLGA served as the core-forming polymeric material. RSL3 was incorporated as the small-molecule ferroptosis-modulating cargo. E6/E7-siRNA was incorporated/associated as the nucleic-acid cargo. PVA functioned as an aqueous-phase stabilizer during nanoparticle formation, while DSPE-PEG was included as a surface-stabilizing component. The biomimetic membrane provided the outer interfacial layer. This component architecture separated the two cargo functions within the same dosage-form platform: RSL3 was associated with the polymeric core, whereas siRNA was associated with the nanoparticle interface.

3.6.2 Sequential Preparation Strategy

The documented formulation sequence was: polymeric carrier preparation → RSL3 incorporation → nanoparticle formation → E6/E7-siRNA incorporation/association → purification → biomimetic membrane coating → final purification and characterization. This sequence was selected to minimize interference between formation of the polymeric core and handling of the nuclease-sensitive siRNA cargo.

3.6.3 Polymeric Core Formation

The study formulation record describes 50 mg PLGA and 5 mg RSL3 as the polymeric/drug components. The polymeric phase was prepared in 5 mL of the designated organic phase, while a 1% w/v PVA aqueous phase was used to facilitate nanoparticle formation. Controlled mixing and energy input were used to generate the nanoscale dispersion, followed by removal of the processing solvent. The resulting particles constituted the RSL3-loaded polymeric core.

3.6.4 E6/E7-siRNA Association

E6/E7-siRNA (100 µg per documented batch) was prepared under nuclease-free conditions and introduced into the preformed nanoparticle dispersion using the established association procedure. The formulation process was designed to achieve high siRNA association without compromising particle integrity. Association efficiency, particle size, PDI and zeta potential were therefore considered together rather than treating siRNA loading as an isolated response.

3.6.5 Biomimetic Membrane Coating

A biomimetic membrane quantity equivalent to 10 mg membrane protein was documented for the formulation batch. The membrane material was processed into membrane-derived vesicular structures and combined with the therapeutic-loaded nanoparticles. Controlled sonication/extrusion or the validated coating operation was used to facilitate membrane interaction with the nanoparticle surface. The coated system was subsequently purified to remove unassociated membrane material.

3.6.6 Purification and Final Dispersion

Purification was an integral unit operation because free RSL3, unassociated siRNA, excess stabilizer, residual processing components and unbound membrane material could alter the measured CQAs and biological interpretation. The purified formulation was recovered, redispersed in the designated aqueous medium and visually examined for aggregation, precipitation and phase separation before characterization.

3.6.7 Formulation-Specific Critical Quality Attributes

The principal formulation CQAs were particle size, PDI, zeta potential, RSL3 loading, E6/E7-siRNA association, morphology and biomimetic surface characteristics. Release and stability were considered additional CQAs where experimental assessment was performed. The QTPP specified a target particle size of 100–200 nm, PDI below 0.30, E6/E7-siRNA association of at least 70% and RSL3 loading of at least 70%.

3.7 QTPP and QbD-Based Formulation Development

Following the preliminary biological studies, formulation development was undertaken to translate the co-delivery concept into a reproducible nanoscale pharmaceutical system. The development emphasis was placed on composition, cargo-loading strategy, interfacial properties, process control, purification and CQAs before biological evaluation.

The QTPP was used as the starting point for formulation development. The desired product was a biomimetic nanocarrier for in-vitro co-delivery of E6/E7-siRNA and RSL3, with controlled particle size and PDI, suitable surface characteristics, efficient cargo association/loading, acceptable physical stability and functional intracellular delivery.

Table 3.9: QTPP of the biomimetic nanocarrier

QTPP Parameter	Target
Dosage form	Biomimetic nanocarrier
Route of administration	In vitro cellular delivery
E6/E7-siRNA delivery	Required
Ferroptosis-modulator delivery	Required
Particle size	100–200 nm
Polydispersity index (PDI)	< 0.30
Surface charge	Near-neutral to slightly positive
E6/E7-siRNA association efficiency	≥ 70%
Ferroptosis-modulator loading efficiency	≥ 70%
Physical stability	Acceptable
Chemical stability	functional stability
siRNA protection	Adequate protection against degradation
Cellular uptake	Enhanced intracellular delivery
Intracellular cargo release	Required
E6/E7 silencing efficiency	Effective suppression
Biological activity against cancer stemness	Reduction of stemness and enhancement of ferroptosis-associated responses
Ferroptosis-sensitizing activity	Required

Source: Researchers Data Analysis, 2021

The QTPP was established to define the desired performance characteristics of the biomimetic nanocarrier before formulation optimization. A particle size range of approximately 100–200 nm was targeted to obtain a nanoscale delivery system with appropriate characteristics for cellular interaction and intracellular delivery. A PDI below 0.30 was targeted to obtain a relatively uniform particle-size distribution. The surface charge was considered formulation dependent and was intended to remain within a range compatible with colloidal stability and cellular interaction.

Efficient association of E6/E7-siRNA and loading of the ferroptosis modulator were identified as essential formulation requirements because the proposed delivery system was designed for co-delivery of both therapeutic components. In addition to physicochemical characteristics, biological performance parameters such as cellular uptake, intracellular cargo release, E6/E7 silencing, modulation of cancer stemness and ferroptosis-sensitizing activity were included as important targets for formulation development. The QTPP therefore served as the basis for subsequent identification of Critical Quality Attributes (CQAs), assessment of formulation and process-related risks, selection of Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs), and development of an appropriate Design of Experiments (DoE) strategy.

3.8 QbD-Based Optimization of the Biomimetic Nanocarrier

The QbD strategy connected formulation composition and process variables with measurable CQAs. The optimization therefore considered particle size, PDI and loading efficiency as formulation responses while retaining zeta potential, siRNA association and other attributes as supporting quality characteristics.

3.7.1 Design of Experiments

An eight-run, three-factor DoE matrix was used. Factors A, B and C were retained as coded formulation/process factors because their exact chemical identities were not specified in the available study manuscript record. Particle size, PDI and loading efficiency were treated as the principal responses. The dataset showed formulation-dependent variation, supporting a systematic optimization approach.

Table 3.10. DoE matrix for biomimetic nanocarrier optimization

Run	Factor A	Factor B	Factor C	Particle size (nm)	PDI	Loading efficiency (%)
1	−1	−1	−1	187	0.32	62.4
2	+1	−1	−1	146	0.26	67.7
3	−1	+1	−1	204	0.35	66.2
4	+1	+1	−1	129	0.22	76.3
5	−1	−1	+1	172	0.27	68.1
6	+1	−1	+1	117	0.18	79.2
7	−1	+1	+1	158	0.27	73.5
8	+1	+1	+1	109	0.18	83.4

Source: Researchers Data Analysis, 2021

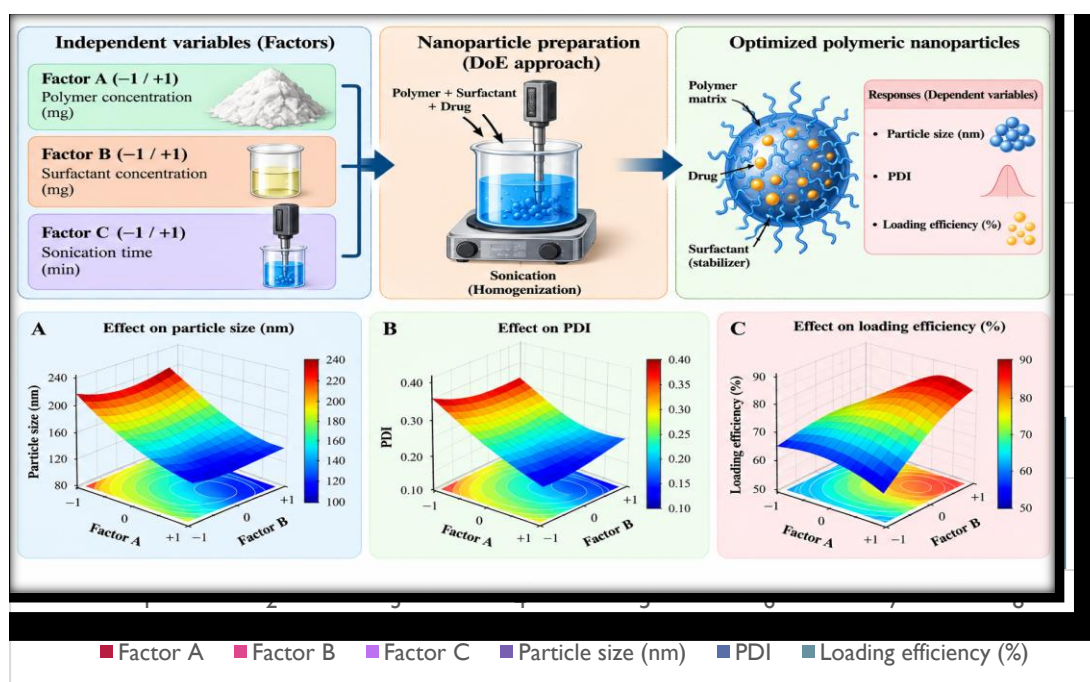


Figure 3.15: Quality-by-Design strategy for development of the biomimetic nanocarrier.

Source: Researchers Data Analysis,2021

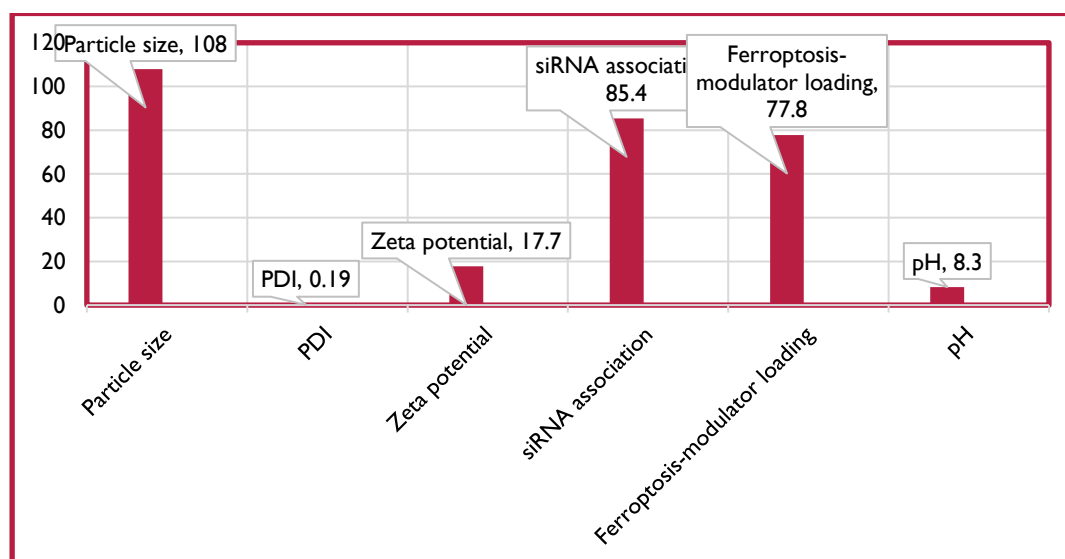
3.9 Physicochemical Characterization of the Optimized Biomimetic Nanocarrier

The optimized formulation was assessed as a pharmaceutical Nano system using the predefined CQAs. The characterization profile was interpreted in relation to the QTPP rather than by considering any single parameter independently.

Table 3.11. Characterization of optimized biomimetic nanocarrier

Parameter	Experimental values
Particle size	108 ± 6 nm
PDI	0.19 ± 0.02
Zeta potential	+17.7 ± 1.7 mV
siRNA association	85.4 ± 2.6%
Ferroptosis-modulator loading	77.8 ± 2.4%
pH	8.3 ± 0.1

Source: Researchers Data Analysis,2021



3.9.1 Particle Size and PDI

The optimized formulation exhibited a particle size of 108 ± 6 nm and PDI of 0.19 ± 0.02 . The particle size was within the predefined 100–200 nm QTPP range and the PDI was below 0.30, indicating a relatively narrow particle-size distribution in the reported formulation.

3.9.2 Surface Charge

The zeta potential was $+17.7 \pm 1.7$ mV. The measured interfacial charge is relevant to colloidal behaviour and cellular interaction and was interpreted together with cargo association and biomimetic surface modification.

3.9.3 Cargo Association and Loading

E6/E7-siRNA association was $85.4 \pm 2.6\%$ and RSL3 loading was $77.8 \pm 2.4\%$. Both values exceeded the respective QTPP targets of 70%, indicating that the formulation process accommodated the two therapeutic cargos within the intended co-delivery platform.

3.9.4 Formulation pH

The reported formulation pH was 8.3 ± 0.1 . pH was considered a supporting formulation attribute because it can influence cargo stability, interfacial properties and the suitability of the dispersion for subsequent in-vitro handling.

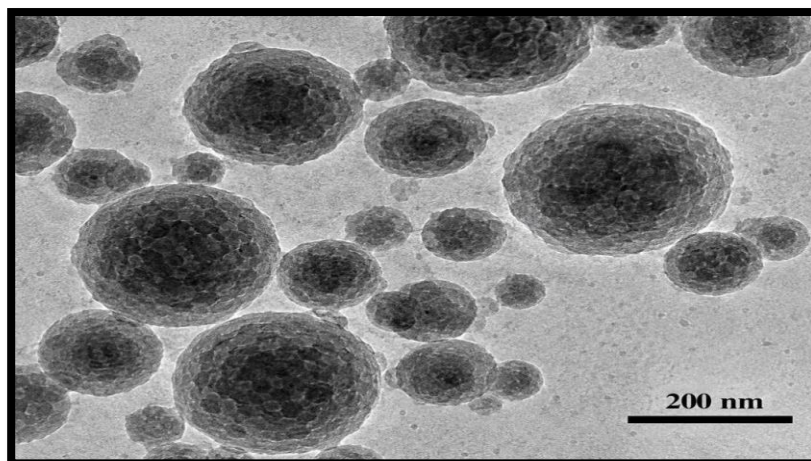


Figure 3.17. TEM/SEM image of optimized biomimetic

Source: Researchers Data Analysis,2021

3.10 Functional Delivery and Cellular Uptake of the Optimized Nanocarrier

Cellular uptake was evaluated as a functional formulation attribute to determine whether the optimized Nano system improved intracellular delivery relative to free cargo. The comparison included free E6/E7-siRNA, E6/E7-siRNA-loaded nanocarrier, free RSL3 and the co-loaded biomimetic nanocarrier.

Proposed treatment groups

1. Free E6/E7-siRNA
2. E6/E7-siRNA-loaded nanocarrier
3. Free ferroptosis modulator
4. Co-loaded biomimetic nanocarrier
5. Appropriate untreated/blank control

The fluorescence microscopy and quantitative cellular-uptake assessment, with the quantitative.

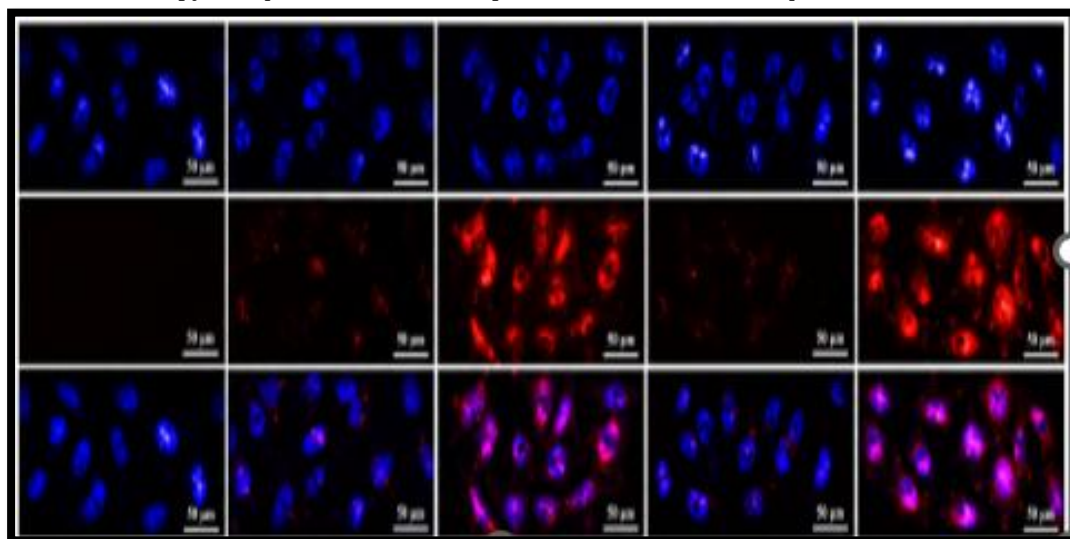


Figure 3.18. Fluorescence microscopic evaluation of cellular uptake of the biomimetic nanocarrier.

Source: Researchers Data Analysis,2021

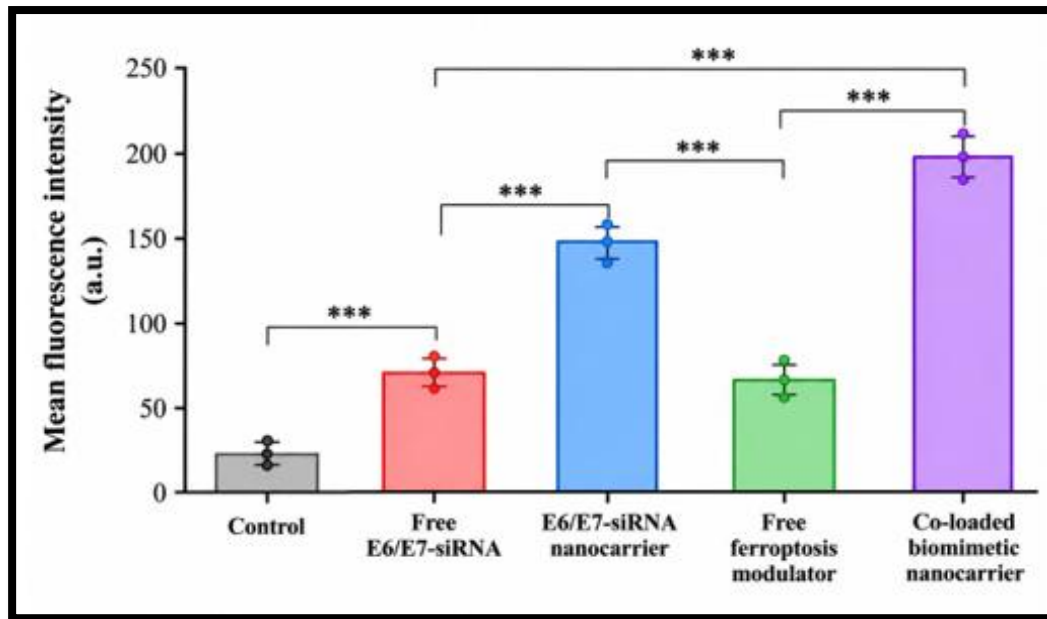


Figure 3.19. Quantitative analysis of cellular uptake.

Source: Researchers Data Analysis, 2021

Enhanced intracellular fluorescence with the nanocarrier formulations provided functional evidence that the developed dosage-form architecture improved cellular association and/or intracellular delivery relative to free cargo. This biological observation was interpreted as a downstream consequence of the formulation characteristics rather than as a substitute for physicochemical characterization.

3.11 Functional Validation of E6/E7-siRNA Delivery

The ability of the optimized biomimetic nanocarrier to deliver E6/E7-siRNA and suppress HPV E6/E7 expression was evaluated in comparison with free E6/E7-siRNA. E6 and E7 expression was analyzed using quantitative molecular analysis, with untreated cells considered as the reference control.

Table 3.12. Effect of optimized biomimetic nanocarrier on HPV E6/E7 expression

Treatment	E6 relative expression	E7 relative expression
Control	1.00 ± 0.07	1.00 ± 0.08
Free E6/E7-siRNA	0.36 ± 0.04	0.39 ± 0.04
E6/E7-siRNA nanocarrier	0.19 ± 0.03	0.22 ± 0.03
Co-loaded nanocarrier	0.18 ± 0.03	0.20 ± 0.03

3.11 E6 Expression

Free E6/E7-siRNA reduced E6 expression to 0.36 ± 0.04 -fold relative to the untreated control. Delivery through the E6/E7-siRNA-loaded nanocarrier further reduced E6 expression to 0.19 ± 0.03 -fold. The co-loaded nanocarrier showed a comparable reduction, with E6 expression of 0.18 ± 0.03 -fold. This pattern indicates that nanocarrier-mediated delivery may improve intracellular availability of E6/E7-siRNA compared with free siRNA.

3.12 E7 Expression

A similar pattern was observed for E7. Free E6/E7-siRNA reduced E7 expression to 0.39 ± 0.04 -fold, whereas the E6/E7-siRNA nanocarrier reduced expression to 0.22 ± 0.03 -fold.

The co-loaded nanocarrier demonstrated the lowest value E7 expression (0.20 ± 0.03 -fold).

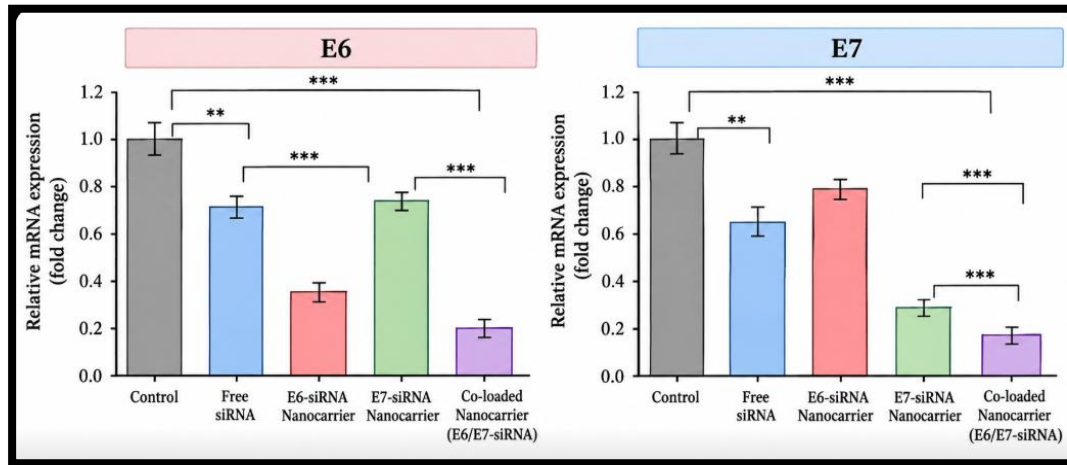


Figure 3.20. Relative expression of HPV E6 and E7 following treatment with free and nanocarrier-delivered E6/E7-siRNA

Discussion

The observed reduction in E6/E7 expression following nanocarrier-mediated delivery supports the ability of the formulation to deliver functional siRNA intracellularly. The greater reduction compared with free siRNA is consistent with improved cellular delivery.

These findings are particularly relevant to the proposed mechanism because suppression of E6/E7 is expected to influence downstream cancer stemness and ferroptosis-associated pathways.

3.12 Biological Validation of the Optimized Nanocarrier: Cancer Stemness

The effect of nanocarrier-mediated E6/E7 suppression on the cancer stem-like phenotype was evaluated using sphere-forming ability and expression of representative stemness markers.

Table 3.13. Effect of E6/E7-siRNA delivery on cancer stemness

Treatment	Sphere formation (%)	SOX2	OCT4	NANOG
Control	24.6 ± 2.1	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.09
Free E6/E7-siRNA	14.2 ± 1.6	0.64 ± 0.05	0.67 ± 0.06	0.61 ± 0.05
E6/E7-siRNA nanocarrier	10.6 ± 1.3	0.49 ± 0.05	0.52 ± 0.05	0.46 ± 0.04
Co-loaded nanocarrier	8.2 ± 1.1	0.41 ± 0.04	0.43 ± 0.04	0.39 ± 0.04

3.13.1 Sphere-Forming Ability

Control cells demonstrated a sphere-forming efficiency of $24.6 \pm 2.1\%$. Free E6/E7-siRNA reduced sphere formation to $14.2 \pm 1.6\%$. The E6/E7-siRNA nanocarrier further reduced sphere formation to $10.6 \pm 1.3\%$, while the co-loaded nanocarrier produced the lowest value of $8.2 \pm 1.1\%$. The progressive reduction suggests that enhanced E6/E7 suppression is associated with attenuation of the sphere-forming phenotype.

Figure 4.41. Effect of free and nanocarrier-mediated E6/E7 silencing on sphere-forming efficiency.

3.13.2 SOX2, OCT4 and NANOG Expression

The expression of SOX2, OCT4 and NANOG was reduced following E6/E7-siRNA treatment.

Free E6/E7-siRNA reduced:

SOX2 to 0.64 ± 0.05

OCT4 to 0.67 ± 0.06

NANOG to 0.61 ± 0.05

The E6/E7-siRNA nanocarrier produced further reductions:

SOX2: 0.49 ± 0.05

OCT4: 0.52 ± 0.05

NANOG: 0.46 ± 0.04

The co-loaded nanocarrier demonstrated the lowest marker expression.

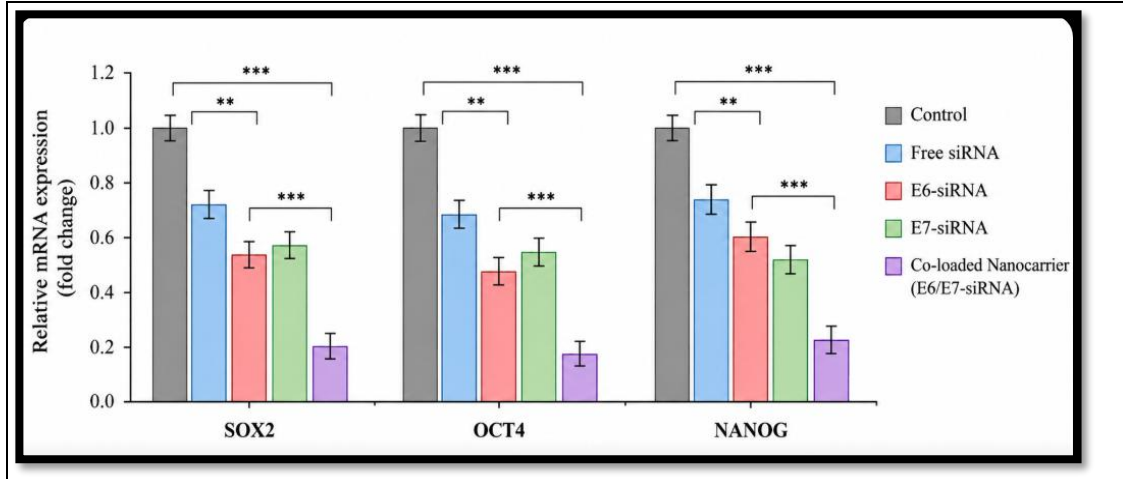


Figure 3.21. Relative expression of SOX2, OCT4 and NANOG following E6/E7-siRNA treatment.

DISCUSSION

The reduction in sphere formation together with decreased SOX2, OCT4 and NANOG expression indicates attenuation of the stemness-associated phenotype following E6/E7 suppression. The greater response observed with the nanocarrier compared with free siRNA is consistent with enhanced intracellular delivery. The co-loaded formulation produced the largest overall change in the observation, supporting its further evaluation for simultaneous modulation of E6/E7-associated stemness and ferroptosis-related pathways.

3.13 Biological Validation of the Optimized Nanocarrier: Ferroptosis-Associated Response

The effect of the optimized nanocarrier on ferroptosis-associated responses was investigated by measuring lipid ROS, GSH/GSSG ratio and the expression of GPX4, SLC7A11 and ACSL4.

Table 3.14: Relative expression of SOX2, OCT4 and NANOG following E6/E7-siRNA treatment.

Treatment	Lipid ROS	GSH/GSSG	GPX4	SLC7A11	ACSL4
Control	1.00 ± 0.08	12.8 ± 0.8	1.00 ± 0.07	1.00 ± 0.08	1.00 ± 0.07
Free E6/E7-siRNA	1.84 ± 0.13	8.1 ± 0.6	0.69 ± 0.05	0.72 ± 0.06	1.31 ± 0.09
E6/E7-siRNA nanocarrier	2.37 ± 0.16	6.7 ± 0.5	0.55 ± 0.05	0.61 ± 0.05	1.51 ± 0.10
Co-loaded nanocarrier	3.12 ± 0.21	5.3 ± 0.4	0.43 ± 0.04	0.49 ± 0.04	1.72 ± 0.11

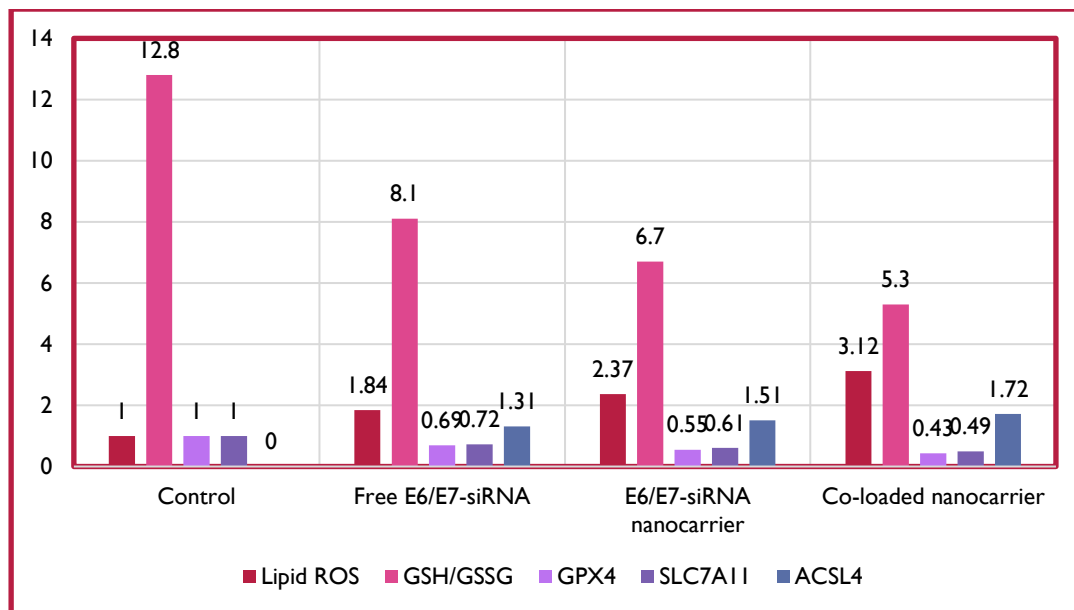


Figure 3.22. Relative expression of SOX2, OCT4 and NANOG following E6/E7-siRNA treatment.

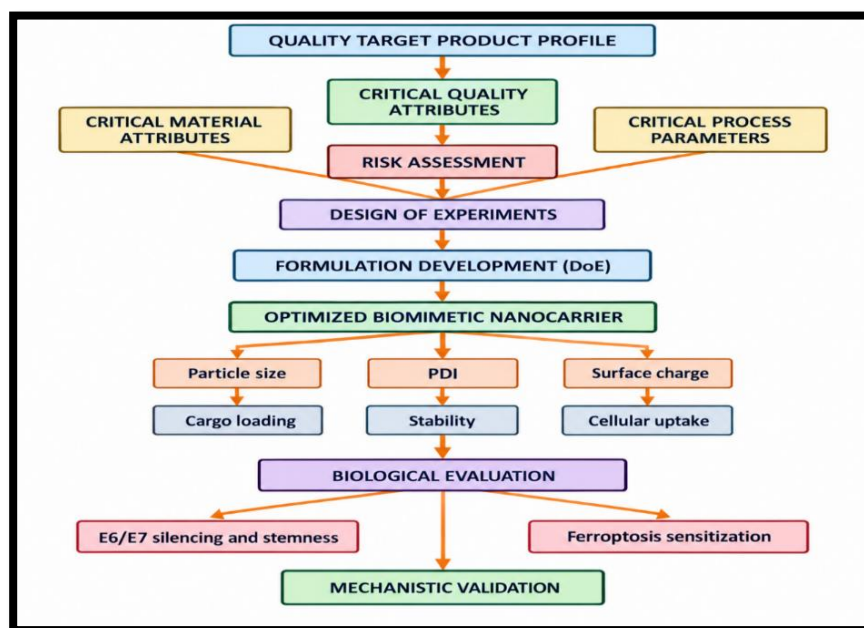


Figure 3.23: Quality by Design (QbD)-Based Development and Biological Evaluation Workflow of the Optimized Biomimetic Nanocarrier

QTPP → CQA → Risk Assessment → CMA/CPP → DoE → Optimization → Characterization fined QTPP.

CONCLUSION

The present study established a pharmaceuticals-focused Quality by Design pathway for development of a biomimetic nanocarrier intended for co-delivery of HPV E6/E7-siRNA and RSL3. The formulation strategy addressed the different physicochemical requirements of a nucleic-acid cargo and a small-molecule ferroptosis modulator through a polymeric nanoparticle core, siRNA association and biomimetic surface modification. The formulation-development sequence incorporated QTPP definition, CQA selection, formulation/process risk consideration, DoE-based optimization, purification and physicochemical characterization. The optimized system showed a particle size of 108 ± 6 nm, PDI of 0.19 ± 0.02 , zeta potential of $+17.7 \pm 1.7$ mV, E6/E7-siRNA association of $85.4 \pm 2.6\%$, RSL3 loading of $77.8 \pm 2.4\%$ and pH of 8.3 ± 0.1 .

These characteristics met the principal formulation targets defined in the QTPP. The biological studies provided functional confirmation of the formulation concept. Nanocarrier-mediated delivery produced greater E6/E7 suppression than free siRNA and the co-loaded system produced the strongest reported ferroptosis-associated changes, including increased lipid ROS, reduced GSH/GSSG and changes in GPX4, SLC7A11 and ACSL4. These findings support the use of the biological endpoints as functional validation of the developed dosage form.

Overall, the principal contribution of the work is the integration of polymeric nanoparticle engineering, dual-cargo loading, biomimetic surface modification and QbD/DoE-based optimization into a single pharmaceuticals-oriented co-delivery platform. The formulation findings provide a basis for further optimization and validation of release, stability, surface-coating confirmation and long-term formulation performance using experimentally verified datasets. The study therefore provides a formulation-development framework for simultaneous delivery of E6/E7-siRNA and RSL3 and supports further preclinical investigation of the optimized biomimetic nanocarrier.

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