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Enhancing Breast Cancer Treatment: Investigating the Influence of Polymer Ratios on Luteolin-Loaded TPGS/Poloxamer Micelles

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Abstract

Luteolin is a widely studied flavonoid recognized for its ability to sensitize multidrug-resistant cells and anti-cancer properties. However, its clinical applications is hindered by challenges arising from its limited solubility and bioavailability. To address this issue, a synergistic approach combiningluteolin with vitamin E TPGS (TPGS) and poloxamer (Pol) was explored to boost tumour apoptosis and suppress P-glycoprotein. This study aimed to optimize luteolin-loaded TPGS/Pol micelles developed using the film hydration method, followed by lyophilization. Various key factors including the drug: polymer ratio and the TPGS: Pol ratio were examined, with encapsulation efficiency (EE) assessed using a UV-Vis spectrophotometer and particle size measured via dynamic light scattering (DLS). The results revealed that the optimized micelle composed of 1:5 drug: polymer ratio and a TPGS: Pol ratio of 3:1, exhibited a particle size below 40 nm, along with EE of 90%. Additionally, there was a notable increase of 459-fold in the solubility of luteolin-loaded micelle in comparison to pure luteolin in water. The TPGS/Pol micelles demonstrated a critical micelle concentration (CMC) of 0.0008 mg/ml, andrelease studies indicated sustained release behaviour for luteolin-loaded micelles. In conclusion, this study has presented the feasibility of TPGS/Pol micelles in improving the therapeutic potential of luteolin, showcasing improvements in EE, particle size, solubility and sustained release behaviour.

Keywords: Luteolin; TPGS; Poloxamer; Optimization; Micelle; Breast cancer.

Introduction

The micellar drug delivery system stands out as a versatile and innovative technology designed to transport poorly soluble drugs to specific target sites. In addition to enhancing the solubility of lipophilic drugs, this system also improves drug bioavailability, targeting precision and release profiles(1,2). Many studies have explored the efficiency of micellar drug delivery systems, particularly in the context of cancer treatment. Conventional chemotherapeutic drugs currently available in the market compromise the quality of life for patients undergoing chemotherapy, owing to their mechanism targeting actively growing cells. However, these drugs inadvertently affect not only tumor cells but also actively growing normal cells, resulting in undesired effects such as alopecia, anemia, and neutropenia(3).

The applicability of micellar drug delivery systems in cancer studies is underscored by their intrinsic targeting

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properties, whether actively or passively achieved, contributing to enhanced drug efficacy and reduced side effects(4,5). Luteolin, a natural bio-compound belonging to the flavone class found in fruits and vegetables like parsley and artichokes, has demonstrated antioxidant, anti-inflammatory, and anti-cancer properties(6,7). Moreover, luteolin shows cytotoxic effects on various cancer cell lines, including lung, colorectal, breast and liver cancer. It demonstrates the ability totrigger cell death, arrest cell cycle progression and inhibit metastasis(8).

Despite its advantageous properties, luteolin's poor solubility in water limits its bioavailability and efficacy, necessitating innovative approaches to fully harness its potential. For instance, luteolin encapsulated in MPEG-PCL micelles has shown a lower inhibition concentration (IC_{50}) and improved pharmacokinetic parameters in contrast to free luteolin(9). This highlights the significance of encapsulating luteolin into micelle: to improve solubility, bioavailability and efficacy.

Vitamin E TPGS, also refer toD-α-tocopheryl polyethylene glycol succinate (TPGS), represents an amphiphilic triblock co-polymer recognized for its multifunctional properties including serving as a solubilizer, surfactant, and additional attribute(10). Extensive research has focused on its application in facilitating drug micellization for cancer therapeutics, leveraging its distinct features such as acting as an inhibitor of P-glycoprotein and inducing apoptosis (Luiz et al., 2021). Similarly, Poloxamer 407, or Pluronics F127, has been explored as a constituent in the construction of encapsulation complexes within micellar nanocarriers for cancer treatment, owing to its amphiphilic nature. Reports indicate that Pluronics exhibit P-gp inhibition in drug-resistant cancer cells(12). Nonetheless, literature suggests that Poloxamer 407's impact on chemo-resistant tumor cells is limited due to its hydrophilicity (HLB:22)(13). To address this limitation, hydrophilic Pluronics have been utilized to enhance circulation time and evade the reticulo-endoplasmic system (RES) within the bloodstream. Therefore, the theoretical synergistic potential of combining these two copolymers with luteolin holds promise in combating breast cancer cells.

In this study, we aim to develop a micellar system comprised of TPGS and Poloxamer 407 (Pol) to encapsulate luteolin and enhance its solubility in water. Our hypothesis proposes that the polymer/drug and the polymer/polymer ratio will significantly influence the physicochemical properties of the micelle, as suggested by prior research. The objectives of this study include investigating the impact of polymer/polymer and polymer/drug ratios on micelle properties, developing micelle with smaller particle size and increased encapsulation efficiency, and characterizing the solubility and release profile of the optimized micelle.

Materials and Methods

Materials

Luteolin and vitamin E TPGS (tocofersolan) were supplied by MedChem Express (Monmouth Junction, NJ, USA). Poloxamer 407 (Pol) was obtained from Sigma-Aldrich (St. Louis, MO, USA), while ethanol was acquired from R&M Chemicals SdnBhd (Subang, Malaysia), while

Development of luteolin-loaded micelle

The development of luteolin-loaded micelles was conductedthrough thin film hydration method, following the procedure outlined in a previous study(14). Luteolin, TPGS and Polwere mixed in ethanol until achieving homogeneity. After removing the solvent utilizinga rotary evaporator, the resulting thin film underwent overnight vacuum-drying. Subsequently, the thin film was immersed in 10 mL of water with continuous stirring and heating until a micellar solution formed. The solution was then subjected to centrifugation and filtration, followed by freeze-drying to produce a solid micellar cake powder, which was kept at 4 °C until further analysis.

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Optimization of drug: Polymer ratio and TPGS: Pol ratio

The impact of drug: polymer ratio and TPGS: Pol ratio of luteolin-loaded micelleson encapsulation efficiency (EE) was explored. The drug: polymer ratio was predefined at 1:2.5, 1:5 and 1:7.5, while the TPGS: Pol ratio was adjusted to 4:0, 3:1, 1:3 and 0:4. The resulting micellar solutions underwent freeze-drying and kept at -4 °C for EE assessment. The micellar cake powder demonstrating optimized EE was then selected for particle size analysis.

Encapsulation efficiency assessment

The method for assessing EE was adapted from Patra et al.(14).A total of 1 mg of micellar cake powder was mixed with 5 mL of ethanol for micelle disruptionto facilitateluteolin release. The absorbance was assessed at a wavelength of 350 nm after dilution with ethanol. Triplicate measurements were performed, and the data are presented as mean ± standard deviation (SD). The percentage of EE was calculated using the following equation:

$$\%\mathrm{EE} = \frac{\text{Luteolin weight in micelle}}{\text{Initial weigth of luteolin}} \times 100$$

Particle size determination

The size of the micelles was measured using dynamic light scattering (DLS) with a Litesizer 100 (Anton Paar, Graz, Austria). The micellar cake powder was dissolved at a concentration of 1 mg/mL and diluted 10 times, followed byfiltrationutilizing a 0.22 µL syringe filter before being transferredinto the cuvette. The measurements were taken at a detection angle of 175°, with the temperature maintained at 25 °C.

Transmission electron microscopy

The Libra 120 Transmission electron microscopy (TEM, Carl Zeiss, Jena, Germany) was employed to reveal the surface morphology of the micelles and to confirm theirparticle size. A copper grid stained with a 2% phosphotungstic acid solution was deposited with a drop of micellar solution and observed under TEM after drying. This step provided additional insights into the micelle's structural characteristics.

Solubility Study

The solubility study was conducted following the method outlined in previous published literature(14), with slight modifications.Distilled water at 25 °C was added with an excess amount of luteolin powder and stirred at 120 rpm in a shaker incubatorfor 72 hours. After centrifugation and filtration through a 0.22 µm syringe filter, 5 mL of ethanol solution was added to 1 mL of the supernatantfor micelle disruption. The absorbance was measuredusing a UV spectrophotometer (Uviline 9400, Secomam, Mainz, Germany)at a wavelength of 350 nm.

Critical micelle concentration determination

The CMC of micelle was assessed using iodine as a hydrophobic probe(Patra et al., 2018; Saxena & Hussain, 2012).A potassium iodide/iodinestandard solution was mixed the serially diluted blank optimized micelle solution with concentrations ranging from 0.1% to 0.000001%. The mixtures were then placed in the dark at room temperature for 12 hours. Absorbance was measured using a UV-Vis spectrophotometer (Uviline 9400, Secomam, Mainz, Germany)at a wavelength of 366 nm, and the CMC was determined as the polymer concentration corresponding to an increase in absorbance, plotted against log polymer concentration.

***In-vitro* drug release study**

Dialysis method was performed to determine and compare the release kinetics of bothluteolin-loaded micelles and free luteolin (14,16).Dialysis membrane bags with sealed ends were separately filled with 1 mg of lut-loaded micelle and free luteolin in ethanol. These bags were then placed into 100 mL release media consisting of 0.5% Tween 80 in phosphate-buffered saline solution of pH 7.4 at 37 °C under 100 rpm of horizontal shaking.

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A total of 1 mL of aliquot of therelease media were withdrawn at predefined intervals, and the absorbance was assessed using a UV-Vis spectrophotometer at a wavelength of 350 nm. Concurrently, 1 mL of fresh release media was replenished each time an aliquot was withdrawn. To simulate the microenvironment of tumour cells, the release kinetic of luteolin-loaded micelles was also assessed in release media of pH6.8, as intracellular tumour cell pH typically ranges from 6.7 to 7.1 (17,18). This comprehensive investigation enables a thorough understanding of the drug release dynamics under conditions that mimic both physiological and tumour microenvironments.

Statistical analysis

The data are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparisons for significance were performedusing JASP SOFTWARE (version 0.16.1). A significance level of $p < 0.05$ was considered statistically significant.

Results and Discussion

The impact of drug: Polymer ratio and polymer/polymer ratio on encapsulation efficiency

EE in nanoparticles is regarded as their capability to efficiently encapsulate and preserve bioactive compounds, proteins or drugs within their structure. This parameter holds significance in determining the efficacy and stability of delivery systems based on nanoparticles. The selection of materials in nanoparticle formulation stands out as a crucial factor influencing encapsulation efficiency. In this context, Table 1 presents that the mixed polymeric micelle outperformed its individual polymeric micelle counterparts with notably higher encapsulation efficiency recorded. This observation aligns with the findings of Chang et al. (19), wherebythe mixed micelles of PEGMEMA 12/PS 595 loaded with curcumin exhibited greater EE than single micelles comprising of PEO–PCL.

Table 1 demonstrates the EE of TPGS/Pol micelles, ranging from 74.3 nm to 92.3 nm. Among the various drug: polymer ratios (1:2.5, 1:5, and 1:7.5), ratios 1:5 (b) and 1:7.5 (c) exhibited higher EE compared to 1:2.5 (a). Particularly noteworthy is the substantial increase in EE at TPGS: Pol ratio 3:1 (B), surpassing other ratios and individual micelles significantly. This trendsuggests that a higher polymer-to-drug ratio correlates with increased EE. Consequently, TPGS: Pol ratio 3:1 emerged as the optimized ratio due to its superior EE compared to other ratios.

The formulations (Bb) and (Cb) were identified as optimized micelles owing to the greater EE obtained. Despite the negligible difference in EE between these two formulations, formulation Bb was selected for subsequent characterization studies. While a previous study proposed that higher EE holds promise in delivering active compounds by enhancing their bioavailability(20), other factors like cost and reproducibility werealso

Table 1: The encapsulation efficiency of luteolin-loaded tpgs/poloxamer micelle with different drug: polymer ratio and polymer: polymer ratio

	Encapsulation Efficiency (%)		
Drug: polymer ratio	1: 2.5 (a)	1: 5 (b)	1: 7.5 (c)
TPGS: Pol ratio			
4:0 (A)	78.6 ± 0.6	80.3 ±0.5	86.2 ± 0.5
3:1 (B)	85.5 ± 1.0	90.7 ± 0.9	92.3 ± 0.6
1:3 (C)	77.7 ± 0.2	79.9 ± 0.3	77.4 ± 0.5
0:4 (D)	77.0 ± 0.7	78.5 ± 0.4	74.3 ± 0.4

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taken into consideration during the selection of an optimized micelle. Micelle(Bb), utilizing a lesser amount of polymers, allows for the production of a larger quantity of micelles for subsequent tests. This strategic selection aligns with the goal of optimizing both performance and resource utilization in nanoparticle-based delivery systems.

The impact of drug: Polymer ratio and polymer: polymer ratio on particle size

In Table 2, the particle sizes of the optimized TPGS: Pol ratio was examined across various drug: polymer ratios. The recorded particle sizes range from 26.72 to 28.65 nm. Notably, the micelle with a drug: polymer ratio of 1:5 (b) exhibits the smallest particle size in comparison to ratios 1:2.5 (a) and 1:7.5 (c). The particle size analysis revealed significant variations among the micelles with different TPGS: Pol ratios, particularly in the 4:0 and 0:4 configurations, as shown in (Table 3). Notably, the micelle with TPGS: Pol ratio of 4:0 exhibited a considerably larger size than the 0:4 counterpart, indicating that TPGS contributes to the formation of smaller micelles compared to Pol. Intriguingly, the combination of both polymers resulted in a larger-sized micelle compared to each polymer individually, suggesting a synergistic effect in micelle size modulation.Significant differences in particle size were also noted between micelles with TPGS: Pol ratios of(Bb) and (Cb), further emphasizing that (Bb) represents the optimized micelle configuration. Consequently, this optimized micelle (Bb) was selected for subsequent tests and characterizations.

Table 2: The particle size of optimized micelle in comparison with other micelles at the same TPGS:Pol ratio (3:1), but different drug: polymer ratio

Drug: Polymer Ratio	Particle Size (nm)
1: 2.5 (a)	26.72 ± 1.55
1: 5 (b)	24.57 ± 0.61
1: 7.5 (c)	28.65 ± 1.32

This observed increase in size, as shown in Table 4, could be attributed to the hydrophilicity of poloxamer 407. In agreement with findings by Wei et al.(21) and Fares et al.(22), the presence of hydrophilic head from poloxamer 407 might contribute to the enlargement of micellar size. Moreover, the proportion of the hydrophilic polymer may influence micelle size, as supported by literature indicating that a lower concentration of hydrophilic polymer tends to reduce micelle size.

This finding establishes the drug: polymer ratio of 1:5 as the optimized configuration, and the formulation (Bb) will be utilized in the subsequent section to refer to the optimized micelle (TPGS: Pol ratio 3:1; drug: polymer ratio 1:5).

The typical size range of nanoparticle is between 1 to 1000 nm, a crucial characteristic with significant implications for theirproperties and behaviour(23), specifically regarding their ability to enhance permeability and retention to improve the penetration of tumour cell. Generally, the particle size of

Table 3: The particle size of optimized micelle in comparison with other micelles at the same drug: polymer ratio (1: 5), but different polymer: polymer ratio

Polymer: Polymer Ratio	Particle Size (nm)
4: 0	18.18 ± 1.01
3: 1	24.57 ± 0.61
1: 3	27.65 ± 1.11
0: 4	22.56 ± 0.66

Table 4: The solubility of luteolin when loaded into micelle in water in comparison with free luteolin in water

	Solubility in water (ug/ml)
Luteolin in water	30.67
Luteolin-loaded micelle in water	2594.02

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micelle must be below 200 nm in order for it to effectively reside within a tumour's blood vessels. The range of nanoparticle sizes conducive to the EPR effect generally falls between 1 to 400 nm (Chentamara et al., 2019).However, if the micelle's size exceeds 200 nm, there is a risk of elimination from the body through the reticuloendothelial system (RES)(24). Thus, thorough monitoring of nanoparticle size is vital to ensure the optimal delivery and efficiency of the drug within the tumour site.

The TEM image shown in Figure 1 illustrates the spherical shape of the micelle, characterized by distinct light grey and black layers, which corresponding to the hydrophilic and hydrophobic regions, respectively. This visualization supports our hypothesis regarding the dissolution and encapsulation of luteolin within the hydrophobic region of the micelle.

Solubility study

The water solubility of luteolin was 30.67 µg/mL, but significantly increased to 2594.02 µg/mL when encapsulated within a TPGS/Pol micelle. This represents a 459 times increase in solubility compared to its solubility in pure water. This phenomenon is further evident by visually examining the solution's physical appearance. In the absence of micelle encapsulation, the presence of luteolin leads to sedimentation, emphasizing the effectiveness of the micelle in preventing such precipitation.This observation highlights the efficacy of the TPGS/Pol micelle in enhancing the solubility of hydrophobic drugs likeluteolin in aqueous environments.

CMC determination

TheCMC was established by monitoring the abrupt rise in absorbance of the KI/I2 solution across diverse dilutions of the optimized blank micelle. The point of CMC determination was identified as the intersection between the steady absorbance reading and the sudden increase in absorbance. As illustrated in Figure 2, the CMC was precisely determined at a log

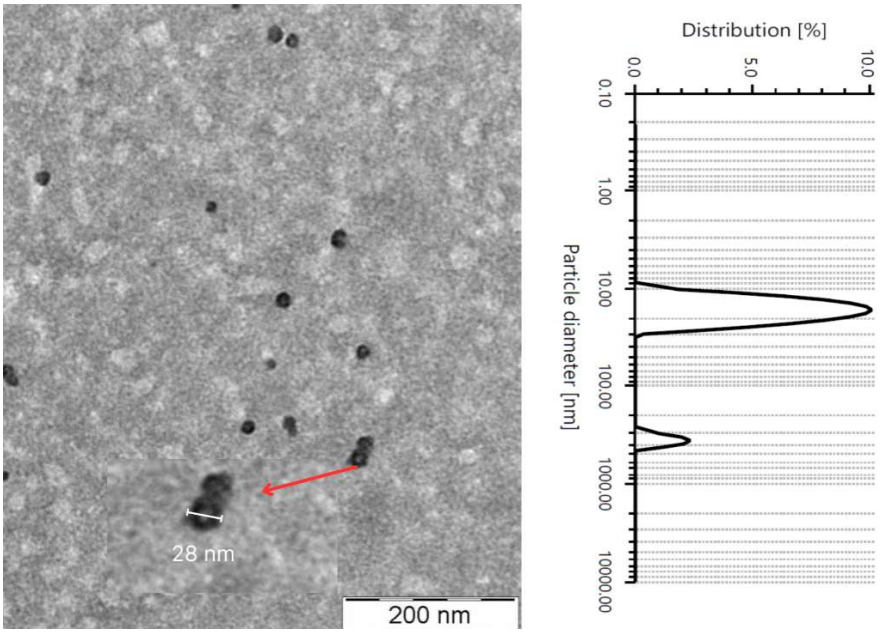

Figure 1: Particle size obtained by transmission electronic microscope (TEM) (left) and dynamic light scattering (DLS) (right)

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concentration of -3.08, corresponding to 0.0008% v/v.

The CMC signifies the lowest surfactant concentration required for self-assembly and encapsulation, transforming into a micelle. Below the CMC, surfactant molecules arrange themselves at the water surface, with the hydrophilic region orients inward, interacting with water molecules, while the hydrophobic region faces outward away from water.Once the surfactant concentration exceeds the CMC, self-assembly occurs, with the hydrophobic region positioning at the micelle's core and encapsulating the hydrophilic region.

Figure 2 demonstrates that the TPGS/Pol micelle exhibited a CMC of 0.0008% w/v. This finding aligns with previous studies, which reported CMC values for TPGS/Pol micelles at 0.0013% w/v (15)and 0.0015% w/v (14). Comparatively, the CMC value for TPGS micelles is 0.00052% w/v, while for Pol micelles it is 0.0575% w/v(25). Therefore, the combination of these two polymers yielded an intermediate value. Moreover, TPGS/Pol micelle exhibited a CMC value that leaned toward the CMC value of pure TPGS micelles, attributing to the greaterproportions of TGPSin the optimized formulation(14,22,25).

The evaluation of CMC holds crucial significance in nanoparticle studies, particularly for micelles. This is attributed to the potential disassembly of micelles in body fluids due to the extreme dilution below its CMC, which eventually affected the intended function of delivering drugs to targeted sites. Hence, micelles with lower CMC values could facilitate the effective drug transport owing to the heightened survivability and stability in body fluids.

In-vitro drug release study

Luteolin released without micelles exhibited a rapid release profile, reaching 100% within less than 4 hours. Conversely, a distinct pattern emerged in the release of luteolin encapsulated within micelles. A rapid initial release was demonstrated in the first 10 hours, followed by a steady increase in the release of luteolin over time in both

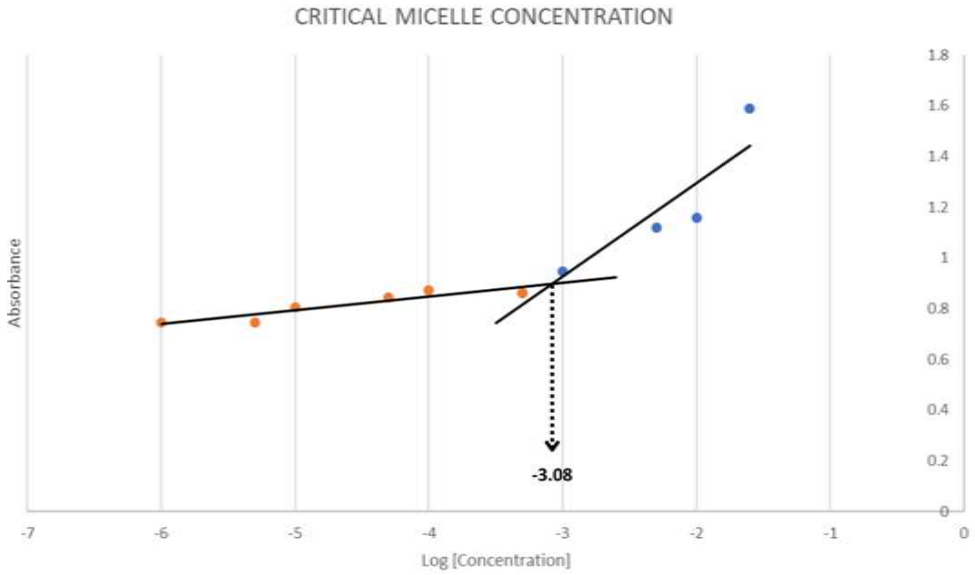

Figure 2: Critical micelle concentration determination by measuring the absorbance of KI/I_2 in diluted optimized blank micelle

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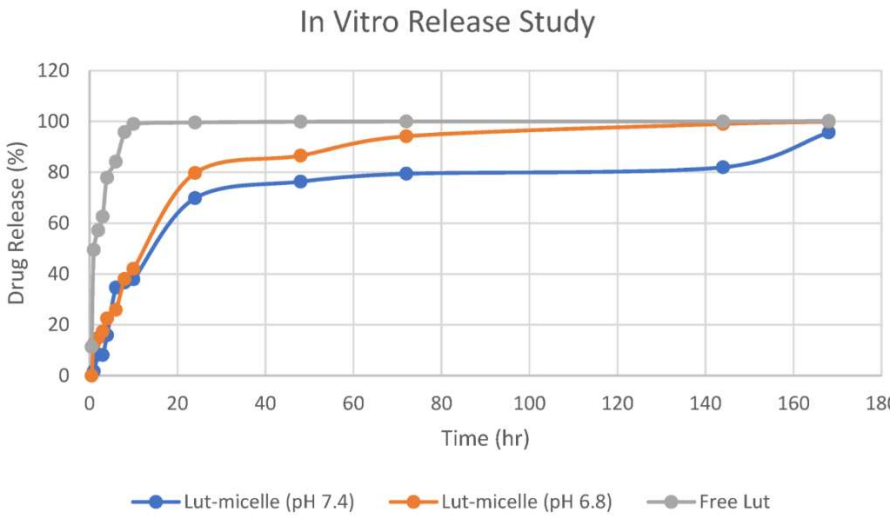

Figure 3: Release profile of free luteolin (grey), luteolin-loaded micelle in pH 7.4 (blue) and luteolin-loaded micelle in pH 6.8 (orange)

media. Remarkably, a continuous steady increase was observed for up to 7 days at physiological pH (pH 7.4). However, pH 6.8 resulted in a greater luteolin release compared to pH 7.4, possibly attributed to luteolin's increased solubility in lower pH environments due to partitioning effects(17). This information suggests that luteolin has the capability to be released liberally in slightly acidic tumour cells while exhibiting prolonged release in body fluids with physiological pH.

Figure 3 shows that free luteolinexhibited a more rapid release compared to luteolin-loaded micelles, with a continuous release observed for up to 7 days. This resultis consistent with prior research, where luteolin without micelles exhibited a burst-like release, whereas loading luteolin into micelles resulted in a sustained release(26–28). The sustained release behavior may result from the hydrolysis or degradation of themicelle, along with the polymer erosion and swelling, facilitating the luteolin to diffuse from micelle into the release medium.

Conclusion

The development and optimization of luteolin-loaded micelles using TPGS and Pol has been successfully achieved. The optimized micelle configuration was attained with TPGS: Pol ratio of 3:1 and a drug: polymer ratio of 1:5. This configuration exhibited remarkable characteristics, including a particle size below 40 nm, an EE of 90% and a CMC of 0.0008% w/v. Additionally, the micelledemonstrated a significantly enhanced solubility of luteolin, emphasizing its potential as a carrier for hydrophobic drugs. Furthermore, the sustained release behaviour of luteolin was demonstrated in the optimized micelle, showcasing controlled release over an extended period. Overall, this research lays a solid foundation for the utilization of TPGS/Pol micelles in drug delivery, particularly for hydrophobic compounds like luteolin, with potential implications for enhancing therapeutic efficacy and addressing solubility challenges in cancer treatment.

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