ATR-FTIR and Raman spectroscopic investigation of the electroporation-mediated transdermal delivery of a nanocarrier system containing an antitumour drug

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Abstract: The aim of the present work was the optimization of the transdermal delivery of a lyotropic liquid crystal genistein-based formulation (LLC-GEN). LLC was chosen as medium in view of the poor solubility of GEN in water. Membrane diffusion and penetration studies were carried out with a Franz diffusion cell, through a synthetic membrane *in vitro*, a chick chorioallantoic membrane *ex ovo*, and *ex vivo* excised human epidermis. Thereafter, LLC-GEN was combined with electroporation (EP) to enhance the transdermal drug delivery. The synergistic effect of EP was verified by *in vivo* ATR-FTIR and *ex vivo* Raman spectroscopy on hairless mouse skin.

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1. Introduction

The transport of molecules through the skin is made difficult by the presence of the stratum corneum (SC). The epidermis is an intricate and highly inhomogeneous structure built up from different layers, but the SC (the outermost layer of the skin consisting of dead flattened cells) causes its barrier function, such as the electrical properties [1, 2]. Transdermal drug delivery has a number of advantages over conventional delivery methods such as oral administration and injection (e.g. elimination of the first-pass metabolism, the minimization

of pain, and the possibility of the sustained release of drugs). However, for the delivery of large molecules through the skin, the use of chemical or physical methods is unavoidable [1].

Electroporation (EP) involves the application of short (microsecond to millisecond) pulses of high voltage (HV) to achieve a transitory structural perturbation of the lipid bilayer membranes. Its main advantages are that it is easy and rapid, and is able to open different kinds of lipid bilayer membranes: artificial (liposomes), cellular (bacterial, yeast, plant and mammalian cells) or even more complex structures (e.g. SC) [3]. Through the use of skin EP, the transdermal delivery of small dyes, drugs, oligonucleotides, DNA plasmids, antigens, hydrophilic molecules, neutral or highly charged compounds, and macromolecules with molecular weights up to 40 kDa can be enhanced by several orders of magnitude [4, 5].

Currently the most broadly developing area of EP is electrochemotherapy, which is an effective way to treat cutaneous and subcutaneous tumours: HV pulses are applied to permeabilize tumour cells to an impermeable cytotoxic drug, which is administered by injection either into a vein or directly into a tumour [6].

Genistein (GEN; 4,5,7-trihydroxyisoflavone), one of the most abundant isoflavones in soybeans, is also called a phytoestrogen because of its structural similarity to the human hormone 17β-oestradiol [7]. GEN, a potent tyrosine kinase inhibitor, has been extensively used in the prevention and treatment of many diseases and disorders, including cancer, cardiovascular diseases, osteoporosis and postmenopausal symptoms [8]. Danciu et al. demonstrated the beneficial effects of GEN as regards tumour size, metastasis potential and melanization in a B16 mouse model of murine melanoma [9]. However, GEN is only poorly soluble in water and this can be a serious problem in terms of formulation [10].

A topically applied GEN-based lyotropic liquid crystal (LLC) formulation was earlier developed and combined with EP to treat murine melanoma lesions [11, 12]. The results showed that this strategy is promising for the recovery of skin lesions of murine melanoma type. LLCs are novel nanosystems usually formed from water and one or two surfactants, possibly with cosurfactants and oils. The main advantages of these systems include their similarity with colloidal systems existing in living organisms, their thermodynamic stability, and the situations that they can be stored for long periods of time without phase separation, they exhibit good penetration and they can facilitate the progressive diffusion of biologically active substances into the skin or systemic circulation. Besides their good solubilizing effects, they are able to incorporate and control the release of drugs of varying size and polar characteristics as they contain lipophilic, hydrophilic and amphiphilic domains [13, 14].

In the present work, the diffusion and penetration of GEN and LLC-GEN through different membranes were first investigated by conventional treatment without EP, and also with the mediation of EP. Measurements were made with a Franz diffusion cell system through a synthetic membrane *in vitro*, chick chorioallantoic membrane (CAM) *ex ovo* and excised human epidermis *ex vivo*. CAM has been introduced as a new and useful biological membrane model for preclinical permeability studies of pharmaceutical substances [15].

Vibrational spectroscopies were used to investigate the effects of EP on animal skin. Infrared (IR) and Raman spectroscopy have recently received increased attention in both the cosmetic and pharmaceutical fields. These methods offer attractive possibilities for the study of EP-mediated drug delivery.

Attenuated total reflection Fourier transform IR spectroscopy (ATR-FTIR) is a suitable tool with which to study the structure of the SC at the molecular level, to characterize its lipids, proteins and water content [16], and to analyse the penetration of drugs into the skin and the biochemical modifications induced by the penetration [17]. ATR-FTIR is essentially a non-destructive sampling technique which combined with tape-stripping serves as a rapid, simple, non-invasive method for studies of the skin. Through sequential tape-stripping, SC layers can be obtained from the deeper regions. The ATR device allows a spectrum to be acquired from a surface of a sample without preparation [18]. FTIR spectrometric studies on GEN have been investigated and reported earlier [19].

Raman spectroscopy is also widely used to study biological samples and to study the skin [20]. It has been utilized to determine the structures of GEN-cyclodextrin inclusion complexes

[21], GEN and other 5-hydroxyisoflavones [22]. The assignment of the Raman spectrum of native skin was reported by Pouliot [23]. Confocal Raman images have been investigated as chemical maps of *ex vivo* pigskin [24] and *ex vivo* human abdominal skin [25].

Following an investigation of the transdermal delivery of our newly developed LLC-GEN formulation through different membranes without EP, LLC-GEN was combined with EP to examine the efficiency of this combination by means of *in vivo* ATR-FTIR and *ex vivo* Raman spectroscopy. With vibrational spectroscopic monitoring, various treatment times and voltages were applied to evaluate the effects of EP on the skin.

2. Materials and methods

2.1. Materials

GEN was purchased from Extrasynthèse (Genay, France; purity >95%), and the non-ionic surfactant Cremophor RH 40 (CRH40; Polyoxyl 40 Hydrogenated Castor Oil USP/NF) from BASF (Ludwigshafen, Germany). The aqueous phase of the systems was purified water (Ph.Eur.6.), and the oil phase was isopropyl myristate (IPM; Merck Kft., Budapest, Hungary). The average particle size of GEN is 12.04 ± 7.74 µm (n = 564).

2.2. Sample preparation

Samples produced with a 2:1 by the oil-surfactant mixture were homogenized with a magnetic stirrer at room temperature, and 10 wt.% of water was then added in small amounts under stirring. For the drug-containing samples, 3 wt.% of GEN as active agent was partly dissolved (1 wt.%) in the oil-surfactant mixture with the aid of a magnetic stirrer.

2.3. Drug diffusion and penetration investigations

2.3.1. Preparation of CAM

Fertilized chicken eggs (*Gallus gallus domesticus*) were prepared for the experiment by cleaning with ethanol, and then placed horizontally in an incubator at 37 °C, with constant humidity. On the third day of incubation, 3–4 mL of albumen was extracted from the most pointed part of the egg, so that the developing CAM could be detached from the inner shell in order to work directly on the membrane. On day 4 of incubation, a window was cut in the surface of the egg, it was resealed with adhesive tape and incubation was continued until the day of the experiment. CAMs were collected in buffered formalin and used for *ex ovo* studies.

2.3.2. Preparation of heat-separated epidermis

With the approval of the Ethical Committee of the University of Szeged, Albert Szent-Györgyi Clinical Centre (Human Investigation Review Board licence number: 83/2008.), excised human skin was obtained from a Caucasian female patient who underwent abdominal plastic surgery. This was. The subcutaneous fatty tissue was separated from the outer skin layers after excision and the skin was stored at −20 °C. For the penetration experiment, after the skin had thawed, the epidermis was separated from the underlying dermis by a heat-separation technique [26]. Individual portions were immersed in water at 60 °C for 90 s. After removal of the skin from the water, it was placed SC side up on a filter paper, and the epidermis (comprising the SC and the viable epidermis) was gently removed from the underlying dermis with the use of forceps and was discarded. The epidermal membrane was floated onto the surface of PBS (phosphate buffer solution, pH = 7.4) for at least 20 min and then placed on a supporting cellulose acetate membrane (Porafil, Machenerey-Nagel, Düren, Germany, and Pall Life Sciences, Washington, NY, USA; pore diameter 0.45 µm) [27, 28].

2.3.3. Franz diffusion cell method

Membrane diffusion and permeability studies of GEN were carried out with a vertical Franz diffusion cell system (Hanson Research, Chatsworth, CA, USA) in a six-unit assembly (effective permeation area 1.33 cm^2) for 24 h (*in vitro* or *ex ovo*) or 48 h (human epidermis) at

37 °C. The donor phase was 0.30 g of sample, which was placed on a cellulose acetate membrane (Porafil, Machenerey-Nagel, Düren, Germany, and Pall Life Sciences, Washington, NY, USA) itself (*in vitro*), or in the case of *ex ovo* and *ex vivo* measurements the CAM and the epidermis were supported with a Porafil membrane filter. The acceptor compartment consisted of 10 wt.% ethanol containing PBS (pH 7.4), which was stirred at 450 rpm throughout the experiment. Samples of 0.8 mL were taken from the acceptor phase by the autosampler (Hanson Research, Chatsworth, CA, USA) and replaced with an equal volume of fresh receiving medium. The absorbance of the GEN content was measured at 261 nm with a Unicam Heλios α Thermospectronic UV-spectrophotometer v4.55 (Unicam, Thermo Fisher Scientific Inc., Waltham, MA, USA). Five parallel measurements were carried out and the amount of GEN penetrating during a time period was plotted. The results were expressed as means ± SEM.

2.3.4. Penetration analysis

Penetration parameters were obtained from the cumulative amount of GEN penetrated per cm^2 versus time plots (Q). The transdermal flux (J) was calculated from the slope of the concentration versus time profile in the receiver compartment, and expressed in $\mu g\ cm^{-2}\ h^{-1}$. For the linear regression analysis, the linear range of the incubation period from 1 to 6 h was selected, and was used to calculate the flux data of the compounds.

2.4. EP parameters

A Mezoforte Duo Mez 120905-D instrument (Dr Derm Equipment Ltd., Budapest, Hungary) was used to generate the electric pulses for the skin EP. The operation of this device is based on a pulsed electromagnetic field generated by an amplitude-modulated sine wave radiofrequency current. The polypropylene-covered treatment headpiece contains a plate electrode 25 mm in diameter indirectly contact with the treated surface. Modulation involves HV pulses 5 ms in duration, followed by a 20 ms break. GEN transport by means of skin EP can be influenced by using 700 V or 900 V with different treatment times (1 or 2 min).

2.5. Animal experiments

12-week-old male SKH-1 hairless mice were housed in plastic cages in a thermoneutral environment with a 12 h light-dark cycle and had access to standard laboratory chow and water *ad libitum*. All interventions were in full accordance with the NIH guidelines and the protocols were approved in advance by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged (licence number: V./145/2013). Prior to the intervention, the animals were anaesthetized with a mixture of ketamine (90 mg (kg bodyweight)$^{-1}$) and xylazine (25 mg (kg bodyweight)$^{-1}$) administered intraperitoneally. At the end of the experiments, the mice were euthanized with an overdose of ketamine (300 mg kg^{-1}).

The mice involved in the study were divided into 5 groups as follows:
Group A: mice treated with dermally applied LLC-GEN for 2 min
(conventional treatment)
Group B: mice treated with LLC-GEN and EP at 700 V for 1 min
Group C: mice treated with LLC-GEN and EP at 900 V for 1 min
Group D: mice treated with LLC-GEN and EP at 700 V for 2 min
Group E: mice treated with LLC-GEN and EP at 900 V for 2 min

For ATR-FTIR measurements, 5 mice were randomly assigned to each experimental condition. Corneocytes were collected from the uppermost layer of their dorsal skin with the use of adhesive tape (D Squames, CuDerm Corporation, Dallas, TX, USA) immediately after treatment. The treated and the untreated dorsal skin of each mouse was stripped with adhesive tape; this procedure was repeated up to 18 strips, an IR spectrum being recorded after every third tape strip. Every first tape with one strip was discarded because of the possibility of surface contamination.

For Raman measurements, 2 mice were randomly assigned to each of groups A, C and E. At the end of the observation period of these groups, the animals were sacrificed (as described above) and full-thickness skin samples were excised from the treated areas. The samples were embedded in Cryomatrix resin (Thermo Fisher Scientific Inc., Waltham, MA, USA) and cryosections of 6 μm were made.

2.6. ATR-FTIR measurements

ATR-FTIR spectra were recorded with an Avatar 330 FT-IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a horizontal ATR crystal (ZnSe, 45°), between 4000 and 400 cm^{-1}, at an optical resolution of 4 cm^{-1}. 128 scans were co-added and all spectral manipulations were performed with the Thermo Scientific's GRAMS/AI Suite software. In order to obtain a reference spectrum of the API, a KBr pellet containing 0.5 mg of GEN was prepared and used. The spectra of the preparations were also recorded. The spectra of five parallel samples, gained from five different animals, were recorded. Each average layer spectrum of the treated SC was corrected with the corresponding average layer spectrum of the untreated SC. No ATR correction was performed.

2.7. Raman measurements

Raman spectra were acquired with a Dispersive Raman Microscope (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a CCD camera and a diode laser operating at 780 nm. The spectra of the CRH40, IPM and GEN were collected with an exposure time of 6 s, with 48 scanning and cosmic ray and fluorescence corrections. Raman measurements were carried out with a laser power of 12 mW at a slit width of 25 μm. The localization of GEN in the skin samples was investigated by confocal Raman mapping. For mapping, the microtomed skin samples as depth sections were rotated by 90° and placed on an aluminium surface with the SC towards the top of the plate. Thus, a 1000 μm length of the sample specimen corresponds to a 1000 μm depth into the skin. The mapping was achieved in a point-by-point mode, using a step size of 10 μm perpendicular and parallel to the SC. Raman maps of skin with dimensions 30-40 μm parallel and 900-1300 μm perpendicular to the SC surface were generated one-by-one. The spectrum acquisition time was 3 s per spectrum, and 24 spectra were accumulated and averaged at each measured point, these parameters ensuring an acceptable signal-to-noise ratio. The mapping was carried out with a laser power of 24 mW at a slit width of 25 μm. The vibrational chemical images were studied by a multivariate curve resolution – alternating least squares chemometric method; the purpose was to identify GEN in the Raman spectra.

2.8. Statistical analysis

Two-way ANOVA was used to identify any significant differences in the total penetrated amount of GEN between the conventional treatment and the combined EP treatments by using GraphPad Prism software (Graph-Pad Software Inc., La Jolla, CA, USA). Differences were regarded as significant if $p < 0.05$* and $p < 0.001$***.

3. Results and discussion

3.1. Drug diffusion and penetration

The membrane diffusion investigations indicated that good drug release and diffusion was attained through the synthetic membrane *in vitro,* 713.26 ± 90.73 μg cm^{-2} being liberated in 24 h (Fig. 1(a)). Somewhat less drug was observed after 24 h (491.26 ± 15.55 μg cm^{-2}) in the case of *ex ovo* penetration (Fig. 1(b)). In the e*x vivo* investigations which revealed not only the amount of drug that diffused through the skin, but also the interaction with the skin and the incidental reservoir function of the SC, only a small amount of drug penetrated through the human epidermis (8.93 ± 1.81 μg cm^{-2} after 24 h and 15.68 ± 3.09 μg cm^{-2} after 48 h), but the penetration profile was similar to that for *ex ovo* penetration and sustained drug release was achieved (Fig. 1(c)). The transdermal flux (J) (reflecting the absorption rate per unit area)

showed that the drug penetration was slowed down by the application of the biological membrane, and that LLC-GEN alone was not sufficient to reach an appropriate plasma concentration.
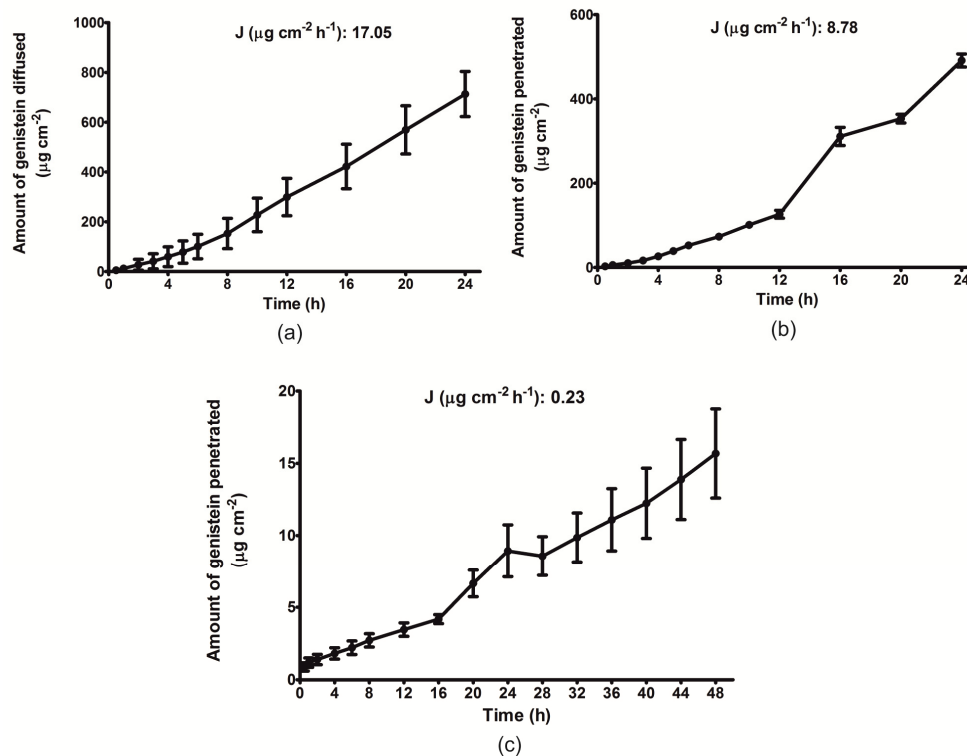

Fig. 1. Amount of GEN diffused *in vitro* (a), or penetrated *ex ovo* (b) or *ex vivo* (c).

3.2. ATR-FTIR spectroscopy

The penetration profile of GEN within the different layers of the SC was established with a combination of tape stripping and ATR-FTIR spectroscopy. The most characteristic peaks in the ATR-FTIR spectrum of GEN were assigned to the C1 = C2 stretch at 1652 cm^{-1}, and the C = O stretch, O7-H22 bend, and ring A C-C stretch at 1518 cm^{-1} (Fig. 2) [19].

Table 1 presents the relative absorbances in the SC layers at 1652 cm^{-1} and 1518 cm^{-1}, which correspond to the amounts of GEN that penetrated into the SC after conventional and EP treatments (Fig. 3). The drug delivery and the onset time for transport depend on the electrical parameters of the pulses and the physicochemical properties of the drug [3]. It was found that EP treatment at 700 V for 1 min EP treatment did not enhance the GEN transdermal delivery relative to the conventional treatment. This may be explained by the high viscosity of the drug carrier, which decreases the efficacy of EP on the skin [29]. Slightly deeper GEN penetration was ensured by EP treatment at 900 V for 1 min, but the total amount of GEN did not exceed that for conventional treatment with LLC-GEN. However, EP treatment at 700 V for 2 min resulted in a deeper penetration of a 2-fold amount of the drug than without EP treatment (revealed at both characteristic bonds of GEN). The best penetration profile of GEN was obtained on EP treatment at 900 V for 2 min, when the total amount of the drug was 3-fold greater as compared with conventional treatment at both assigned bonds (p < 0.001*** and p < 0.05*), but the GEN distribution was not consistent. The presence of GEN was not seen in the 5th and 6th tape strips, whereas the drug appeared again in the 7th strips. This phenomenon could be explained by the electrical breakdown of

the SC layers. Furthermore the amount of drug that penetrated increased with increasing voltage of the pulses [30, 31].
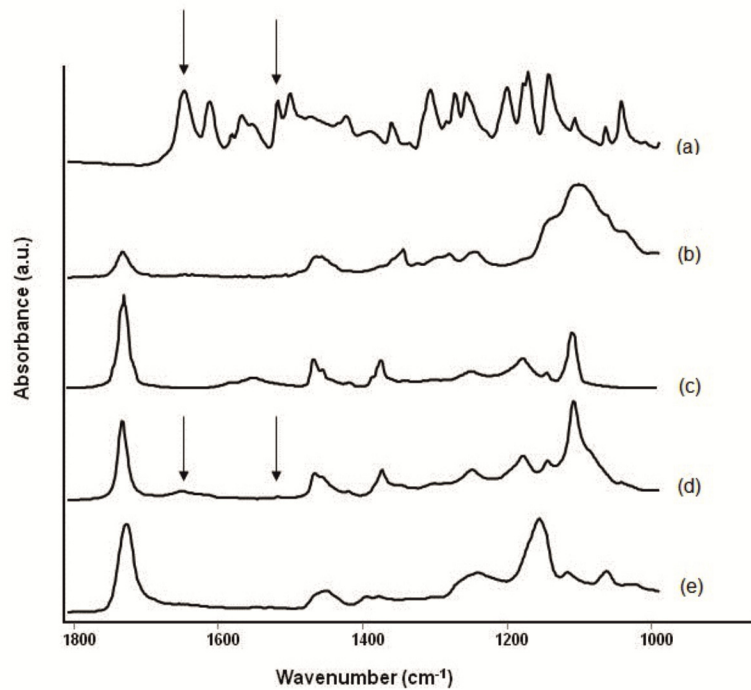

Fig. 2. ATR-FTIR spectra of pure GEN (a), Cremophor RH 40 (b), isopropyl myristate (c), LLC-GEN composition (d) and non-treated SC (e).

Table 1. Intensities of GEN in the different layers of the treated SC (average value ± SD, n = 3) (CT = conventional treatment).

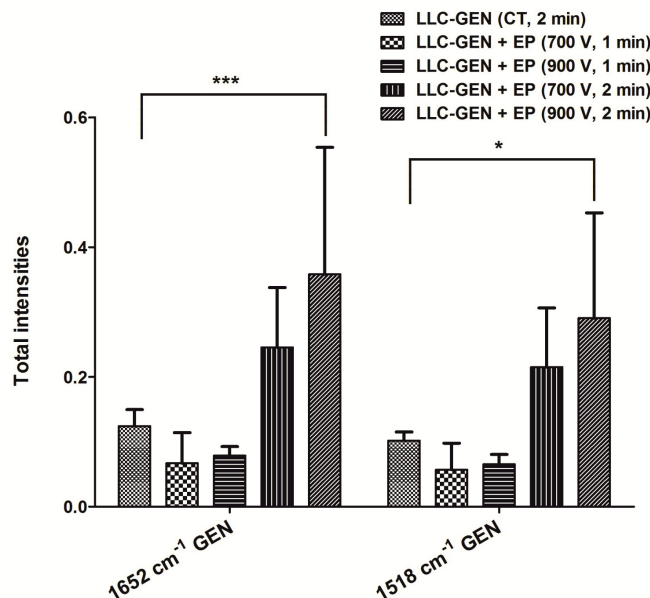| No. of tape strips	Treated with LLC-GEN (CT, 2 min) Absorbance at		Treated with LLC-GEN + EP (700 V, 1 min) Absorbance at		Treated with LLC-GEN + EP (900 V, 1 min) Absorbance at		Treated with LLC-GEN + EP (700 V, 2 min) Absorbance at		Treated with LLC-GEN + EP (900 V, 2 min) Absorbance at	
	1652 cm^{-1} GEN	1518 cm^{-1} GEN	1652 cm^{-1} GEN	1518 cm^{-1} GEN	1652 cm^{-1} GEN	1518 cm^{-1} GEN	1652 cm^{-1} GEN	1518 cm^{-1} GEN	1652 cm^{-1} GEN	1518 cm^{-1} GEN
1	0.06 ± 0.03	0.05 ± 0.02	0.05 ± 0.05	0.04 ± 0.04	0.04 ± 0.01	0.03 ± 0.00	0.15 ± 0.02	0.12 ± 0.02	0.22 ± 0.10	0.18 ± 0.08
2	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.08 ± 0.06	0.07 ± 0.05
3	0.03 ± 0.02	0.02 ± 0.01	-	-	0.03 ± 0.00	0,03 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.05 ± 0.00
4	-	-	-	-	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01
5	-	-	-	-	-	-	0.01 ± 0.00	0.01 ± 0.00	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	0.02 ± 0.02	0.02 ± 0.01
8	-	-	-	-	-	-	-	-	-	-

Fig. 3. Total intensities of GEN at 1652 cm^{-1} and at 1518 cm^{-1}. Conventional treatment (CT) was used as a control. $p < 0.001$*** and $p < 0.05$* vs. EP-treated group.

3.2. Raman spectroscopy

The deeper skin penetration of LLC-GEN containing the lipophilic ingredients CRH40 and IPM was studied in Raman scattering experiments. Raman chemical mapping was employed to confirm the localization of GEN at various depths of the skin specimens. Since ATR-FTIR demonstrated that EP treatment at 900 V ensured deeper penetration than that at 700 V, EP treatments at 900 V for 1 and 2 min were chosen for comparison with conventional treatment by Raman spectroscopy.

Figure 4 depicts the experimental Raman spectra of pure CRH40, IPM and GEN in the wavenumber range 2000-200 cm^{-1}. As model solution, GEN was dissolved in CRH40 and IPM containing LLC (GEN content 3 wt.%) to observe the physical state of GEN. Six common characteristic ranges can be seen in the spectra of CRH40 and IPM, with peaks centred at 1733, ~1442-1437, 1300, 1063 and 843-823 cm^{-1}, and the spectra of CRH40, IPM and the LLC-GEN composition are very similar. Table 2 lists the Raman bands of the components and the LLC-GEN composition. The lipid components used in this study have similar chemical structures and therefore similar Raman bands. The Raman spectrum of the LLC-GEN is basically the sum of the individual spectra, with minor shifts at around 1440 and 1079 cm^{-1} (Fig. 4(c)), indicating that the CH$_2$-CH$_3$ bonds of the LLC ingredients were affected by the interaction. Only a few Raman peaks of GEN were observed in the spectrum of LLC-GEN, at around 1617, 1062, 991, 887 and 789 cm^{-1}. For GEN, two characteristic peaks, centred at 1617 and 789 cm^{-1}, were assigned to the vibration of the C = C bond in ring B and the C-C-C, C-C and C = O bonds in ring C of the isoflavonoid molecule. These bands, free from interference from LLC ingredient bands, were chosen to confirm the localization of GEN in the skin specimen.

Raman spectra were acquired from skin treated with LLC-GEN and from non-treated intact *ex vivo* skin (Fig. 5), both perpendicular to the SC at equivalent depth (1000 μm). In the Raman spectrum of the treated skin, characteristic peaks of GEN and the LLC ingredients were observed at around 1670-1650 and 1470-1410 cm^{-1}, respectively. The band of interest
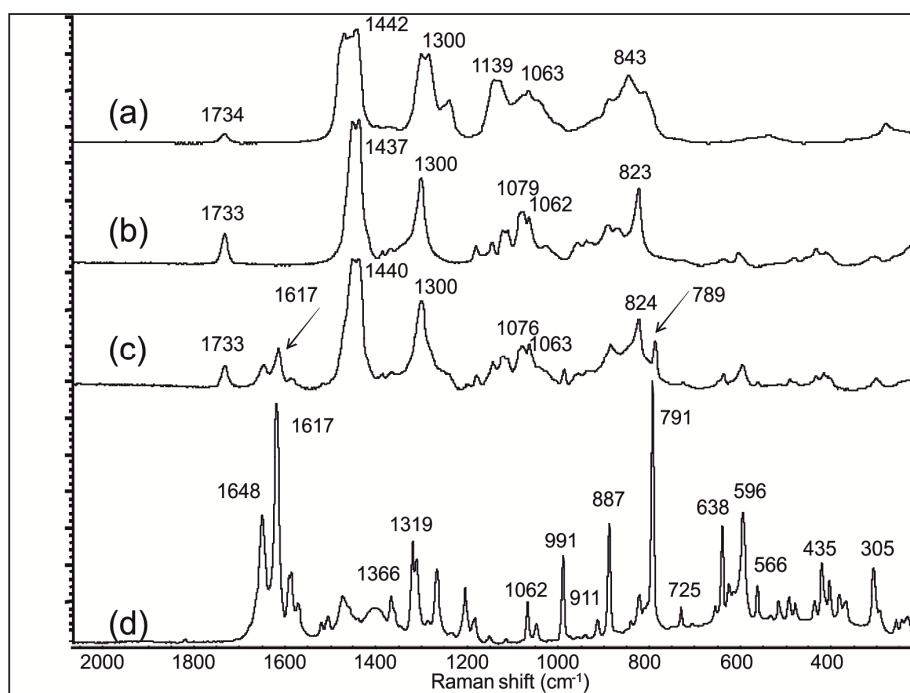
Fig. 4. Experimental Raman spectra of Cremophor RH 40 (a), isopropyl myristate (b), LLC-GEN composition (c) and pure GEN (d).

Table 2. Observed Raman peaks and peak assignments of individual components and LLC-GEN (*str* = stretching, *def* = deformation, *tw* = twisting).

Assignment	Cremophor RH 40	Isopropyl myristate	LLC-GEN	GEN
str C = O (ester)	1734	1733	1733	-
str C = C (ring A)	-	-	1648	1648
str C = C (ring B)	-	-	1617	1617
def CH2-CH3	1442	1437	1440	-
str C = C	-	-	-	1366
-	-	-	-	1319
def CH2 and *tw* CH3	1300	1300	1300	-
str C-C	1139	-	-	-
		1079	1076	-
str O-C	1063	1062	1063	1062
str C = C and C-C-C (ring A)	-	-	991	991
str C = C (ring A) and C-C-C (ring C)	-	-	887	887
str C-CH (aromatic ring)	843	-	-	-
	-	823	824	-
str C-C-C (C ring), *str* C = O	-	-	789	791
str C-C-C and C = C (ring A)	-	601	596	596
str C-C-C (rings A and C)	-	-	-	566

centred at 1440 cm⁻¹ was assigned to the vibration of the amide functional group due to C = O stretching and –CH₂, -CH₃ bending of the skin lipid constituents. The C = C stretching

observed at 1617 cm^{-1} arose predominantly from the GEN isoflavonoid. The chemical mapping therefore confirmed the presence of the characteristic GEN band at around 1617 cm^{-1}. This peak is close to the vibrations of the native skin components. The skin component peaks at 1608 and 1453 cm^{-1} are well known to be associated with the C = C vibration of phenylalanine, the CH$_2$ scissoring of lipids and the CH$_2$, CH$_3$ deformation of proteins in the native skin.
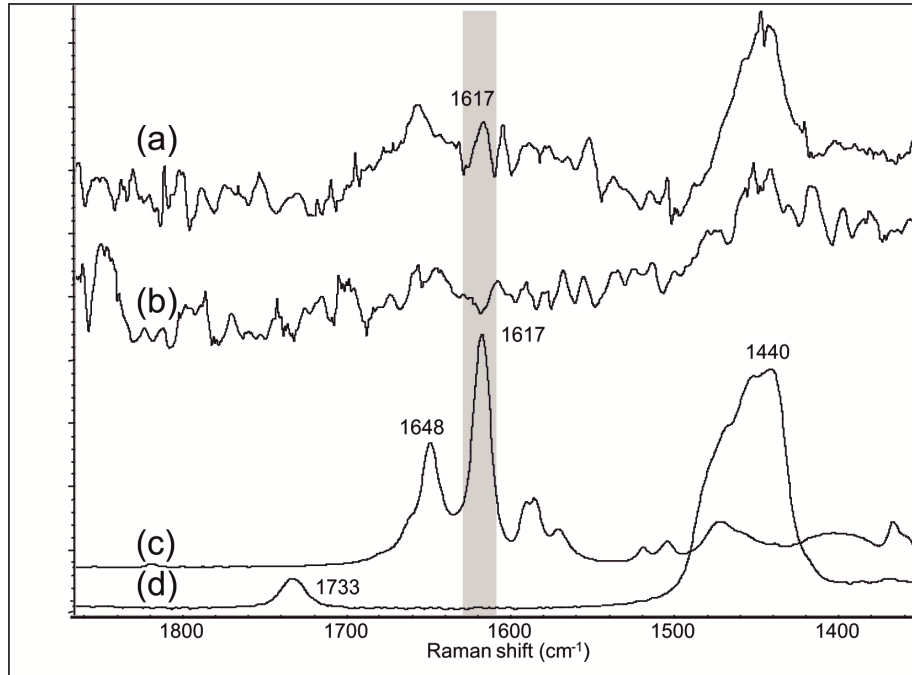

Fig. 5. Raman spectra of GEN-treated skin (900 V, 2 min, 1000 µm depth) (a), non-treated skin specimen as control (1000 µm depth) (b), pure GEN (c) and GEN-free LLC composition (d) in the range 1900-1350 cm^{-1}.

Figure 6 displays qualitative distribution maps of the LLC components and GEN in animal skin specimens after different EP protocols, at 10-fold magnification. It was of interest to determine whether GEN diffusion is limited to the SC or to the epidermis or dermis. As discussed in the Materials and methods section, as the skin specimens were planar, there was no need for the z-axis and adjustment of the depth values. 1300 µm deep sections of the skin specimen were investigated, from corresponding top to bottom to the SC → epidermis → dermis. In order to confirm GEN diffusion, the chemical maps were resolved. The characteristic bands obtained for GEN at around 1617 cm^{-1} were used to visualize the spatial distribution of GEN from the Raman chemical mapping. The resolution of the chemical map and hence the identification of the ingredients present were achieved by using a multivariate curve resolution – alternating least squares chemometric method. This method furnishes the response profiles of different components in complex samples, and provides information about the composition.

The presence of bands due to GEN and the LLC components in the upper layer of the skin (0-1000 µm) clearly indicates a good distribution following conventional treatment of the LLC-GEN composition (Fig. 6(a)). After the 900 V process for 1 min, GEN was accumulated in the middle section of the dermis (Fig. 6(b)). The penetration of GEN into the dermis was slowed by the dermis a being dense network of collagen fibres. The GEN and LLC component contents of the skin specimen (to a depth of 1300 µm) were relatively low after the 900 V EP process for 2 min (Fig. 6(c)), indicating that the GEN penetrated into the deep

layer of the dermis. Although the difference between the EP processes for 1 and 2 min may seem minor, the slower diffusion of GEN may be presumed because of the enrichment of the LLC components (as vehicles of GEN) in the 1000-1300 μm deep section. In the absence of the EP process, the epidermal and upper dermal regions serve as a tight barrier, impeding deep GEN delivery. Thus, slow diffusion of the GEN took place.
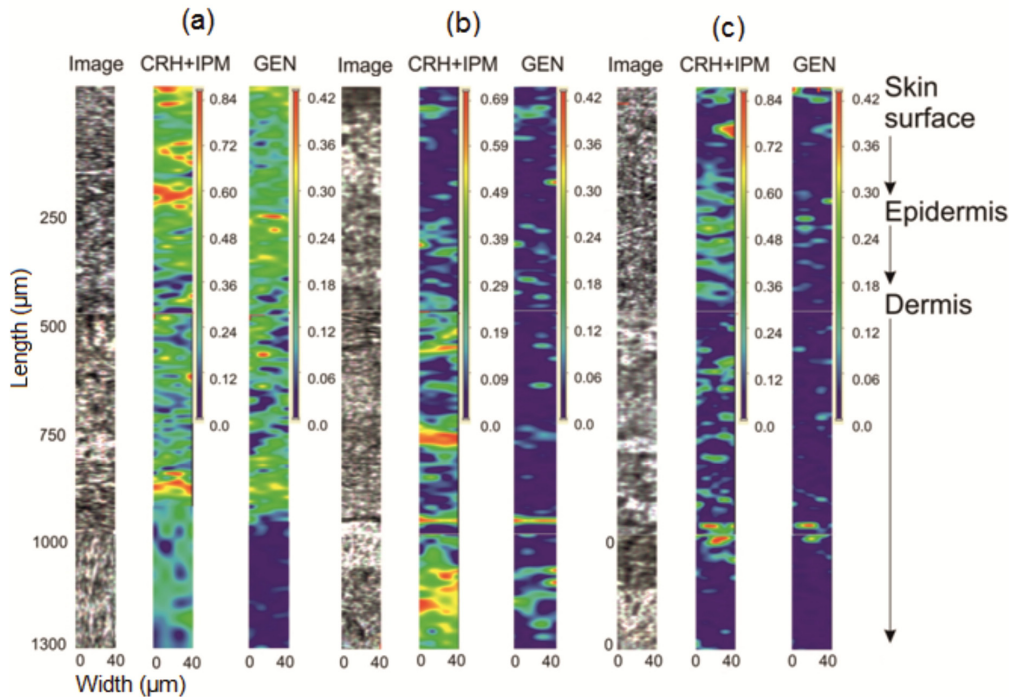

Fig. 6. Qualitative Raman maps of GEN distribution in animal skin specimens following different EP protocols (at 10-fold magnification). Conventionally treated skin as control (a), skin treated with EP at 900 V for 1 min (b) and skin treated with EP at 900 V for 2 min (c). Image: LLC-GEN composition, CRH + IPM: Cremophor RH 40 + isopropyl myristate, GEN: Genistein. Colour coding of content: red>yellow>green>blue.

4. Conclusions

In summary, the results of this study have demonstrated that our newly developed LLC formulation is a suitable carrier for the poor water soluble GEN. The penetration through the skin was ensured already by LLC-GEN alone application. However the combination of LLC nanocarrier system with EP was resulted rapid and more effective transdermal drug transport than alone LLC-GEN formulation. The synergistic effect of EP was verified by both the ATR-FTIR and the Raman spectroscopy. It could be concluded from our study that combination of LLC an EP process is very promising, but the optimization of drug penetration is necessary.

Acknowledgments