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Design and Characterization of a Transethosome-Based Gel for Cutaneous Administration of Genistein

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ABSTRACT

In this study, lipid nanovesicular systems such as ethosome and transethosome dispersions were studied as potential vehicles for the cutaneous administration of genistein, an isoflavone with antioxidant and chemopreventive properties. The lipophilicity of genistein requires specialized delivery systems suitable for transdermal administration. Genistein loading in ethosomes and transethosomes, prepared by water injection and ethanol injection methods, was investigated. The evaluation of the dispersion macroscopic stability enabled the selection of transethosomes, produced by the ethanol injection method, loaded with genistein 0.25 mg/mL. The morphology of the vesicles was studied by cryogenic electron microscopy and by small-angle x-ray scattering, while the size distribution and the stability of genistein were evaluated for 3 months by photon correlation spectroscopy and high-performance liquid chromatography, respectively. The results showed vesicle mean values of 111–145 nm, polydispersity indexes of 0.13–0.2, and drug entrapment capacity of 96% w/w. Transethosomes maintained physical-chemical stability both as size distribution and genistein content. The zeta potential value ranged between –19 and –24 mV, while the pH of the formulation was around 5.5, suitable for skin application. Photochemiluminescence studies confirmed the antioxidant activity of genistein-loaded transethosomes. The diffusion kinetics of genistein, studied in vitro by Franz cells, demonstrated that transethosomes increased the drug permeation threefold with respect to the genistein suspension. In view of a possible skin application, the transethosomes were thickened with 0.5% xanthan gum, selected through leakage and spreadability studies. Furthermore, tape stripping studies performed within 6 h after formulation application on the skin demonstrated a gradual increase of genistein content in the *stratum corneum* in the case of transethosome gel, compared to the conventional gel. The genistein-loaded transethosome gel could exert a long-lasting protection, crucial for preventing UV-induced skin damage and photoaging.

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1 | Introduction

Lipid nanovesicular systems have gained a huge interest in pharmaceuticals due to their drug delivery potential [1]. Indeed, the spontaneous assembly of natural lipids, such as phosphatidylcholine, gives rise to bi-layered vesicles with a supramolecular organization resembling the physiological membranes [2]. These nanovesicular systems, such as liposomes, transferosomes, ethosomes (ETs), and transethosomes (TEs), typically composed of natural phospholipids such as phosphatidylcholine, are effective in delivering both hydrophilic and lipophilic drugs [3, 4]. Since the 1960s, liposome applications as drug delivery systems have been widely recognized, offering the possibility to control drug release under administration by different routes [5]. However, the transdermal potential of liposomes can be hampered due to their inability to reach the deeper layers of the skin [6, 7]. Indeed, some authors suggest that the vesicles remain confined to the more superficial layers, while their affinity with the lipids of the stratum corneum allows their fusion and formation of a depot from which the active compounds are gradually released [8–10]. Other authors demonstrated that intact liposomes can reach the deeper layers only through skin appendages (hair follicles and sweat glands) [9, 11]. ET has been described for the first time by Tuitou et al. [12] in order to overcome the limitations of conventional liposomes in delivering active compounds through the skin. Their composition mainly differs from liposomes due to a high percentage of ethanol (20%–45%), in addition to phospholipids and water [13]. Ethanol confers many advantages; indeed, it allows (i) to reduce vesicle size; (ii) to enhance the vesicle stability and flexibility with respect to classic liposomes; (iii) to increase the entrapment efficiency of lipophilic drugs; and (iv) to confer a negative surface charge to the vesicles, preventing their aggregation and drug leakage [13]. Moreover, the ethanol penetration enhancer behavior promotes the vesicle penetration through the skin toward the deepest layers [14].

The second generation of ET, typified by TE, was firstly produced by Song et al. in 2012 [15] to further increase the permeability of nanovesicles. In TE the presence of edge activators, such as glycerol or surfactants, can further increase the penetration ability, making the vesicles even more malleable than ET [16]. Edge activators intercalate in the lipid bilayer of the vesicles, altering its packing, thus changing the nanovesicle size, permeability, and flexibility [17].

Genistein (GEN) is an isoflavone with estrogen-like action, possessing a plethora of biological activities, such as antioxidant, anti-inflammatory, and antimicrobial effects, and is widely studied for its chemopreventive and anticancer action against several types of tumors [18–20]. GEN can prevent skin damage, particularly UV-induced photodamage [21, 22]. Vu et al. [23] demonstrated that GEN administration was effective in preventing UVB-induced skin carcinogenesis in mouse models, resulting in even more efficacy when administered topically than orally. Moreover, topical application of GEN efficaciously protected against UVB-induced skin photodamage on human skin in a clinical trial and photodamage in hairless mouse skin [21].

However, GEN's low solubility and metabolic susceptibility hinder its therapeutic application [24]. Thus, in the present study, to overcome GEN limitations, its delivery through vesicular nanosystems, such as ET and TE, has been proposed by virtue of their

promising potential for topical drug delivery. GEN has already been investigated for its incorporation into nano-based delivery systems for dermatological purposes [25], but to the best of our knowledge, its delivery via ET and TE has not been considered yet.

Accordingly, a preformulatory study was conducted to select a nanovesicular system for GEN delivery through the skin. ET and TE have been prepared by two different methodologies, water injection (WI) and ethanol injection (EI). The selected colloidal dispersions were then characterized in terms of size, zeta potential, and morphology. Entrapment capacity (EC), as well as in vitro permeation of GEN, was evaluated. A TE gel based on x-gum was obtained to enable GEN application and permanence on the skin, evaluating leakage, spreadability, and rheology. Small angle x-ray scattering (SAXS) was employed to gain insight on the supramolecular organization of the formulations. The formulation antioxidant activity was evaluated in vitro by the photochemiluminescence (PCL) test, while the in vitro cytotoxicity was evaluated on HaCat cells. Finally, in vivo evaluations on healthy human volunteers, based on patch tests and tape stripping, were performed to prove the safety and the potential of the nanovesicular systems for the transdermal delivery of GEN [26].

2 | Materials and Methods

2.1 | Materials

GEN (purity $\geq 98\%$) was purchased from Cayman Chemical Company (Michigan, USA). Phosphatidylcholine from soybean (PC) (purity 94%) was acquired by A.C.E.F. Spa (Fiorenzuola d'Arda [PC], Italy). Polysorbate 80 (polyoxyethylene sorbitan monooleate, Tween 80, TW80) and xanthan gum (X-Gum) were bought from Merck Life Science S.r.l. (Milan, Italy). STRAT-M membranes were purchased from Merck Life Science S.r.l. (Milan, Italy). Solvents were of HPLC grade, and all other chemicals were of analytical grade.

2.2 | Preparation of ETs and TEs

Two different approaches were employed, namely the WI and the EI methods [27, 28].

In both cases, a PC solution (30 mg/mL) in ethanol was prepared under magnetic stirring at room temperature (IKA RCT basic, IKA-Werke GmbH and Co. KG, Staufen, Germany). For TE, polysorbate 80 (Tween 80, 10 mg/mL) was solubilized in the PC-ethanol solution.

To obtain the vesicles via the WI technique, the aqueous phase was incrementally added to the ethanolic lipid phase under stirring.

In the EI method, the procedure was reversed: The PC-ethanol solution (with or without Tween 80) was gradually added to bidistilled water under the same stirring conditions.

The water/ethanol ratio was 70:30 (v/v), and magnetic stirring was maintained at 750 rpm for 30 min at room temperature.

For both WI and EI, drug-loaded formulations (0.2–0.5 mg/mL of GEN) were obtained by solubilizing GEN in the ethanol solution before mixing. All steps were carried out in the dark to avoid photodegradation.

2.3 | Cryogenic Electron Microscopy (CRYO-EM)

Sample vitrification was performed using a Vitrobot mkIV (Thermo Fisher Scientific) at 4°C and 95% humidity. A 3 µL aliquot of the suspension was applied to each grid, followed by double-sided blotting (3 s, blot force 3) before plunge-freezing in liquid ethane. Cryo-EM data were collected at the National Cryo-EM Centre SOLARIS (Kraków, Poland) using a Titan Krios G3i microscope (Thermo Fisher Scientific) operating at 300 kV. Images were acquired with a K3 direct electron detector and a BioQuantum Imaging Filter (Gatan) utilizing a 20 eV slit width in counting mode. Data collection was managed via EPU 2.10 at a magnification of $\times 105,000$, resulting in a pixel size of 1.7 Å and a total dose of 41.09 e[−]Å^{−2} at a nominal defocus of -2.0 µm.

2.4 | Photon Correlation Spectroscopy (PCS)

Vesicle size and polydispersity index (PDI) were assessed through PCS using a Malvern Zetasizer (633 nm laser), Zetasizer Nano-S90 instrument (Malvern Panalytical Ltd., Malvern, UK) equipped with a 5 mW helium–neon laser (633 nm), at 25°C and a detection angle of 90°. Samples were diluted tenfold with bidistilled water before measurement [27, 28]. Data processing was based on the “CONTIN” algorithm [29], and all determinations were conducted in triplicate, with results expressed as mean $\pm$ standard deviation.

2.5 | Zeta Potential

The zeta potential of the samples was measured at 25°C using a Zetasizer Pro (Malvern Panalytical, UK) equipped with a 633 nm He–Ne laser. Samples were diluted 1:10 (v/v) in bidistilled water and analyzed in DTS1070 capillary cells. The instrument calculated zeta potential from the electrophoretic mobility of the particles detected through laser light scattering.

2.6 | Entrapment Capacity

To determine drug entrapment, colloidal dispersions (500 µL, 24 h postpreparation) were transferred to Microcon centrifugal filter units equipped with YM-10 membranes (NMWCO 10 kDa; Sigma-Aldrich, St. Louis, MO, USA). Centrifugation was performed at 4000 rpm for 20 min using a Spectrafuge 24D Digital Microcentrifuge (Infitek Inc., Spokane, WA, USA). To quantify the entrapped fraction, the vesicles retained on the membrane were diluted tenfold with ethanol and subjected to magnetic stirring for 30 min to facilitate complete bilayer disruption and drug release. Simultaneously, a 100 µL reference aliquot of the bulk dispersion was processed using the same protocol to establish the total drug content [27, 28]. All samples were filtered through 0.22 µm nylon syringe filters before HPLC analysis. The EC was calculated as follows:

$$EC = \frac{D}{T_D} \cdot 100, \quad (1)$$

where D corresponds to the amount of GEN retained within the vesicular fraction, and T_D is the total GEN content in the formulation.

2.7 | HPLC Analysis

Quantitative determinations were performed on a Perkin Elmer Series 200 HPLC system (PerkinElmer, Waltham, MA, USA)

equipped with a micropump, autosampler, and UV detector. Separation was achieved on a Hypersil BDS C18 stainless steel column (15 $\times$ 0.46 cm, 5 µm particle size; Thermo Fisher Scientific S.p.A., Milan, Italy). The mobile phase, consisting of acetonitrile/water (40:60, v/v) + 0.1% trifluoroacetic acid (TFA), was delivered at a constant flow of 1 mL/min. The UV detector was set at 260 nm, with an injection volume of 5 µL. Under these chromatographic conditions, the retention time of GEN was approximately 4.1 min.

2.8 | Measurement of pH

A Crison Basic C20 pH meter (Crison Instruments, S.A., Alella, Barcelona, Spain) equipped with a pH 50 12-Hach electrode was employed to measure the formulation pH.

2.9 | Stability Studies

Physical-chemical stability of formulations was studied by evaluating size distribution, Z-potential, and GEN loading in samples stored at 4°C and protected from light for 3 months, repeating analyses at different time intervals. Specifically, size distribution and Z-potential were evaluated by Zetasizer Nano-S90 and Zetasizer Pro, while GEN chemical stability was evaluated by HPLC.

2.10 | In Vitro Permeation Test (IVPT)

Permeation experiments were conducted using Franz vertical diffusion cells (orifice diameter 0.9 cm; PermeGear Inc., Hellertown, PA, USA) equipped with STRAT-M synthetic membranes [27]. These membranes are composed of two polyethersulfone layers over a polyolefin backing and impregnated with synthetic lipids, providing a reliable biomimetic model of the stratum corneum barrier [30].

The receptor compartment contained 5 mL of an ethanol/water mixture (50:50, v/v) to ensure sink conditions and was maintained under continuous magnetic stirring (500 rpm) at 32°C for the entire experimental period. In the donor compartment, 1 mL of the test formulation was applied, and the system was sealed to minimize evaporation. At predetermined intervals (hourly from 0 to 24 h), 0.5 mL aliquots were withdrawn from the receptor phase and analyzed by HPLC; each sample removal was compensated with an equal volume of fresh receiving medium.

The cumulative permeated amount of GEN, expressed as µg/cm², was plotted against time. In accordance with Fick's law of diffusion, the drug concentration in the receptor is negligible compared to that in the donor, so the concentration gradient approximates the donor concentration. The linear portion of the permeation profile corresponds to the steady-state region, from which the flux (J_{ss} , µg·cm^{−2}·h^{−1}) was determined as the slope. The permeability coefficient (K_p) was calculated from the relationship as follows:

$$K_p = \frac{J_{ss}}{C_d}, \quad (2)$$

where C_d is the GEN concentration in the donor phase.

The lag time (T_{lag}), representing the period required to reach steady-state permeation, was obtained from the x-intercept of the regression line.

2.11 | Gel Preparation

TE dispersions were converted into semisolid forms by the addition of X-Gum. Twenty-four hours after preparation, both unloaded and GEN-loaded TE were thickened by slowly sprinkling X-Gum into the colloidal dispersion while maintaining continuous magnetic stirring to prevent the formation of lumps and ensure homogeneity of the system. Three concentrations of the polymer were assessed (0.5%, 0.75%, and 1% w/w) [27].

All operations were performed at room temperature; for GEN-containing formulations, the procedure was conducted in the dark to avoid light-induced degradation. As a control gel for *in vivo* studies, GEN was dispersed directly in water and then thickened with X-Gum following the same gradual mixing procedure.

The obtained TE-based gels were characterized one day after preparation by means of spreadability and leakage assays.

2.12 | Spreadability Test

The TE gel spreadability was determined by examining the area covered by the formulation when a constant weight was applied [27]. In brief, 100 mg of the gel was placed at the center of a glass Petri dish (3 cm in diameter) and pressed with a glass plate carrying a 50 g weight. After 10 s, the diameter of the spread area was recorded. The spreadability (S) was calculated using the formula as follows:

$$S = \frac{m \cdot l}{t}, \quad (3)$$

where m represents the applied weight in grams, t is the duration of pressure (10 s), and l is the diameter of the area occupied by the gel in centimeters. Each measurement was repeated three times for every formulation tested.

2.13 | Leakage Test

To test TE gel leakage, their sliding ability on agar gels was evaluated [27]. Agar gels were previously prepared by agar solubilization (1.5% w/w) in hot water (95°C) under magnetic stirring. The hot agar solution was poured into petri dishes (3 cm diameter) and allowed to cool. Once the room temperature was reached, gelation of the solution occurred, making it suitable for the test. In parallel, gels were dyed by directly dissolving 1 mg of Coomassie blue (Merck Life Science S.r.l., Milan, Italy).

The test was conducted by placing 50 mg of formulation at one end of the agar slide and then positioning it vertically (90° angle with the supporting surface). The agar surface was preheated at 32°C–35°C to mimic physiological conditions. Afterward, the distance traveled by the gel in 10 s was measured. Gel leakage, expressed as the running distance of the gel, was measured three times for each formulation.

2.14 | Rheological Analysis

TE_{EL}, TE_{EL}-gel, TE-GEN_{EL}, and TE-GEN_{EL}-gel were subjected to rheological analysis, performed by an Anton Paar MCR702e MultiDriver Rheometer (Anton Paar GmbH, Graz, Austria). Each sample was placed between two appropriate cone-plate geometries (diameter 50 mm, cone truncation 0.1 mm, and cone angle 1°). Once the shear rate profile is defined, the rheometer

applies the corresponding shear stress to the sample, and the viscosity is obtained using the measured shear stress and the applied shear rate. The viscosity (expressed as Pa·s) was reported as a function of the shear rate (expressed as 1/s). Measurements were performed at 25°C and 32°C, with 20% humidity, keeping these parameters constant throughout the experimental section.

2.15 | SAXS

SAXS experiments were carried out at the Austro-SAXS beamline of the Elettra Synchrotron in Trieste, Italy. Each sample was introduced into a quartz capillary with a 1.5 mm diameter and kept at a controlled temperature of 32°C using a Peltier-based KPR sample holder (Anton Paar, Graz, Austria). Data collection involved 10 s exposures, repeated for 10 consecutive frames per sample. The raw 2D scattering images were first corrected for background and detector response, then radially averaged to produce the scattering intensity, $I(Q)$, as a function of the scattering vector Q , defined as $Q = 4\pi \sin\theta/\lambda$, where 2θ represents the scattering angle and λ the x-ray wavelength. The instrument configuration allowed exploration of a Q -range spanning 0.1–4 nm^{−1}. All measurements were recorded using a Pilatus3 1M detector, featuring 172 × 172 μm² pixels and an x-ray energy of 8 keV ($\lambda = 0.154$ nm) [27].

2.16 | In Vitro Cytotoxicity Evaluation by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

HaCaT cells (AddexBio, San Diego, CA, USA) were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Milan, Italy), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ until reaching approximately 80% confluence.

For treatments, formulations were diluted in culture medium to obtain final working GEN concentrations ranging from 0.5 to 50 μg/mL.

Cells were seeded in 96-well plates at a density of 2×10^4 cells/well in 200 μL of culture medium. After 24 h, the cells were treated for 48 h with varying concentrations (0.5–50 μg/mL) of either unloaded or GEN-loaded formulations. Post-treatment, the medium was replaced with 50 μL of serum-free medium and 50 μL of MTT solution (0.5 mg/mL). Following a 3 h incubation at 37°C and 5% CO₂, formazan crystals were solubilized by adding 100 μL of DMSO and incubated for an additional 15 min under the same conditions. The plates were then subjected to orbital shaking for 5 min, after which absorbance was measured at 590 nm with a reference wavelength of 670 nm using a spectrophotometer. Cell viability was calculated as a percentage relative to untreated control cells (CTRL) [31].

2.17 | PCL Test

The antioxidant activity of GEN-loaded formulations against superoxide anion radicals (O₂•[−]) was evaluated via the PCL method, as described by Popov and Lewin [32]. Measurements were conducted using a Photochem instrument (Analytik Jena, Leipzig, Germany), which detects the inhibition of luminol-induced chemiluminescence by radical-scavenging

antioxidants. The ACL (lipid-soluble) protocol was employed, utilizing Trolox as the reference standard. The light emission kinetics were monitored for 180 s, and the resulting areas under the curve (AUC) were processed using PCLsoft software. To establish the baseline, double blank measurements were performed using a mixture of 2.3 mL ACL diluent, 0.2 mL reaction buffer, and 0.025 mL luminol solution. Samples (TE_{EI}, TE_{EI} gel, SOL GEN, TE-GEN_{EI}, and TE-GEN_{EI} gel) were appropriately diluted in methanol prior to analysis. All assays were conducted in triplicate, with results expressed as micromoles of Trolox equivalent per gram of sample (Te)/g.

2.18 | In Vivo Evaluation

2.18.1 | Patch Test

The patch test was performed at the facilities of COMPLIFE ITALIA S.r.l. (Pavia, Italy). Twenty male and female volunteers with healthy, intact skin were recruited for patch testing in accordance with the principles of the Helsinki Declaration 1964. Thirty percent of volunteers with sensitive skin were included. Subjects with spots or marks in the test area, with dermatological problems, on local or systemic drug therapy, with a previous clinical history of dermatitis, or with a positive history of atopy were excluded. The tested formulations (TE-GEN_{EI}-gel and GEN-gel) were applied using the Finn Chamber, an 8 mm diameter aluminum disc. The Finn Chamber was affixed to the skin with a tape that had previously been tested for its safety and ensured the occlusive application of the product. Enough product was applied to fill the chamber (50 µg). The formulations were applied on the back and left in contact with the skin surface for 48 h. Skin reactions were analyzed at 15 min, 1 h, and 24 h after removal of the Finn chamber. A Finn chamber containing an absorbent paper disc soaked in demineralized water was also applied as a negative control. The erythematous reactions were categorized into five groups, according to the degree of erythema: absent, light, clearly visible, moderate, and severe. The edematous reactions were similarly categorized into five groups, according to the degree of edema: absent, light, clearly visible, moderate, and severe. The mean irritation index (IIM) was calculated for each experimental time, represented by the sum of the mean erythema value and the mean edema value recorded. This index was used to classify the product under investigation as non-irritating, slightly irritating, moderately irritating, or highly irritating.

2.18.2 | Tape Stripping

The percutaneous absorption of the TE-GEN_{EI}-gel and GEN-gel formulations was monitored in vivo using the tape stripping method, a relatively non-invasive technique enabling the stripping of *stratum corneum* samples from the skin [33, 34]. The in vivo study was approved by the Ethics Committee of the Area Vasta Emilia Centro (protocol 8/2025/Sper/UniFe). Thirty male and female volunteers with healthy, intact skin were recruited for tape stripping after giving written consent. Subjects with spots or marks in the test area, with dermatological problems, on local or systemic drug therapy, with a previous clinical history of dermatitis, or with a positive history of atopy were excluded. The study was carried out in a partly air-conditioned room at a temperature of 22°C ± 2°C. The anatomical sites chosen were seven areas (10 cm²) of the inner forearm. Three areas, A, B, and C, were treated with 200 µL of TE-GEN_{EI}-gel formulation each,

while areas D, E, and F were treated with 200 µL of GEN-gel formulation each. The gels were spread uniformly by means of a plastic spatula, and then the sites were left under non-occlusive conditions. Area G was the negative control. Tape stripping was conducted at three distinct time points after removing the residual formulations by gently wiping with clean cotton balls. One hour post-application of the products (t 1), tape stripping was performed on areas A, D, and G. Three hours post-application (t 3), tape stripping was executed on areas B and E. Six hours post-application (t 6), tape stripping was carried out in areas C and F. Rectangular sections of adhesive tape were applied to the skin, adhering precisely to the previously demarcated areas of the forearm (2 cm × 5 cm). The stratum corneum of the treated area was removed through 11 successive tape strippings using “3M” (3M, Minneapolis, MN, USA) invisible adhesive tape. The tape was applied to the test site using forceps, uniformly flattened twice, and removed gently with moderate and even traction. Due to the potential for surface contamination and to eliminate surface lipids, the first (uppermost) tape strip was discarded. Subsequent tapes were then placed, adhesive side downwards, into a glass container with 15 mL of MeOH (HPLC grade) and subsequently subjected to 30 min of treatment at 99% power in an ultrasonic bath (CP104, Vetrotecnica S.r.l, Italy) at room temperature. Solutions were then purified via centrifugation (10 min at 4000 rpm) to separate small horny layer particles. The supernatant was then filtered using nylon syringe filters (0.22 µm) and analyzed by HPLC analysis to determine GEN concentration. Statistical evaluations were conducted using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data normality was assessed and confirmed via the Shapiro–Wilk test. Given the longitudinal nature of the data (measurements obtained from the same subjects across multiple time points), a one-way repeated measures ANOVA was employed. The Geisser–Greenhouse correction was applied to account for potential violations of sphericity. Post hoc analysis was performed using Holm–Sidak’s multiple comparisons test, with individual variances calculated for each specific comparison.

3 | Results and Discussion

3.1 | Preparation of ETs and TEs

A preformulative study was conducted to select an appropriate nanosystem for GEN loading, using the WI or EI methods for ET and TE preparation [34]. The composition of ET and TE, reported in Table 1, was based on previous studies conducted by our research group [35, 36], employing the non-ionic surfactant TW80 as an edge activator in the case of TE [37].

All formulations produced in the absence of GEN appear homogeneous and free of separation phenomena, more translucent in the case of TE. Conversely, regarding GEN-loaded forms, both methods induced phase separation in the case of ET, whilst the loading of GEN up to 0.25 mg/mL in TE did not induce any phase separation, using WI or EI methods. It is likely that in the case of ET, excessive amounts of GEN could alter the phospholipid packing, leading to vesicle rupture and phase separation, whilst, in the case of TE, TW80 presence could stabilize the system, improving GEN solubilization. For this reason, both ET-GEN_{WI} and ET-GEN_{EI} were not considered for further studies.

TABLE 1 | Composition of ET and TE.

Formulation	PC ¹ (% w/w)	Ethanol (% w/w)	TW80 ² (% w/w)	H ₂ O (% w/w)	GEN ³ (% w/w)	Macroscopic aspect
ET _{WI}	0.9	29.1	—	70.0	—	Homogeneous
ET _{EI}	0.9	29.1	—	70.0	—	Homogeneous
TE _{WI}	0.9	28.8	0.3	70.0	—	Homogeneous
TE _{EI}	0.9	28.8	0.3	70.0	—	Homogeneous
ET-GEN _{WI}	0.9	29.1	—	70.0	0.025	Phase separation
ET-GEN _{EI}	0.9	29.1	—	70.0	0.025	Phase separation
TE-GEN _{WI}	0.9	28.7	0.3	70.0	0.025	Homogeneous
TE-GEN _{EI}	0.9	28.7	0.3	70.0	0.025	Homogeneous

¹Soy phosphatidylcholine.²Tween 80.³Genistein.

Table 2 reports the size distribution parameters and zeta potential values of TE evaluated the day after preparation.

All formulations showed a homogeneous size distribution, confirmed by the PDI values, always around or below 0.2, with smaller Z-Average values in the case of TE_{EI} [28, 38]. In the EI method, the solution of PC in ethanol is rapidly injected into the water phase. Since ethanol and water are highly miscible, their rapid mixing leads to a quick decrease in the solubility of PC, causing a high degree of supersaturation. When PC rapidly precipitates out of the solution due to this decrease in solubility, it tends to form many nucleation sites almost simultaneously. This rapid and widespread nucleation results in the formation of many small vesicles rather than fewer, larger ones [39].

In the case of TE-GEN_{WI} and TE-GEN_{EI}, GEN loading did not affect TE vesicle Z-Average and PDI (Table 2). Notably, as found for empty vesicles, the EI method led to smaller Z-Average and PDI values [40, 41].

Zeta potential values were always around −20 mV, suggesting vesicle electrostatic repulsion. Indeed, some studies suggest that ethanol can modify the net charge of the nanovesicular system, leading to a degree of steric stabilization [42, 43]. This increased negative charge on the surface of the forming vesicles can lead to electrostatic repulsion between them, preventing aggregation and growth into larger sizes [28, 38]. Z-potential values were slightly affected by GEN loading, maintaining a negative surface charge on the polar head region of PC.

The pH values of TE-GEN_{WI} and TE-GEN_{EI} were 5.34 ± 0.06 and 5.65 ± 0.32 , respectively, thus compatible for cutaneous application (stratum corneum physiological pH 4.1–5.8 [44]).

The assessment of EC was evaluated the day after preparation, using the ultrafiltration method. Typical chromatograms and validation data are reported in Supporting Information, Figure S1 and Table S1. As expected, due to the lipophilic character of GEN, an almost complete association with the nanovesicles was observed ($EC = 96.00\% \pm 2.20$, $n = 7$). Indeed, due to their lipid-based composition, TE have a high affinity for lipophilic drugs, particularly the presence of polysorbate 80 further improves the vesicle's capability to entrap lipophilic compounds, such as GEN [35, 36]. No differences were detected between the EI and WI methods.

Cryo-EM enabled us to gain insights into the morphology of TE-GEN_{WI} and TE-GEN_{EI} (Figure 1).

In both cases, spherical, unilamellar vesicles were detected, as previously found in the case of TE with the same composition [45]. The structural organization of the vesicles is probably affected by TW80 that intercalates into the phospholipid bilayer, exposing the polar head outward, creating a steric hindrance that prevents the assembly of multiple bilayers [46]. Vesicle mean diameters perfectly agree with PCS data. Notably, TE morphology was not affected by the preparation methods.

TE-GEN_{EI} were selected for further studies, being characterized by the smallest mean diameter and PDI values with respect to the other nanovesicular systems.

3.2 | Stability Studies

The stability of TE_{EI} and TE-GEN_{EI} was evaluated in samples stored for 3 months at 4°C and protected from light. Size

TABLE 2 | Z-average, dispersity index, and Z-potential of the indicated formulations.

Formulation	Z-average (nm) $\pm$ SD	Polydispersity index $\pm$ SD	Z-potential $\pm$ SD
TE _{WI}	136.15 ± 10.99	0.14 ± 0.01	-21.86 ± 2.80
TE _{EI}	107.78 ± 3.48	0.13 ± 0.01	-23.80 ± 6.34
TE-GEN _{WI}	144.65 ± 9.26	0.20 ± 0.08	-24.62 ± 5.36
TE-GEN _{EI}	111.99 ± 2.61	0.14 ± 0.01	-19.59 ± 5.01

Note: Data are the mean of 4 independent determinations on different batches.

Abbreviation: SD, standard deviation.

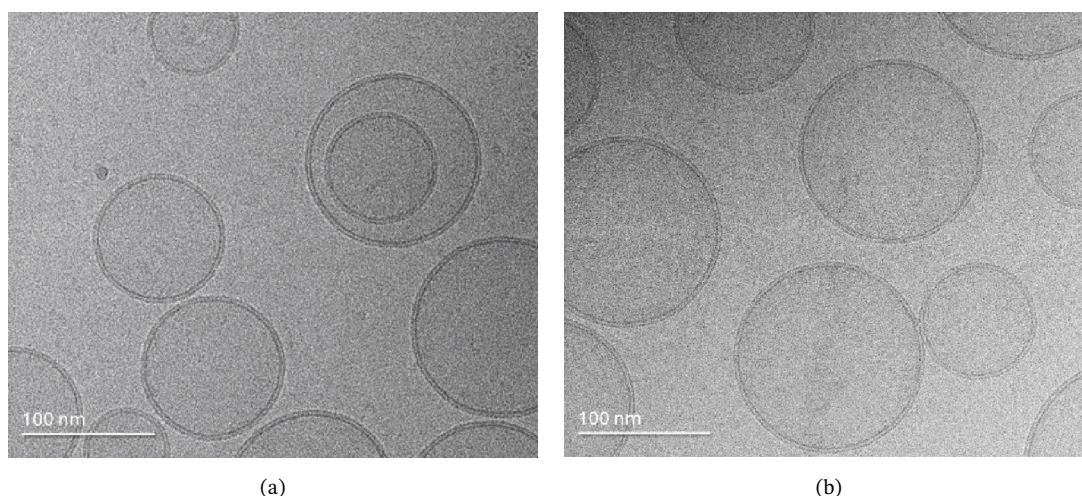


FIGURE 1 | Cryogenic electron microscopy (Cryo-EM) images of TE-GEN_{WI} (a) and TE-GEN_{EI} (b). Bar corresponds to 100 nm.

distribution of both TE_{EI} and TE-GEN_{EI} evaluated by PCS evidenced strong size stability of vesicles, maintaining almost unvaried Z-Average and PDI also after 3 months (Table 3). The vesicle Z-potential underwent a tendency toward more negative value fluctuations over time, especially in the case of TE-GEN_{EI}. Notably, GEN content was maintained also after 90 days of storage; indeed in TE-GEN_{EI}, 95% of the initial GEN content was detectable.

3.3 | IVPT

In this study Franz cells assembled with STRAT-M membrane were employed to predict the effect of TE vesicles on GEN diffusion through the skin. According to the European Medicines Agency Guideline (EMA), for permeation study, the assessment of compound diffusivity is typically accomplished using the Franz cell system, associated with a natural or artificial membrane mimicking the skin [47]. Although natural membranes derived from human (e.g., cadaver, surgical, or cosmetic sources) or animal (e.g., porcine or rodent) skin yield results highly indicative of in vivo drug permeation, their application is constrained by numerous disadvantages. These limitations include elevated cost, constrained availability, ethical restrictions, inter-sample variability, potential for contamination, and stringent storage protocols. Consequently, synthetic membranes are commonly employed in quality control applications to evaluate topical drug diffusion, primarily due to their excellent reproducibility and widespread commercial accessibility. In this respect, skin surrogates could be relied on as a prescreening in formulative studies. The STRAT-M membrane is a synthetic

skin-mimicking engineered with specific barrier characteristics, including appropriate lipophilicity and porosity, to replicate the *stratum corneum*. Structurally, it consists of multiple polymer layers: two outer layers of polyether sulfone stacked upon a lower polyolefin layer, with the addition of synthetic lipids to complete its skin-like composition. Different studies demonstrated the STRAT-M suitability to reproduce skin permeability, suggesting its use as an alternative to skin [30, 47–49].

Moreover, according to EMA, in the case of Franz cells for permeation studies, the receptor media should be an aqueous buffer for water-soluble drugs, while for poorly water-soluble drugs, hydroalcoholic media can be used. In this respect the receptor compartment of the Franz cell contained a water-ethanol solution (50:50) to allow the establishment of the “sink condition” and to sustain permeant solubilization [47].

TE-GEN_{EI} and a GEN suspension in EtOH/H₂O 30:70 (SUSP-GEN) (GEN 0.25 mg/mL in both cases) were compared. The passive diffusion of drugs through the membrane is related to (i) the preferential distribution between the membrane/skin or the vehicle and (ii) the drug passage across the membrane/skin, described by K_p , depending on the drug and vehicle physical-chemical features [50]. GEN diffusion profiles are shown in Figure 2, while Table 4 reports IVPT parameters.

GEN diffusion through the membrane was faster in the case of TE-GEN_{EI} with respect to SUSP-GEN. In both cases a lag time was observed, slightly longer in the case of SUSP-GEN with respect to TE-GEN_{EI}. The K_p value was 3.3-fold higher in the case of TE-GEN_{EI} with respect to SUSP-GEN, suggesting the vesicle

TABLE 3 | Variation of physical-chemical parameters of TE_{EI} and TE-GEN_{EI} analyzed the day after preparation (d_0) and after 3 months (d_{90}).

Formulation		Z-average (nm) $\pm$ SD	PDI $\pm$ SD	Z-potential $\pm$ SD	GEN content (mg/mL) $\pm$ SD
TE _{EI}	d_0	107.78 $\pm$ 3.48	0.13 $\pm$ 0.01	−23.80 $\pm$ 6.34	—
	d_{90}	116.53 $\pm$ 4.08	0.16 $\pm$ 0.02	−25.04 $\pm$ 1.32	—
TE-GEN _{EI}	d_0	111.99 $\pm$ 2.61	0.14 $\pm$ 0.01	−19.59 $\pm$ 5.01	0.250 $\pm$ 0.002
	d_{90}	116.15 $\pm$ 1.52	0.15 $\pm$ 0.04	−34.69 $\pm$ 4.31	0.240 $\pm$ 0.008

Note: Data are the mean of 4 independent determinations on different batches.
Abbreviation: SD, standard deviation.

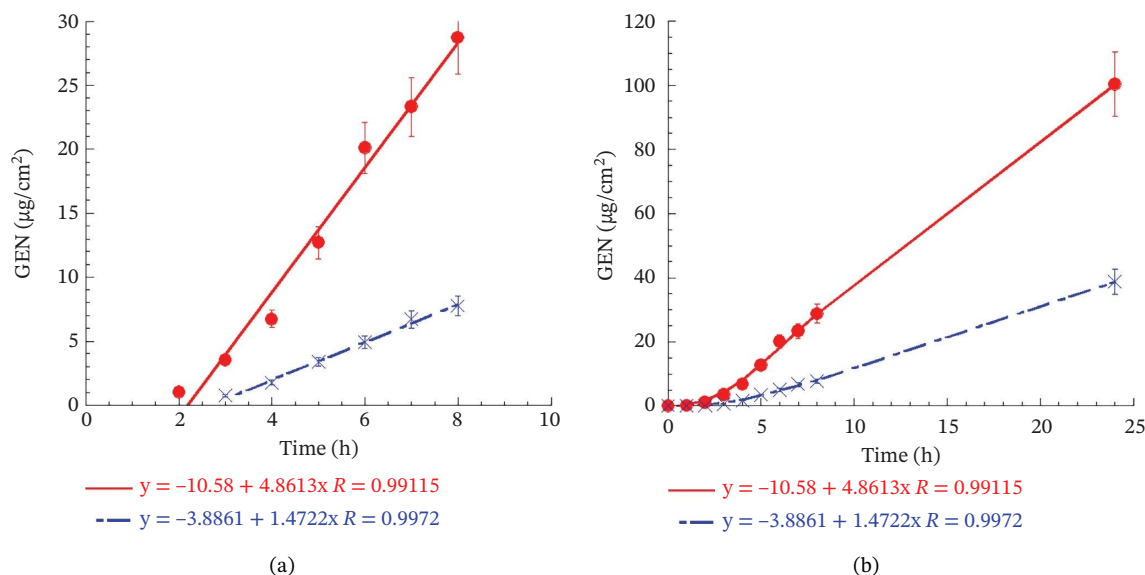


FIGURE 2 | GEN diffusion kinetics from TE-GEN_{EI} (red circles) and SUSP-GEN (blue squares) as determined by Franz cells associated with the Strat-M membrane. (a) Linear part of the profile (up to 8 h) (b) up to 24 h. Data are the mean of 4 independent experiments ± SD.

TABLE 4 | IVPT parameters of the indicated formulations.

IVPT parameters	TE-GEN _{EI}	SUSP-GEN
J_{ss}^1 (μg·cm ⁻² ·h ⁻¹)	4.86 ± 1.05	1.47 ± 0.68
K_p^2 (cm·h ⁻¹) × 10 ³	19.44 ± 3.64	5.88 ± 2.74
T_{lag}^3 (h)	2.17 ± 0.72	2.62 ± 0.56
A^4 (μg·cm ⁻²)	102.50 ± 3.96	30.02 ± 8.97

Note: GEN concentration was always 0.25 mg/mL. Data are the mean of 4 independent Franz cell experiments ± SD.

¹Steady-state flux per unit area.

²Permeability coefficient.

³Lag time.

⁴Cumulative amount of GEN diffused at 24 h.

transdermal potential. Notably, the amount of GEN diffused after 24 h (A value) was almost threefold higher in the case of TE-GEN_{EI} with respect to SUSP-GEN.

3.4 | Transethosomal Gel Preparation and Characterization

To allow cutaneous application of TE, X-Gum was employed as a thickening agent. Namely, TE gels were prepared by direct addition of X-Gum (0.5%–1%, w/w) to TE_{EI}. Homogeneous whitish systems were obtained, with increasing viscosity and opacity directly related to X-Gum concentration. Spreadability and leakage assays (Table 5) enabled the selection of the x-gum

concentration of 0.5% w/w, leading to the higher spreadability and leakage values.

A rheological analysis was performed to evaluate the viscosity curves, comparing TE and their corresponding thickened forms, loaded or unloaded with GEN. Analyses were performed at 25°C and 32°C to assess the behavior at room temperature and under physiological conditions. Figure 3 shows viscosity curves obtained for all tested formulations.

All samples show shear-thinning behavior, with viscosity decreasing as the shear rate increases. Moreover, a moderate decrease in viscosity is observed for all samples when the temperature increases from 25°C to 32°C. In all the investigated

TABLE 5 | Spreadability and leakage values of TE_{EI} gels.

Formulation	X-gum (%) (w/w)	Spreadability ¹ (g cm·s ⁻¹)	Leakage ² (cm)
TE _{EI} gel	0.5%	21.75 ± 0.5	3.23 ± 0.42
	0.75%	20.83 ± 0.29	1.84 ± 0.51
	1%	18.67 ± 0.29	0.77 ± 0.06

Note: Data are the mean of 3 independent measurements ± SD.

¹Calculated as reported in Equation (1).

²Running distance along the slide.

samples, viscosity decreases with an increase in the shear rate, confirming a non-Newtonian flow with shear-thinning behavior [51]. Such results are attributed to the deformation and/or disruption of the particle aggregates under shear, reducing internal resistance to flow and consequently lowering the apparent viscosity. As expected, formulations thickened with X-Gum (Figures 3(b) and 3(d)) exhibit a viscosity approximately 10 times higher than the corresponding samples without the gelling agent (Figures 3(a) and 3(c)), across all shear rates.

3.5 | SAXS Results

To further get an insight into the inner supramolecular organization, SAXS measurements were performed. Namely, TE_{EI}, TE-GEN_{EI}, TE_{EI}-gel, and TE-GEN_{EI}-gel were considered. The measurements were carried out at 25°C and 32°C to compare the behavior of the formulation at room temperature and under physiological conditions. Figure 4 reports the SAXS spectra.

SAXS data clearly revealed distinct microstructures in the presence of the thickening agent with respect to plain samples. In the case of TE_{EI} and TE-GEN_{EI} (Figures 5(a) and 5(b)), the scattering profiles are characteristic of lipid bilayered vesicles, with a broad peak centered around 1.5 nm^{-1} . According to [28, 45], this feature corresponds to a bilayer thickness of approximately 4.2 nm. Upon addition of X-Gum (Figures 5(c) and 5(d)), the SAXS profiles undergo significant changes and appear dominated by the scattering from the gel matrix [27]. These profiles can be accurately fitted using a worm-like fractal flexible cylinder model, a mass fractal model composed of flexible cylinders as structural building blocks [52] commonly applied to SAXS data from gels and hydrogels [53]. These findings suggest that in these samples the vesicles are fully embedded within the three-dimensional polymeric network of X-Gum.

Importantly, temperature variation between 25°C and 32°C did not produce any noticeable changes in the SAXS patterns, indicating a high stability of the supramolecular organization

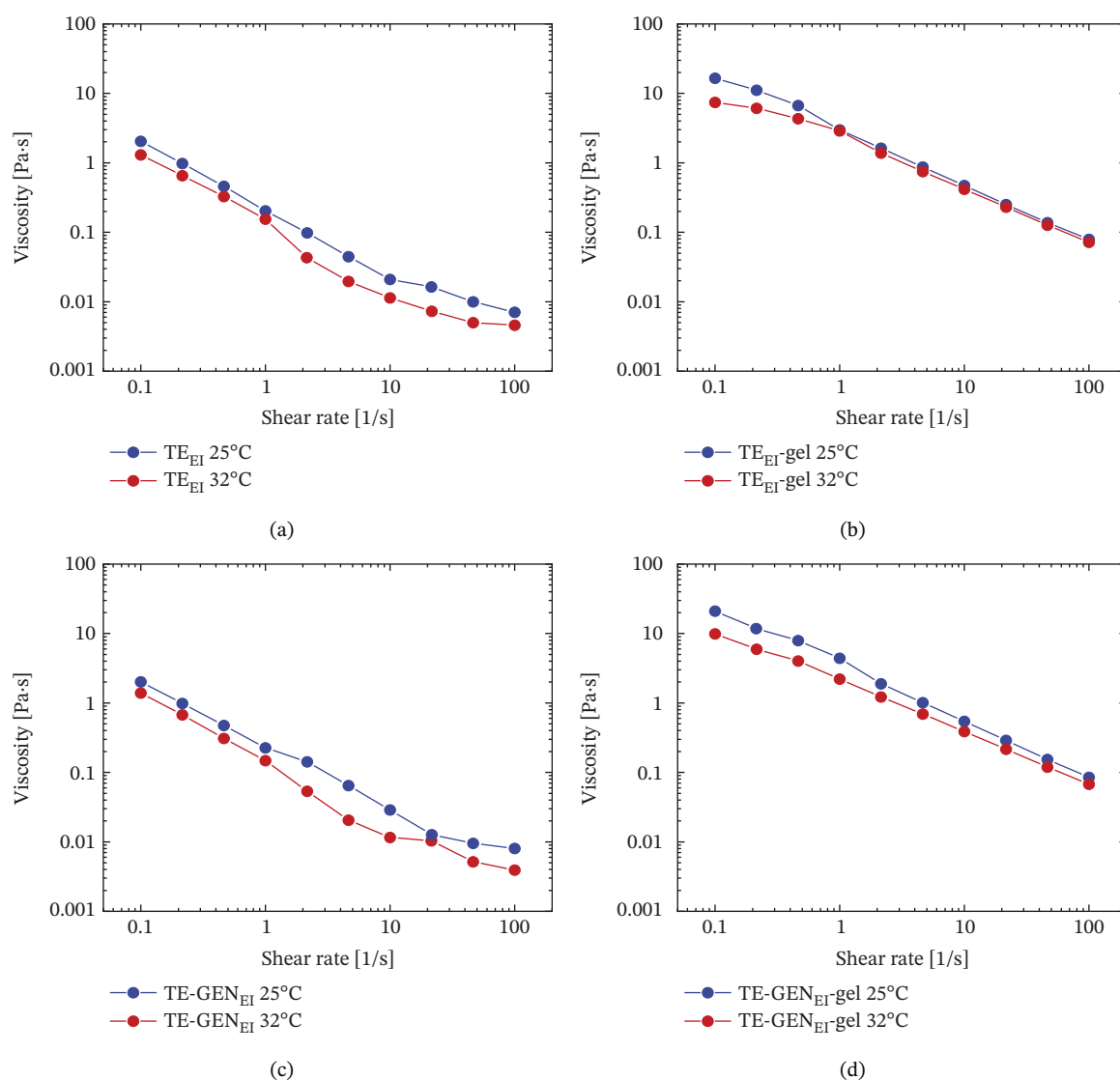


FIGURE 3 | Viscosity curves analyzed for TE_{EI} (a), TE_{EI}-gel (b), TE-GEN_{EI} (c), and TE-GEN_{EI}-gel (d). The blue curves refer to the measurement carried out at 25°C, while the red curves are at a temperature of 32°C.

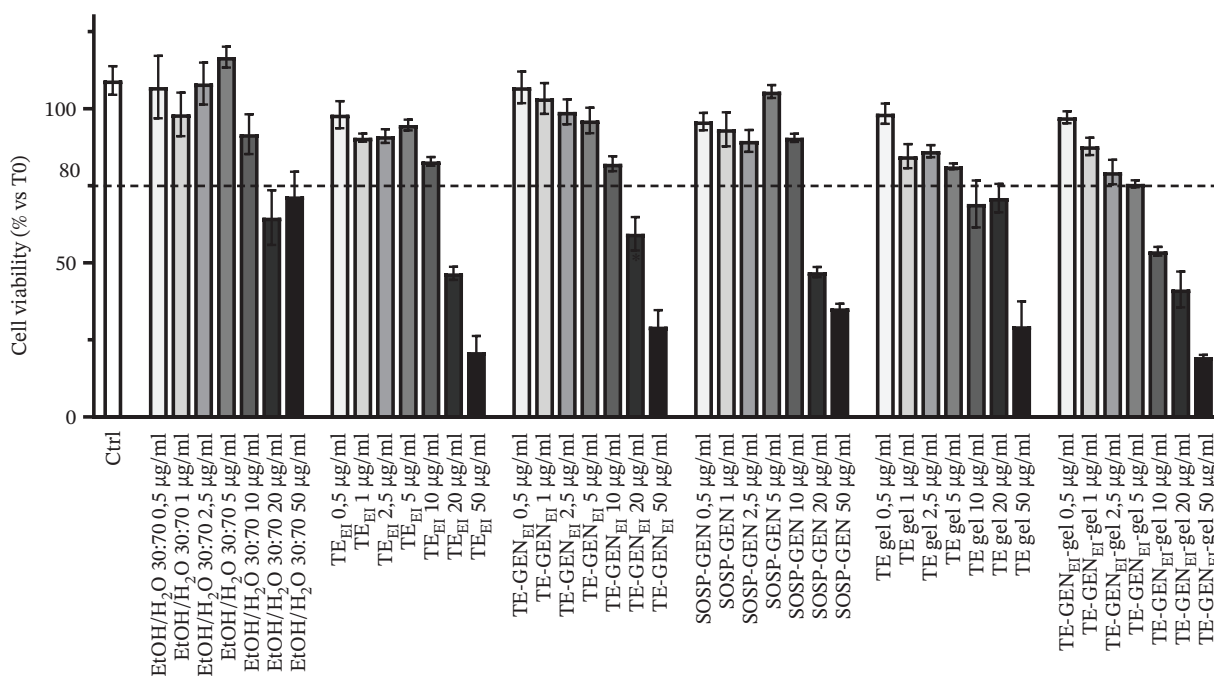


FIGURE 4 | HaCaT cell viability evaluated by MTT assay after 48 h of treatment with GEN-loaded or unloaded formulations. Data represent the results of two independent experiments performed in triplicate. Results are expressed as a percentage of the control condition and are presented as mean $\pm$ SD.

under both storage and physiological conditions. Furthermore, no significant differences were observed between the GEN-loaded and unloaded formulations, demonstrating that drug incorporation did not alter the overall structural features of the delivery system.

3.6 | Cytotoxicity Evaluation

The potential cytotoxicity of the formulations was assessed using an MTT assay on HaCaT cells. Keratinocytes were treated with different concentrations of GEN-loaded or unloaded formulations (i.e., 0.5, 1, 2.5, 5, 10, 20, and 50 $\mu\text{g/mL}$), and cell viability was evaluated 48 h post-treatment. Specifically, TE-EI, TE-GEN-EI, SUSP-GEN, the mixture EtOH/H₂O, 30:70 v/v (i.e., the continuous phase of SUSP-GEN), TE-EI-gel and TE-GEN-EI-gel were considered. Results were compared to the control (CTRL) to verify the concentrations leading to a decline in vitality below the 80% threshold.

As shown in Figure 4, the EtOH/H₂O 30:70 groups maintained high viability levels (> 90%) across most tested concentrations (0.5–10 $\mu\text{g/mL}$), indicating minimal toxicity. The different treatment groups exhibited a dose-dependent cytotoxic effect, with the highest doses (20–50 $\mu\text{g/mL}$) reducing cell viability to approximately 60%, relative to the CTRL condition. Notably, GEN-loaded formulations displayed higher cytotoxicity than SUSP-GEN, but only for concentrations higher than 10 $\mu\text{g/mL}$.

3.7 | Antioxidant Capacity Evaluation

The in vitro PCL assay effectively characterized the antioxidant capacity of both TE-GEN-EI and TE-GEN-EI-gel, quantifying their ability to scavenge superoxide anion radicals.

For comparison, the antioxidant activity of free GEN in ethanol solution (SUSP-GEN) and of the empty formulations (i.e., TE-EI

and TE-EI-gel) as controls, were also evaluated (Table 6). As expected, GEN in solution displayed a very high ACL value ($\approx 200 \mu\text{mol Te/g}$), in line with the strong radical-scavenging properties of isoflavones [51]. In contrast, ACL values expressed per gram of formulation for SUSP-GEN and GEN-loaded vesicles were much lower, which mainly reflects the relatively low GEN loading fraction in the colloidal systems rather than an intrinsic loss of activity [52]. When the signal of the corresponding empty formulations is subtracted, the GEN-related ACL values of TE-GEN-EI and TE-GEN-EI-gel (0.068 and 0.062 $\mu\text{mol Te/g}$, respectively, two-tailed p value 0.3035) are comparable to that of SUSP-GEN (0.071 $\mu\text{mol Te/g}$) (the two-tailed p values are 0.5111 and 0.1980, respectively), indicating that, at equal drug content, encapsulated GEN maintains its antioxidant capacity.

This behavior suggests that, although encapsulation can in principle reduce the immediate accessibility of GEN to the reactive species by locating the isoflavone within or at the interface of the lipid bilayer, the flexible vesicles used here still allow an efficient interaction between GEN and the radicals generated in the PCL assay. Similar trends have been reported for other polyphenol-loaded liposomal systems, where the apparent antioxidant capacity per gram of dispersion is reduced relative to the pure compound but is preserved when normalized to the actual polyphenol loading. For instance, hydroxytyrosol-loaded liposomes retain or even enhance the antioxidant activity of the free molecule while providing improved stability and controlled release [54]; oligomeric procyanidin liposomes show comparable Trolox-equivalent antioxidant capacity to the free extract when expressed per amount of encapsulated antioxidant [55], and quercetin-loaded liposomes are specifically optimized to maintain high TEAC values despite partial shielding of the flavonoid by the bilayer [56]. In the case of GEN, asolectin-based liposomes

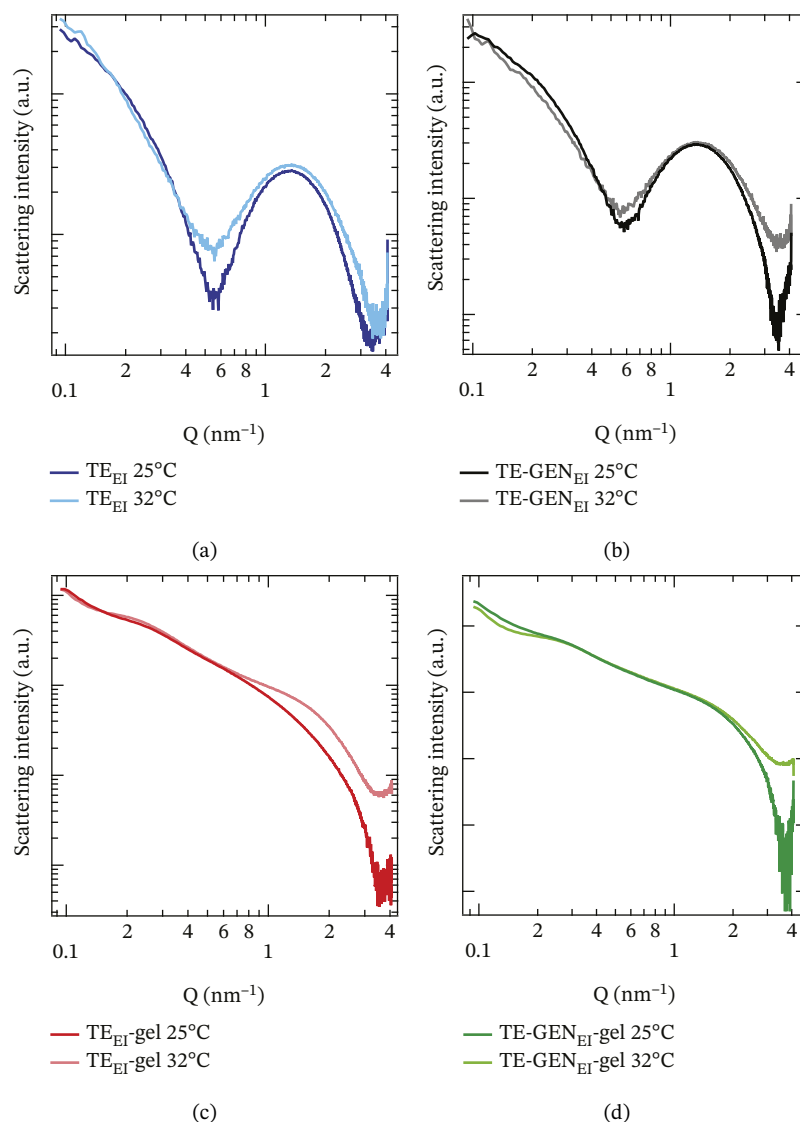


FIGURE 5 | SAXS diffraction spectra of TEEI (a), TE-GEN_{EI} (b), TE_{EI}-gel (c), and TE-GEN_{EI}-gel (d). Analyses were performed at 25°C and 32°C.

TABLE 6 | PCL test results.

Formulation	ACL (μmol Te/g) ¹	ACL (μmol Te/g) ¹ —blank ²
GEN	200.53 ± 13.74	—
SUSP-GEN	0.071 ± 0.005	—
TE-GEN _{EI}	0.137 ± 0.004	0.068 ± 0.0037
TE _{EI}	0.070 ± 0.005	—
TE-GEN _{EI} -gel	0.123 ± 0.005	0.062 ± 0.0047
TE _{EI} -gel	0.061 ± 0.003	—

Note: Data are the mean of 3 independent experiments ± SD.

¹Antioxidant capacity of lipid-soluble compounds.

²Blank is considered the ACL signal of corresponding empty formulations (TE_{EI} for TE-GEN_{EI} and TE_{EI} gel for TE-GEN_{EI}-gel).

have been shown to preserve or increase its in vitro antioxidant and biological activities, an effect attributed to the specific location of GEN within the phospholipid membrane and the consequent modulation of lipid dynamics [57, 58]. Overall, our PCL results are consistent with these observations: The modest reduction in ACL values when moving from free GEN to

vesicular formulations is largely explained by drug loading and the contribution of the vehicle, including the intrinsic, low-level antioxidant activity of polysorbates such as Tween 80 [59], while GEN encapsulation in TE-GEN_{EI} and TE-GEN_{EI}-gel does not mask its antioxidant profile and preserves its radical-scavenging performance within a vesicular delivery system.

3.8 | In vivo Studies

In vivo studies were carried out on human volunteers to evaluate the possible irritating effect of the vesicular gel (TE-GEN_{EI}-gel) on the skin, as well as GEN distribution. An X-Gum GEN gel (GEN-gel) was employed for comparison. Precisely, GEN-gel was produced by dispersion of GEN in water, followed by addition of X-Gum under magnetic stirring. The formulation possesses the same amount of GEN and X-Gum as the TE-GEN_{EI} gel.

3.8.1 | Patch Test

A patch test was conducted to evaluate the irritating potential of the selected formulations when applied on intact human skin in a single dose. The number of edematous and erythematous reactions caused by the GEN-gel and TE-GEN_{EI}-gel, recorded for each experimental time, is reported in Table 7 as the IIM, suggesting that both formulations can be classified as non-irritant when applied to human skin.

3.8.2 | Tape Stripping

A tape stripping study was performed on 30 healthy volunteers to evaluate TE-GEN_{EI}-gel's capability to promote GEN permeation through the skin, employing GEN-gel for comparison [33, 34]. The in vivo study was approved by the Ethics Committee of the Area Vasta Emilia Centro (protocol 8/2025/Sper/UniFe). The amount of GEN in the *stratum corneum* was assessed by the tape stripping method associated with HPLC, comparing the transdermal potential of the nanovesicular gel versus the plain one under cutaneous application. In Figure 6, the amounts of GEN found in the superficial layers of the *stratum corneum* at three different experimental time points (1–3–6 h) are reported. The mean values $\pm$ standard deviations of each experimental time for each formulation and the outcomes of the statistical analysis performed using a repeated-measures one-way ANOVA with Geisser–Greenhouse correction, followed by Holm–Sidak post hoc multiple comparison tests (with individual variances calculated for each comparison), are reported in Table S2.

At t 1 from removal of the formulations, the amount of GEN found in the *stratum corneum* was higher for the areas treated with GEN-gel than for those treated with TE-GEN_{EI}-gel.

In the following time points (t 3 and t 6), in the case of GEN-gel, a gradual reduction of the GEN amount was observed. Conversely, in the case of the areas treated with TE-GEN_{EI}-gel, a gradual GEN increase was observed.

Regarding GEN-gel, the initial GEN accumulation in the superficial skin layers, tending to decrease over time, could be possibly ascribable to the progressive degradation of the active compound. Indeed, the test was conducted under non-occlusive conditions, exposing the formulation to the natural light in the external environment. Since GEN is a photosensitive molecule [18, 60], when applied by X-Gum-gel, it could rapidly degrade, resulting in a possible loss of activity in the long term. In this regard, further studies will be required to support this hypothesis. Different considerations should be done about the TE-GEN_{EI}-gel trend. On one hand, TE vesicular structures could protect GEN from photodegradation; on the other, the GEN progressive increase over time could be ascribable to a fast vesicle penetration through the *stratum*

TABLE 7 | Number of edematous and erythematous reactions caused by tested formulations, expressed as mean irritation index (IIM).

Reactions	GEN-gel	TE-GEN _{EI} -gel
IIM erythematous 15 min	0.05	0.05
IIM edematous 15 min	0.00	0.00
IIM erythematous 1 h	0.05	0.05
IIM edematous 1 h	0.00	0.00
IIM erythematous 24 h	0.00	0.00
IIM edematous 24 h	0.00	0.00

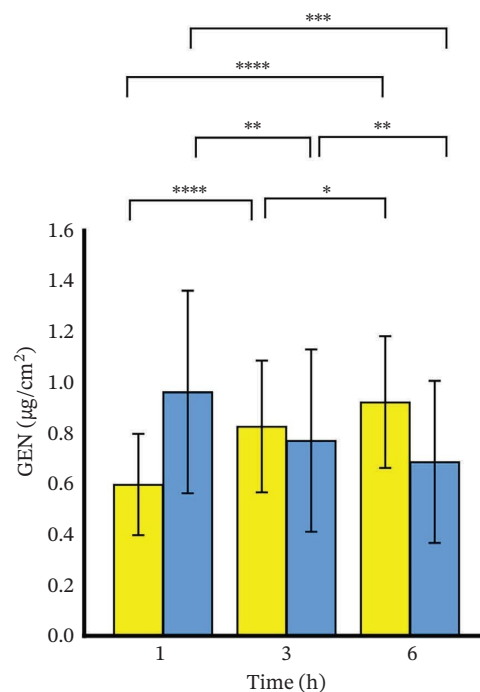


FIGURE 6 | Comparative evaluation of GEN amount present at 1, 3, and 6 h in *stratum corneum* as determined by tape-stripping experiments under skin application of TE-GEN_{EI}-gel (yellow) and GEN-gel (blue). Data were obtained from tape stripping performed on 30 healthy volunteers. Data represent the mean of 30 subjects $\pm$ SD, **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05.

corneum, followed by a GEN content release. Indeed, a previous *ex vivo* evaluation about the fate of TE applied on human skin explants [45] demonstrated by transmission electron microscopy the vesicle presence in the *stratum corneum*, both in the intercellular space and inside corneocytes, after 1 h of incubation. After 3 h, TE presence in the epidermis was less frequent, until absence after 6 h, suggesting complete vesicle degradation [45]. In this respect, the increasing amount of GEN detected in skin samples by tape-stripping can be related to the TE vesicle progressive degradation inside the *stratum corneum* sustaining the release of loaded GEN. Taken together, these results demonstrate the potential of TE-GEN_{EI}-gel as a transdermal vehicle, able to gradually release GEN in the skin, suggesting its possible suitability for prolonged protective action required to prevent UV-induced skin damage.

4 | Conclusions

In conclusion, the formulative study enabled the selection of the method and the composition to produce nanovesicular systems suitable for GEN skin administration. The novel TE-GEN_{EI}-gel is a stable, well-tolerated smart nanovesicular system containing GEN, which successfully maintained GEN antioxidant activity and significantly enhanced its permeation into the *stratum corneum* with respect to a simple suspension (SUSP-GEN). The formulation significance lies in its potential as a tool for enhancing GEN therapeutic activity, improving its delivery challenges through a nanovesicle formulation, topically suitable by thickening with X-gum. The potential promising application of TE-GEN_{EI}-gel concerns the treatment of skin damage and photoaging, though further research is required to confirm its protective efficacy against UV exposure in vivo.

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Ethics Statement

All procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the Ethics Committee of the Area Vasta Emilia Centro (protocol 8/2025/Sper/UniFe).

The privacy rights of human subjects have been observed, and informed consent was obtained for experimentation with human subjects.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available from the authors upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. (*Supporting Information*)

Figure S1 shows the typical chromatograms corresponding to the GEN content in the total TE-GEN_{El} sample deriving from disaggregation and to the drug content in the lipid phase corresponding to the retentate after ultrafiltration. In Table S1, the validation data for the EC of GEN in TE-GEN_{El}, evaluated by the ultrafiltration method, are reported, including recovery tests and mass balance calculations. Table S2 reports the tape stripping data along with the results of the corresponding statistical analysis.