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EFFECT OF PEGYLATED TRANSFERSOMES CONTAINING MACROMOLECULAR PROTEIN ON TRANSDERMAL DELIVERY

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Abstract: Hydrophilic protein macromolecules are limited to passively penetrate through the skin. The objective of this study was to investigate the effect of various concentrations of PEGylation (1, 5 and 10%) grafted liposomes for delivering macromolecular protein through the skin. PEGylated transfersomes composed of phosphatidylcholine (PC), cholesterol (Chol), DSPE-PEG2000, Tween20 and d-limonene were prepared for loading bovine serum albumin (BSA) as macromolecular protein model. The characterizations of these nanocarriers were evaluated such as size, PDI, zeta potential and %loading capability (%LC). The in vitro permeation study was performed by using Franz diffusion cells through porcine skins. BSA-FITC content was analyzed using a fluorescence spectrophotometer. For the results, all formulations showed narrow size distributions (PDI<0.3). The sizes and zeta potential of all transfersomes were 43.90±1.75, 44.39±7.20 and 42.98±0.32 nm, and -9.95±0.22, -12.53±1.96, -12.93±1.44 mV, respectively, which increasing the PEGylation decreased the particle size and increased negative charge. %LC of all transfersomes was in the range of 9.70-13.33. The permeation study showed that the skin treated with 5% transfersomes has higher macromolecular protein delivery through skin than other formulations, because higher hydrophilic PEG content might provide higher conformational change on the surface of the transfersomes, leading to increase the skin hydration and then enhance the hydrophilic BSA-FITC penetrated through the skin. In conclusion, the concentration of PEGylation on the liposomes structure significantly affects the physicochemical properties ($p < 0.05$) leading to improve the skin permeability of hydrophilic protein macromolecules.

Keywords: PEGylation, transfersome, BSA, protein, skin

INTRODUCTION

Proteins are macromolecular compounds and important therapeutic agents. These molecules are sensitively degraded in gastrointestinal system by oral route and short half-life resulting to frequency repeat in the parenteral route of administration and low patient compliance (Alfredson and Zeller, 2012). Transdermal drug delivery system has been reported to improve these limitation and convenience to use (Zylberberg and Matosevic, 2016).

Skin is the largest organ and has multilayer to protect the body from exogenous molecule in the environment. Stratum corneum (SC) is the uppermost layer of the skin, which it composed of corneocytes and differentiated non-nucleated cells. SC is mainly barrier function for penetration of compounds through the skin (Godin and Touitou, 2007). Transdermal drug delivery using vesicle carrier with penetration enhancers can entrap and

deliver drug pass through the skin. Moreover, transdermal formulations can design to sustain release of active molecules from topical formulations and transport active compounds through the membrane barrier for modulating of systemic absorption (Zylberberg and Matosevic, 2016).

In the 1960s, Bangham reported liposomes for the first time. Liposomes are one type of lipid-based vesicle carrier, which amphiphilic phospholipid and cholesterol are the most component. Shape of liposomes is spherical unilamellar or multilamellar vesicles and size between 0.025 μm to 2.5 μm. In pharmaceutical and cosmetic industries, liposomes have been used for biodegradability, biocompatibility, low toxicity and both entrapment of hydrophilic and hydrophobic molecules (Dragicevic and Maibach, 2018; Akbarzadeh et al., 2013). The advantages of dermal liposomal drug delivery such as reducing serious side effects, avoiding first pass metabolism, improving stability of the drugs, enhancing physiological and pharmacological response and good compliance of patients (Zylberberg and Matosevic, 2016). Conventional liposome, the first generation of liposome, based on neutral phospholipid bilayer revealed poor stability and rapid clearance in the body (Sercombe et al., 2015). Subsequently, liposomes were developed to improve stability and longer-circulation time by remodeling their structure for example coating these liposomes shell with inert hydrophilic polymers (Zylberberg and Matosevic, 2016). Polyethylene glycol (PEG) as hydrophilic polymer has been presented to improve sterically-stabilized liposomes and enhance prolong circulation time, because covalent bond of liposome to PEG protects the active moiety from the recipient's immune system and changes hydrodynamic size to reduce its renal clearance. When PEG is grafted on the structure of liposomes, it affects the negatively charged of the liposomes and can entrap positively charged compounds and other macromolecules to improve the stability (Sercombe et al., 2015; Papahadjopoulos et al., 1991; Bulbake et al., 2017).

Transfersomes were also reported by Cevc and et al., which the deformable structure made more elastic vesicle to penetrate through pore or channel of the SC by hydration gradient. Some of studies were reported that protein or peptide can be transferred through the skin by transfersomes (Ashtikar et al., 2016). The objective of this study was to investigate the effect of various concentrations of PEGylation (1, 5 and 10%) grafted transfersomes for delivering macromolecular protein through the skin. PEGylated transfersomes composed of phosphatidylcholine, cholesterol, polyethylene glycol2000-distearoyl-glycerol-phosphoethanolamine (PEG2000-DSPE), Tween20 and d-limonene were prepared for loading bovine serum albumin (BSA) as macromolecular protein model. The characterizations of these nanocarriers were evaluated such as size, PDI, zeta potential and %loading capability (%LC). The in vitro permeation study was performed by using Franz diffusion cells through porcine skins. BSA-FITC contents were analyzed using a fluorescence spectrophotometer.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC) and *N*-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethylene, sodium salt (DSPE-PEG2000) were a gift from Lipoid GmbH, Ludwigshafen, Germany. Cholesterol (Chol), bovine serum albumin (BSA), BSA-fluorescein isothiocyanate conjugate bovine (BSA-FITC) and d-limonene were purchased from Sigma-Aldrich, MO, USA. Tween 20 was obtained from Ajax Finechem, Auckland, New Zealand. Other chemicals were analytical grade.

Preparation of PEGylated transferliposomes

The liposomal formulations were prepared by thin film hydration and sonication methods **(**Rangsimawong *et al*., 2014**).** The proportional compositions of thin film for all formulations were shown in **Table 1**. In brief, PC, Chol and DSPE-PEG2000 were dissolved in chloroform: methanol (2:1) and mixed together in test tubes. The solvent was evaporated to make lipid film by nitrogen gas and kept in desiccator for drying over 6 h. The 0.2% (w/v) of BSA-FITC dissolved in phosphate buffer saline (PBS pH 7.4) was added into the test tubes, followed by vortexes to formulate multi-lamellar liposomes, and then added tween 20 (2%(w/v)) and *d*-limonene (1%(w/v)), respectively. All formulations were sonicated on ice by probe-sonicator for 30 min (2 cycles) to reduce the size of liposome vesicles.

Table 1. The composition of all formulations

Formulations	**PC (mM)**	**Cholesterol (mM)**	**DSPE-PEG2000 (mM)**
F1 (transfersomes)	10	2	0
F2 (1%PEGylated transfersomes)	9.88	2	0.12
F3 (5%PEGylated transfersomes)	9.40	2	0.60
F4 (10%PEGylated transfersomes)	8.80	2	1.2

Characterization of PEGylated transfersomes

The particle size, polydispersity index (PDI) and zeta potential of PEGylated transfersomes were determined using a Dynamic Light Scattering (DLS) particle size analyzer (Zetasizer Nano-ZS, Malvern Instrument, Worcestershire, U.K.) with temperature 25 °C and a 4 mW He-Ne laser at a scattering angle of 173. Each formulation was diluted by ultrapure water before measuring. All formulations were performed in triplicate.

Drug loading capacity (%LC)

The BSA entrapped transfersomes were determined by using Amicon® Ultra-4 centrifugal filter devices. The filters were thoroughly rinsed out by addition of 2 ml of ultrapure water, followed by centrifugation for 15 min at 4000 rpm, 4 °C. After the filter devices were dried, the 2 ml of each sample was added. Centrifugation was performed for 15 min at 4000 rpm, 4 °C. The samples were collected from filtrate and Triton X-100 was added in ratio 1:1. The BSA content using lowry method and measured by a microplate reader (VICTOR NivoTM Multimode Plate Reader, PerkinElmer, Germany). The percent loading capacity was calculated according to equation 1:

$$\%LC = (\text{drug encapsulation}/ \text{total lipid component}) \times 100 \quad (1)$$

In vitro skin permeation study

Abdominal porcine skins were thawed in PBS pH 7.4 at room temperature before use. Subcutaneous fat was removed by medical scissors. The skin penetration was determined using Franz diffusion cells. The skins were inserted between the acceptor and the receiver compartments containing PBS pH 7.4 with a magnetic stirrer. Temperature was maintained at 32 °C during experimentation. An acceptor was added by 2 ml of each sample. The 500 µl of the receiver compartment was collected at different time intervals (2, 4, 6, 8 and 24 h) and equal volume of fresh PBS pH 7.4 was added to replace. The permeated BSA-FITC was analyzed by fluorescence microplate reader. The cumulative permeation profiles were plotted and the slope of the linear portion represented the permeated flux of BSA-FITC.

Fluorescence intensity of skin permeation

After *in vitro* skin permeation for 4 h, abdominal porcine skins treated with BSA-FITC loaded F1 (transfersomes) and BSA-FITC loaded F3 (5% PEGylated transfersomes) were imaged under a fluorescence microscope (LEICA DFC450 C, Heerbrugg, Germany). The results were calculated from fluorescence intensity according to equation 2:

$$\text{Fluorescence intensity} = FI - FI_0 \quad (2)$$

when FI_0 is fluorescence intensity at time 0 h and FI is fluorescence intensity at time 4 h**.**

RESULTS AND DISCUSSION

Physicochemical properties

As shown in Table 2, all formulations showed nanometer scale sizes in the range of 42.98 to 44.39 nm. The PDI values of all formulations were less than 0.3 representing a narrow size distribution and the zeta potentials of all formulations were negative charges. Although the physical properties of PEGylated transfersomes depend on the concentration of PEG graft, particle size, PDI and zeta potential of all formulations was no significantly difference. This indicated that Tween 20 as edge activator in transfersomes affected on the physical properties of liposome vesicles more than PEG grafted onto liposomes (data not shown).

For loading capacity, PEGylated transfersomes were significantly higher the %LC than transfersomes, but no significant difference between various concentration of PEG on liposomes structure. BSA is a hydrophilic macromolecular protein and entrapped in aqueous phase of liposomes. Hydrophilic PEG grafted liposomes, that can both entrap BSA in aqueous cavity and absorb BSA with the PEG graft (Melis *et al*., 2014), exhibited high BSA loaded liposome vesicles.

Table 2. The physicochemical characterization of all formulations. The results were represented mean ± S.D. (N=3). * indicates significant difference from transfersomes (p<0.05).

Formulation	Size (nm)	PDI	Zeta potential (mV)	%LC
F1 (transfersomes)	43.63 ± 0.18*	0.275 ± 0.00	-14.37 ± 2.56*	2.42 ± 0.30
F2 (1%PEGylated transfersomes)	43.90 ± 1.75	0.286 ± 0.02	-9.95 ± 0.22	13.33 ± 0.79*
F3 (5%PEGylated transfersomes)	44.39 ± 7.20	0.259 ± 0.00	-12.53 ± 1.96*	9.69 ± 0.98*
F4 (10%PEGylated transfersomes)	42.98 ± 0.32*	0.181 ± 0.00	-12.93 ± 1.44	12.72 ± 0.33*

In vitro skin permeation

In this experiment, we used BSA-FITC as a model macromolecular protein. The results were shown in Fig.1(A,B). The F3 (5% PEGylated transfersomes) was the highest BSA-FITC penetrated through the skin, followed by F1 (liposomes), F2 (1% PEGylated transfersomes) and F4 (10% PEGylated transfersomes), respectively. The appropriate concentration of PEG grafted transfersomes can improve skin penetration, because it increases the stretching property to support expanding of elastic vesicles membrane and bind water molecules for increasing hydration of the stratum corneum to supply permeation through the skin (Chen *et al*., 2013).

The quantity of DSPE-PEG2000 affects the different liposome structures. In PEG grafted liposomes up to 4 mol%, the structure of PEG shows mushroom-like structure. PEG

grafted liposomes in concentration above 4 mol% exhibited the brush-like structure of PEG. For PEG grafted liposomes more than 8 mol%, the liposomes start a transition process from liposomes to micelles and the size is reduced (Garbuzenko *et al*., 2005; Immordino *et al*., 2006). Besides, in the literature reported that varies concentration of PEG grafted lipid surface affected to absorption of BSA (Yan *et al*., 2005). In this study, the 5 mol% PEG on transfersome structure (F3) might be the suitable structure for delivering macromolecular protein through the skin.

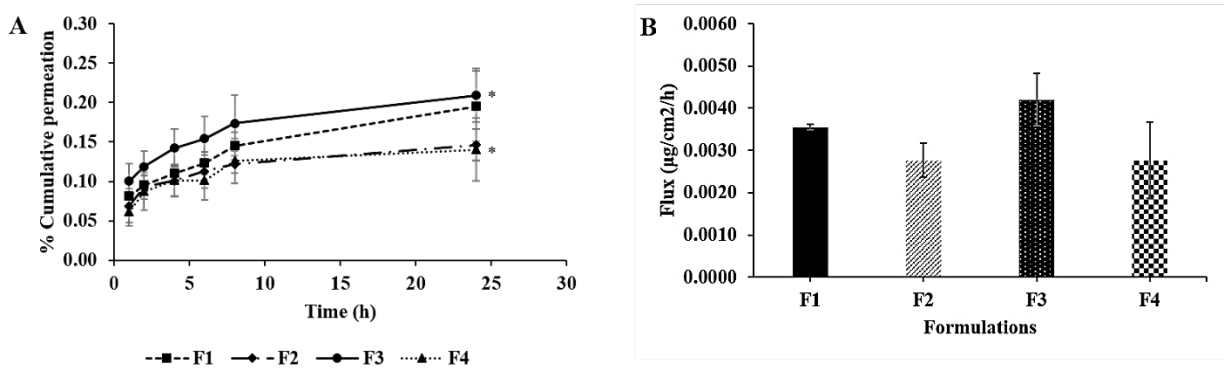


Figure.1 (A) The percentages cumulative permeation of all formulations through the skin by Franz diffusion cells. **(B)** The flux of drug permeation of all formulations. Each value shows mean ± S.D. (N=3). * represents significantly different values between 2 groups ($\rho < 0.05$).

Fluorescence intensity of skin permeation

To confirm the skin permeability of F3 (5% PEGylated transfersomes), the fluorescence intensity of skin treated with F3 after in vitro skin permeation for 4 h, abdominal porcine skins treated with BSA-FITC loaded F1 and BSA-FITC loaded F3 were imaged under a fluorescence microscope. The results are shown in **Fig. 2**. The fluorescence intensity of skin treated with F3 was higher BSA-FITC deposited in the skin than that F1 did. This indicated that 5% (w/v) PEGylated transfersomes improved the permeation of hydrophilic macromolecule protein into the skin.

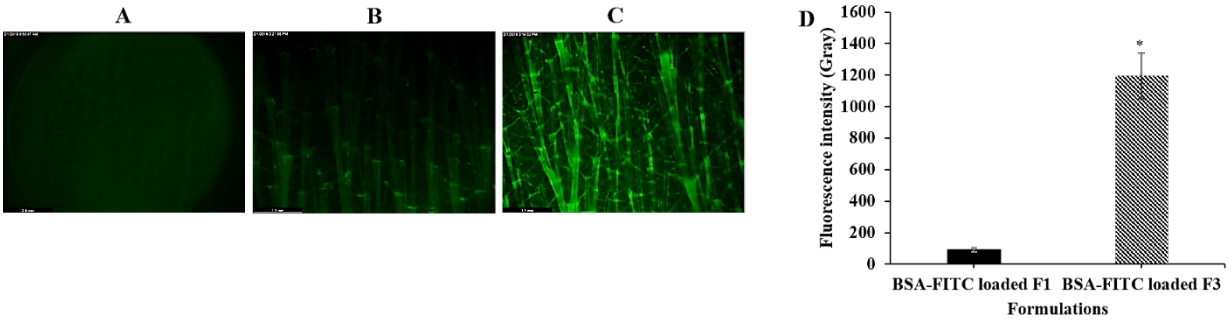


Figure.2 Fluorescence images of the skin: **(A)** untreated skin (control), **(B)** skin treated with F1 (transferliposomes) containing BSA-FITC for 4 h., **(C)** skin treated with F3 (5% PEGylated transfersomes) containing BSA-FITC for 4 h, and **(D)** The fluorescence intensity of BSA-FITC loaded F1 (transferliposomes) compared with BSA-FITC loaded F3 (5% PEGylated transfersomes). * indicates significant difference between formulations.

Statistical analysis

The data was shown as the mean ± S.D. and statistical analysis was analyzed by one-way analysis of variance (ANOVA) and student's t-test. *P-value*s less than 0.05 were indicated that the data was significant difference.

CONCLUSION

Various concentrations of PEG-DSPE on the liposomes structure affect the physicochemical properties and the skin permeability. The degree of PEGylation on the transfersomes significantly affect the entrapment of macromolecule proteins into the vesicles The 5% PEGylated transfersomes was a suitable physicochemical properties and the highest macromolecular protein delivery into and through the skin. Therefore, the optimal formulation of 5% PEGylated transfersomes can improve the skin permeability of hydrophilic protein macromolecules.

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