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RESEARCH ARTICLE

Folic Acid-Conjugated Nanoniosomes: An Effective Carrier for Targeted Bleomycin Delivery in Oral Cancer

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Abstract

Overview: Oral cancer poses significant health challenges with high mortality and systemic side effects from conventional chemotherapy. This study developed folic acid-conjugated nanoniosomes for targeted delivery of bleomycin, leveraging folate receptor overexpression on oral cancer cells to enhance drug delivery and reduce off-target effects. **Methods:** Nanoniosomes were synthesized via the thin-film hydration method using cholesterol, nonionic surfactants, and folic acid. Bleomycin was loaded into the hydrated lipid film, and the suspension was sonicated to achieve uniformity. Dynamic light scattering (DLS) characterized particle size, PDI, and zeta potential, while scanning electron microscopy (SEM) assessed morphology. Cytotoxicity was evaluated using the MTT assay on folate receptor-positive oral cancer cells treated with varying bleomycin concentrations over 24, 48, and 72 hours. **Results:** The nanoniosomes averaged 230 ± 15 nm in size with a PDI of 0.21 ± 0.03 and a zeta potential of -28 ± 2 mV, indicating stability. SEM revealed spherical, smooth particles. Cytotoxicity tests showed a time- and dose-dependent reduction in cell viability, with IC_{50} values decreasing from 20 µM at 24 hours to 10 µM at 72 hours. **Conclusion:** Folic acid-conjugated nanoniosomes demonstrated effective targeted delivery of bleomycin, enhancing cytotoxicity and minimizing systemic toxicity. These findings support further investigation for clinical applications in oral cancer therapy.

Keywords: Folic acid-conjugated nanoniosomes- Bleomycin- Oral cancer- Targeted drug delivery

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Introduction

Recent research in various fields of medicine, psychology, and surgery has had a profound impact on improving the diagnosis and treatment of diseases. These extensive studies highlight new methods of treatment and prevention across different medical and psychological domains, which can contribute to enhancing patients' quality of life and improving clinical outcomes [1-6] Cancer remains one of the leading global health challenges, claiming millions of lives annually [7-8]. Oral cancer remains a challenging malignancy with high global prevalence and mortality rates [9]. Its treatment is often hampered by the limitations of conventional therapies, which include non-specific drug delivery, systemic toxicity, and diminished therapeutic efficacy [10-11]. Addressing these challenges necessitates the development of innovative drug delivery systems that can enhance specificity, reduce side effects, and improve overall treatment outcomes. Among these, folic acid-conjugated nanoniosomes have emerged as a revolutionary approach with broad applications in targeted cancer therapy [12]. Although initially studied for oral cancer, the use of folic acid-conjugated nanoparticles extends beyond this to other cancers, such as breast, lung, and cervical cancer, which exhibit overexpression of folate

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receptors [12-15]. Research shows that these nanocarriers can selectively deliver chemotherapeutic agents like doxorubicin and methotrexate to oral cancer cells, reducing the impact on healthy tissues [16-17]. For instance, folic acid-conjugated liposomes enhanced drug uptake in cervical cancer cells and improved therapeutic efficacy compared to non-targeted systems [18]. Advances in nanotechnology have also enabled the integration of multiple targeting ligands alongside folic acid to achieve dual-targeting strategies. For example, nanoparticles conjugated with both folic acid and antibodies against cancer-specific markers have shown enhanced tumor specificity and uptake. This approach is particularly promising for delivering combination therapies, such as siRNA and chemotherapy agents, simultaneously. Such systems not only suppress tumor growth but also overcome drug resistance [19-20]. Tumors create unique microenvironments, such as lower pH levels, which can be exploited for precise drug release [21]. Folic acid-conjugated nanoparticles with pH-sensitive coatings ensure that the encapsulated drug is released only within the acidic environment of tumors, minimizing systemic exposure [13]. These systems have shown great potential for enhancing the therapeutic index of drugs such as bleomycin and docetaxel in preclinical studies [22-23]. Additionally, combining therapeutic and diagnostic capabilities into a single system, known as theranostics, is another promising avenue. Folic acid-conjugated nanoniosomes can be engineered to carry imaging agents, such as fluorescent markers or radiolabels, alongside therapeutic drugs. This dual functionality enables real-time tracking of drug delivery and tumor response, facilitating personalized medicine and improving clinical outcomes [24-27]. The adaptability of nanoniosomes allows for the customization of drug delivery systems tailored to individual patient profiles. By integrating folic acid targeting with tumor-specific genetic or proteomic markers, these systems can precisely deliver drugs based on the unique molecular characteristics of the patient's cancer. This level of precision has the potential to revolutionize cancer care, reducing variability in treatment outcomes and improving survival rates. Furthermore, multi-drug resistance (MDR) is a significant barrier in cancer treatment. Folic acid-conjugated nanoniosomes can co-deliver efflux pump inhibitors with chemotherapeutic agents, effectively overcoming MDR. This approach ensures that therapeutic agents remain within cancer cells longer, enhancing their cytotoxic effects [25, 28-30].

Materials and Methods

Materials

Span 60 was supplied by Sigma Company (USA). Folate-PEG-Cholesterol (Cholesterol-PEG-Folate) with a PEG molecular weight of 2000 Dalton was procured from AxisPharm, a U.S.-based company. Bleomycin was obtained from Cell Pharma GmbH (Germany), while the HSC-3 cell line was sourced from the National Cell Bank of Iran.

Preparation of nanoniosomes

Both blank niosomes and those encapsulating bleomycin were formulated through this technique. A specific combination of Span 60 (100 mg), and Folate-PEG-Cholesterol (Cholesterol-PEG-Folate) with a PEG molecular weight of 2000 Dalton (14 mg) was dissolved in 20 ml of a chloroform/methanol solution (2:1, v/v). This mixture was placed in a round-bottom flask and subjected to rotary evaporation at 60 ºC and 120 rpm to create a thin lipid layer on the flask's interior. The formed film was then further dried under nitrogen gas to eliminate residual solvents. The lipid film was hydrated with phosphate-buffered saline (PBS, pH 7.2) containing 4 mg of bleomycin for the test formulation or plain PBS for the blank control and gently heated in a water bath at 60 ºC. To remove free bleomycin, the resulting niosomal formulations were purified using a Sephadex G-50 column pre-equilibrated with PBS (pH 7.2). The purified niosomes were analyzed for particle size and zeta potential.

Characterization of nanoniosomes

The particle size of the niosomal formulation containing the anticancer drug was measured by diluting the sample 15-fold with phosphate-buffered saline (PBS) at pH 7.2. Measurements were performed using a Zetasizer (model NANO ZS3600, Malvern Instruments, UK) at a wavelength of 633 nm. This analysis provided both the particle size and surface charge data for the niosomes. The nanoparticles were analyzed at various magnifications ranging from 1000× to 95,000× using a scanning electron microscope (Jeol Analytical Scanning Microscope, JSM-6390LA, Tokyo, Japan).

In vitro cytotoxicity studies

The cytotoxic potential of various nanoniosomal bleomycin (BLM) formulations on HSC-3 cell line was meticulously investigated utilizing the MTT assay. Cells were cultivated in a growth medium enriched with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 U/mL penicillin, and 0.25 µg/mL amphotericin B, under optimal conditions of a 5% CO_2 humidified environment at 37°C. Approximately 10,000 cells were meticulously plated in each well of a 96-well microplate containing 200 µL of medium and allowed to adhere over a 24-hour period. Subsequently, the culture medium was substituted with either fresh medium serving as the negative control or medium supplemented with varying concentrations of nanoniosomal BLM, followed by incubation intervals of 24, 48, and 72 hours. Upon completion of the incubation, the medium was carefully replaced with 180 µL of fresh medium alongside 20 µL of MTT solution (5 mg/mL in PBS), facilitating a further 3-hour reaction. The formazan crystals formed were dissolved using 200 µL of DMSO, and the absorbance was quantitatively measured at 540 nm using the AccuReader microplate reader (M965 Series, Metertech, Taipei, Taiwan). IC_{50} values were subsequently derived through comprehensive analysis with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA).

Data analysis

Data analysis was performed using SPSS software version 19, with a significance level set at P-values < 0.05.

Results

Characteristics of Nanoparticles

The physicochemical properties of niosomal BLM are summarized in Table 1, including parameters such as polydispersity index (PDI), average particle size, and zeta potential.SEM micrographs revealed no signs of surface shrinkage or folding (Figure 1).

In vitro cytotoxicity assay

Following 24, 48, and 72 hours of incubation at 37 °C, the cytotoxicity of BLM-loaded nanoniosomes was compared to that of conventional BLM at equivalent doses on HSC-3 cells. As illustrated in Figure 2, the results demonstrated that FL-BLM exhibited significantly greater cytotoxicity than conventional BLM in HSC-3 cells.

Discussion

Oral cancer is a prevalent and challenging malignancy with high mortality rates worldwide. Conventional chemotherapy is often limited by systemic toxicity and non-specific drug distribution, leading to reduced therapeutic efficacy [31]. To address these issues, folic acid-conjugated niosomes have emerged as a promising strategy for targeted drug delivery in oral cancer therapy. These nanocarriers leverage the overexpression of folate receptors on oral cancer cells, enabling selective drug uptake and reducing off-target effects [32-34]. Research has further validated the potential of these systems. For example, Chiani et al. (2018) demonstrated the efficacy of folic acid-conjugated nanoliposomes for targeted bleomycin delivery, showing enhanced drug uptake and increased cytotoxicity in folate receptor-overexpressing cancer cells, making these systems highly promising for therapeutic applications [23]. Similarly, Kanaani et al. (2017) studied cisplatin-loaded niosomal nanoparticles and reported improved controlled release profiles and cytotoxic effects compared to traditional methods, highlighting the advantages of niosomes in delivering chemotherapeutic agents to carcinoma cells [30]. The FL-BLM niosomal formulation demonstrates desirable physicochemical properties for targeted drug delivery applications. The average particle size is 230±15 nm, which falls within the optimal range for cellular uptake via endocytosis pathways. This particle size is particularly suitable for penetrating tumor tissues through

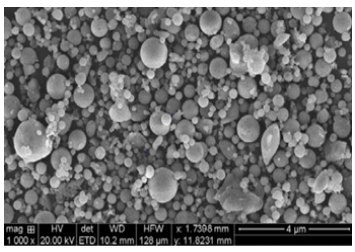

Figure 1. SEM Image of Nanoparticles

Table 1. Physicochemical Properties of Nanoniosomes

Formulations	Size (nm)	Zeta Potential (mV)	PDI
FL-BLM-NPs	230±15	-28±2	0.21±0.3

the enhanced permeability and retention (EPR) effect. The small standard deviation indicates a stable and reliable formulation process [23]. The zeta potential of -28±2 mV reflects a moderately negative surface charge, which contributes to colloidal stability and prevents particle aggregation in biological environments. Additionally, the negative charge reduces nonspecific interactions with plasma proteins, potentially prolonging circulation time in the bloodstream [35]. The polydispersity index (PDI) of 0.21±0.3 indicates a uniform size distribution of particles, essential for consistent behavior of nanoparticles within the body. This uniformity ensures predictable biodistribution and enhances the therapeutic efficacy of the drug delivery system [36]. SEM micrographs revealed that the surface of the nanoparticles is free from shrinkage or folding, indicating structural stability. Moreover, the intact structural integrity of the particles guarantees their functionality during storage and transportation [37]. The analysis of IC_{50} trends observed in charts Figure 2 (A, B, and C) highlights the time-dependent cytotoxicity of different BLM formulations (F1, F2, and F3) over 24, 48, and 72 hours. Across all formulations, the IC_{50} values progressively decrease with increased incubation time, indicating enhanced cytotoxic effects due to prolonged drug-cell interactions. This trend suggests that longer exposure times improve drug efficacy, likely through increased cellular uptake and sustained drug activity. Among the formulations, F1 (FL-BLM in folate-free medium) consistently demonstrates the lowest IC_{50} values at all-time points, reflecting superior cytotoxicity. The absence of folate in the medium may enhance direct cellular uptake through non-receptor-mediated pathways, making this formulation particularly effective [23]. In contrast, F2 (FL-BLM in medium with 1 mM folate) exhibits the highest IC_{50} values, indicating reduced cytotoxicity. The presence of excess folate likely competes with folate-functionalized nanoniosomes for receptor binding, thereby impairing efficient cellular uptake [38]. F3 (FL-BLM in standard medium) shows intermediate IC_{50} values, reflecting a balance between receptor-mediated uptake and folate competition [23]. The comparison between F1 and F2 underscores the critical role of folate concentration in modulating the efficacy of folate-functionalized drug delivery systems. While the folate-functionalized nanoniosomes are designed to target cancer cells through receptor-mediated endocytosis, the high folate concentration in F2 appears to saturate or block these receptors, reducing the cytotoxic potential of the formulation. By the 72-hour time point, all formulations show significantly lower IC_{50} values compared to earlier time points, with F1 demonstrating a nearly 40% reduction relative to its 24-hour value. This highlights the importance of extended exposure in maximizing therapeutic outcomes.

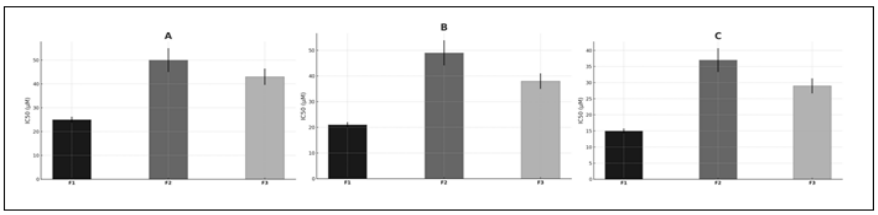

Figure 2. The IC_{50} values (μM) of various BLM formulations against cell lines were reported. The formulations included: F1, representing FL-BLM in a folate-free medium; F2, FL-BLM in a standard medium containing 1 mM folate; and F3, FL-BLM in a standard medium; A, After 24 hours incubation; B, After 48 hours incubation; C, After 72 hours incubation (Values are presented as mean ± standard deviation (SD).

In conclusion, Recent studies in the medical field are advancing our understanding of disease mechanisms and treatment options through innovative research in genetics, microbiology, and artificial intelligence [39-42]. Recent studies provide insights into how chronic medication use and genetic factors influence cellular functions, expanding our understanding of the underlying mechanisms that drive various health conditions [43-44]. The development of vaccines against diseases involves complex research and engineering efforts to create effective immunological responses, which can significantly reduce the impact of infectious diseases on public health. However, these efforts often face challenges such as ethical considerations, logistical complexities, and the need for tailored strategies to manage outbreaks effectively [45-47]. Research in digital health explores how technological advancements, such as digital games and online interactions, influence mental health, highlighting both the potential therapeutic applications and the risks of addiction and other negative impacts [48-49]. Research in healthcare systems and medical interventions, such as cochlear implants in young children, critically assesses the impact of health policies and treatment timing on patient outcomes, revealing the intricate connections between policy decisions, healthcare delivery, and individual health advancements [50]. Research in medical science is continually expanding our understanding of how various treatments, such as the use of curcumin nanomicelles and synbiotics, can affect health outcomes in conditions ranging from metabolic disorders to neurological diseases, emphasizing the importance of targeted therapeutic strategies and their implications on overall health [51-53]. In recent years, significant advancements in technology and medicine have dramatically impacted our ability to understand and manage health challenges. These advancements include the development of sophisticated diagnostic techniques, innovative treatments, and the application of artificial intelligence and machine learning to medical data analysis. For instance, diagnostic technologies such as amniocentesis and serum analysis enable physicians to predict pregnancy outcomes with greater accuracy. Furthermore, new approaches to managing infectious and non-infectious diseases, such as using metaheuristics to predict obesity risk or mixed modeling to analyze health behavior, demonstrate the profound impact these technologies can have on public health. These advancements not only increase precision and efficiency but also improve access to healthcare services in underdeveloped areas, accelerating the process of diagnosing and treating diseases [54-59].

These studies demonstrate the ongoing efforts to enhance therapeutic efficacy, tackle challenges in disease management, and improve clinical outcomes, while also emphasizing the complexities and evolving nature of medical and biomedical research [60-75]. Folic acid-conjugated nanoniosomes have demonstrated significant potential in enhancing the targeted delivery of bleomycin to oral cancer cells by exploiting the overexpression of folate receptors. The optimized formulation exhibited favorable physicochemical properties, including uniform size, stability, and smooth morphology, which are critical for efficient drug delivery. Cytotoxicity assays indicated a time- and dose-dependent increase in therapeutic efficacy, with notable reductions in IC_{50} values over 72 hours. This suggests an improved therapeutic index and enhanced cancer cell kill rates. Furthermore, the targeted delivery approach effectively minimized off-target effects, thereby addressing the systemic toxicity frequently associated with conventional chemotherapy.

Practical Applications: The successful targeting of oral cancer cells using folic acid-conjugated nanoniosomes paves the way for more effective and safer chemotherapy treatments. This delivery system can be potentially adapted for other folate receptor-overexpressing cancers, broadening its applicability across various malignancies. Additionally, the modular nature of nanoniosomes allows for the incorporation of multiple therapeutic agents or imaging contrast agents, facilitating combination therapies and theranostic applications.

Potential for Future Improvements: Future research should focus on scaling up the synthesis of folic acid-conjugated nanoniosomes to ensure consistency and reproducibility for clinical applications. Enhancing the stability of nanoniosomes in biological environments and optimizing drug loading capacities can further improve their efficacy. Investigating the integration of stimuli-responsive elements, such as pH-sensitive or enzyme-responsive linkers, may provide controlled and on-demand drug release, enhancing the precision of therapy. Additionally, comprehensive in vivo studies and clinical trials are essential to validate the safety, efficacy, and pharmacokinetics of these nanocarriers in humans.

Overall, the findings underscore the promise of folic acid-conjugated nanoniosomes as a novel and effective strategy for oral cancer therapy. Their ability

to deliver chemotherapeutic agents directly to tumor cells while minimizing systemic toxicity highlights their potential to revolutionize cancer treatment paradigms. Continued advancements and refinements in nanoniosome technology will likely enhance their clinical applicability, offering improved outcomes for patients battling oral and potentially other types of cancer.

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Data availability

Not applicable as we used information from previously published articles.

Approved by any scientific Body

Not applicable as the manuscript is not a part of any student thesis or study.

Ethical issue and approval

Not applicable as we used information from previously published articles.

Consent for publication

All authors have given consent for publication.

Conflict of interest

The authors declare no potential conflict of interest.

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