Delivery of tocopherol phosphate nanomaterials into the skin to protect against ultraviolet radiation

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Delivery of tocopherol phosphate nanomaterials into the skin to protect against ultraviolet radiation

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A thesis submitted for the degree of Doctor of Philosophy (PhD) in Pharmaceutical Sciences

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Contribution

I hereby declare the work presented in this thesis was performed exclusively by me.
List of publications

Journal articles


Abstracts


Abstract

Topically applied vitamin E (α-tocopherol, α-T) can provide protection from ultraviolet radiation induced skin damage and photoaging. However, α-T is oxidized and due to its hydrophobicity it is not easily delivered into viable skin layers where it elicits its photoprotective effect. Alpha-tocopherol phosphate (α-TP), a chemically stable, water-soluble derivative of α-T, could have similar effects as α-T as well as it may be able to more easily reach epidermis. The aim of this thesis was to investigate the skin penetration of α-TP and its photoprotection in vitro. At concentration of 35 mM, α-TP was found to form aggregates with 9 nm hydrodynamic size at a critical aggregation concentration of 4.2 mM when dissolved in 20% ethanol, 20% propylene glycol, 60% tris buffer at pH 7.4. The AFM images showed that at low concertation’s (~1 mM), α-TP formed spherical liposomes (diameter of 50 nm, height of 17 nm) and at high concentrations (6 mM) α-TP formed rods (diameter of 300-570 nm, height of 12 nm). The α-TP nanomaterial at pH 7.4 showed a 5-fold higher total skin deposition than α-T. In stratum corneum (SC) lipid monolayer model, the mean molecular area (MMA) per molecule of pure SC lipids at surface pressure of 30 mN/m (30.18 Å²/molecule) increased with the addition of 30% α-TP (38.12 Å²/molecule), this may be due increase the repulsive forces upon increasing the percentage of α-TP and thus weaken the attractive forces between SC lipids head groups and disrupt their packing.

The 24 h pre-incubation of HaCaT keratinocytes with α-TP in low serum media significantly suppressed the cell viability reduction induced by UVA1 (226 J/cm²) by 14% compared to irradiated vehicle control and whereas α-T did not. In vitro α-TP displayed weak antioxidant activity using the DPPH and ORAC assay, but the radical-scavenging activity in HaCaT keratinocytes using pre-irradiation treatment with 100 μM α-TP significantly reduced the ROS generation by ~ 24% compared to the irradiated vehicle control. Adding SLN to the α-TP topical formulation to mimic sunscreen products enhanced the release of α-TP from a 2% lotion and 2% gel by 2-fold and 11-fold, respectively possibly by deaggregation. The α-TP could be delivered into the viable epidermis where it showed photoprotective effects and it seemed feasible to include the α-TP in topical sunscreen (e.g. SLN) formulation to add or maximize the protection.
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<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activation protein-1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>NanoSiO2NH2</td>
<td>Amine-modified silica nanoparticles</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>AOxs</td>
<td>Antioxidants</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis protease activation factor-1</td>
</tr>
<tr>
<td>A</td>
<td>Area</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>APPH</td>
<td>2,2’-azobis-2-methyl propanimidamine dihydrochloride</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>BP-3</td>
<td>Benzophenone-3</td>
</tr>
<tr>
<td>NanoSiO2COOH</td>
<td>Carboxy-modified silica nanoparticles</td>
</tr>
<tr>
<td>CE</td>
<td>Cellulose ester</td>
</tr>
<tr>
<td>CRCE</td>
<td>Centre for Radiation, Chemical and Environmental Hazards</td>
</tr>
<tr>
<td>Cer/Chol/PA</td>
<td>Ceramide/Cholesterol/Palmitic acid</td>
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<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>CPE</td>
<td>Chemical permeation enhancer</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variance</td>
</tr>
<tr>
<td>Cs</td>
<td>Compressibility</td>
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<tr>
<td>ks</td>
<td>Compressibility modulus</td>
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<tr>
<td>IC(_{50})</td>
<td>Concentration for 50% inhibition</td>
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<tr>
<td>CAC</td>
<td>Critical aggregation concentration</td>
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</table>
CMC  Critical micelle concentration
lec  Critical chain length of the hydrophobic tail
Cpp  Critical packing parameter
CPD  Cyclobutane pyrimidine dimers
COX-2 Cyclooxygenase 2
CK10 Cytokeratin 10
DNA  Deoxynucleic acid
Kcps  Derived count rate per second
DEJ  Dermal-Epidermal Junction
DCF  2’, 7’-dichlorofluorescein
DCFDA  2’,7’-dichlorofluorescin diacetate
DMSO  Dimethyl sulfoxide
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DPPH  1,1-diphenyl-2-picryl-hydrazyl
DPG  Dipotassium glycyrrhizinate
DPA  Dipyridylamine
DMEM  Dulbecco’s modified Eagle’s medium
DLS  Dynamic light scattering
A0  Effective hydrophilic head group surface area
Vo  Effective volume of the hydrophobic tail
G’  Elastic modulus
EGF  Endothelial growth factor
ER  Enhancement ratio
ETOH  Ethanol
ECM  Extracellular matrix
Af   Final absorbance
FBS  Fetal bovine serum
FDA  Food and drug administration
Hz   Frequency
FWHM Full width at half maximum
FDO  Furildioxime
GAA  Glacial acetic acid
GSH  Glutathione
HO   Haem oxygenase
HPLC High performance liquid chromatography
HRF  High resonance frequency
n    Hill slope
HaCaT Human adult low calcium temperature
HT29 Human colon adenocarcinoma cells
HCT116 Human colorectal cancer
HGF-1 Human gingival fibroblast
TPH-1 Human monocytic leukaemia cell line
HCl  Hydrochloric acid
HAT  Hydrogen atom transfer
H2O2 Hydrogen peroxide
HPC  Hydrogenated phosphatidylcholine
HIF-1 Hypoxia-inducing factor-1
IL-1β Interleukin-1β
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ICH</td>
<td>International council for harmonisation</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardisation</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>IVL</td>
<td>Involucrin</td>
</tr>
<tr>
<td>IPM</td>
<td>Isopropyl myristate</td>
</tr>
<tr>
<td>LIP</td>
<td>Labile iron pool</td>
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<tr>
<td>$t_{\text{lag}}$</td>
<td>Lag time</td>
</tr>
<tr>
<td>LB</td>
<td>Langmuir Blodgett</td>
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<tr>
<td>L-AA</td>
<td>L-ascorbic acid</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
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<td>LOQ</td>
<td>Limit of quantitation</td>
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<td>LO$^*$</td>
<td>lipid alkoxy radicals</td>
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<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
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<tr>
<td>LOO$^*$</td>
<td>Lipid peroxyl radicals</td>
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<tr>
<td>LC</td>
<td>Liquid condensed</td>
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<tr>
<td>LE</td>
<td>Liquid expanded</td>
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<tr>
<td>MM</td>
<td>Malignant melanoma</td>
</tr>
<tr>
<td>MPPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>Vmax</td>
<td>Maximum surface pressure</td>
</tr>
<tr>
<td>MMA</td>
<td>Mean molecular area per molecule</td>
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<td>MCF-7</td>
<td>Michigan Cancer Foundation-7</td>
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<tr>
<td>ME</td>
<td>Microemulsion</td>
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<td>MED</td>
<td>Minimum erythemal dose</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
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<td>NOX-1</td>
<td>NADPH oxidase</td>
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<td>Nanoemulsion</td>
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<td>NLC</td>
<td>Nanolipid carrier</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate hydrogen</td>
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<td>NHEK</td>
<td>Normal human epidermal keratinocytes</td>
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<tr>
<td>Nf-Kb</td>
<td>Nuclear factor-B</td>
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<td>NER</td>
<td>Nucleotide excision repair</td>
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<tr>
<td>N</td>
<td>Number</td>
</tr>
<tr>
<td>$\eta_c$</td>
<td>Number of carbon atoms in the hydrophobic tail</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
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<td>OAT</td>
<td>Organic anion transporter</td>
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<td>ODC</td>
<td>Ornithine decarboxylase</td>
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<td>8-oxoGua</td>
<td>8-Oxo-7,8-dihyoguanine</td>
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<td>ORAC</td>
<td>Oxygen Radical Absorbance Capacity</td>
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<td>Peroxyl radicals</td>
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<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>PDI</td>
<td>Polydispersity index</td>
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<td>KBr</td>
<td>Potassium bromide</td>
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<td>PG</td>
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<td>PGE$_2$</td>
<td>Prostaglandin $E_2$</td>
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<td>Abbreviation</td>
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<tr>
<td>PHE</td>
<td>Public Health England</td>
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<td>PIH</td>
<td>Pyridoxal isonicotinyl hydrazone</td>
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<td>6-4 PP</td>
<td>(6-4) Pyrimidine-pyrimidone</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RHE</td>
<td>Reconstructed human epidermis</td>
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<td>Sucrose mono-palmitate</td>
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<td>Serine palmito-transferase</td>
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<td>Si$_3$N$_4$</td>
<td>Silicon nitride</td>
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<td>SET</td>
<td>Single electron transfer</td>
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<td>NaBr</td>
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<td>Sodium cholate</td>
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<tr>
<td>SSR</td>
<td>Solar simulating radiation</td>
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<td>SLN</td>
<td>Solid lipid nanoparticles</td>
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<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SED</td>
<td>Standard erythemal dose</td>
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<td>$A_0$</td>
<td>Starting absorbance</td>
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<td>Storage modulus</td>
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<td>Stratum corneum</td>
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<td>SPF</td>
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<td>Sunscreen</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>$\pi$</td>
<td>Surface pressure</td>
</tr>
<tr>
<td>TBHP</td>
<td>Tertiary butyl hydroperoxide</td>
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</table>
3D  Three dimensional
T   Time
K   Time to reach 50% of maximum surface pressure
TiO₂  Titanium dioxide
α-T  α-tocopherol
α-TA  α-tocopherol acetate
α-TE  α-tocopherol equivalent
δ-TG  δ-tocopherol glycoside
α-TP  α-tocopherol phosphate
TPM  Tocopherol phosphate mixture
TPGs  Tocopherol polyethylene glycols
TGF-β  Transforming growth factor-β
TGM  Transglutaminase
TFA  Trifluoroacetic acid
TEAC  Trolox equivalent antioxidant capacity
T80  Tween 80
UVR  Ultraviolet radiation
Nano₅O₂  Unmodified silica nanoparticles
VE  Vitamin E
WLM  Wormlike micelle
XP  Xeroderma pigmentosum
ZnO  Zinc oxide
Chapter 1. Introduction
1.1 General introduction

Cosmeceutical products applied to the skin can comprise either natural or synthetic actives, which are topically applied as a lotion, cream, or gel product, with the intention of having a beneficial effect within local viable skin layers (Draelos, 2009; Lintner et al., 2009; Roy et al., 2013). However, the actives must pass the skin’s outermost layer, the stratum corneum (SC), to elicit their beneficial effects; thus, their efficacy can be limited by their physiochemical properties. It is possible that an active may remain on the skin surface, it may be retained in the SC, or it may permeate into the viable skin layers (Forster et al., 2009). Therefore, an understanding of delivery is essential in developing cosmeceutical products when they are applied to the cutaneous tissue.

Sun protection products are examples of cosmeceuticals that deliver actives into the epidermis. They are part of global sun care market, which is estimated to be worth US $24.91 billion by the end of 2024 (Transparency Market Research, 2016). Consumer increased awareness of the potential damage caused by overexposure to ultraviolet radiation (UVR) has driven an increase in demand for sun protection products in the last few years. One of the most common cosmeceutical actives that can protect from the sun’s damage is the antioxidant α-tocopherol (α-T) (Manela-Azulay and Bagatin, 2009). However, α-T has limitations; it is (i) chemically unstable, (ii) susceptible to photodegradation, and (iii) too hydrophobic to pass into the epidermis. These limitations hinder its effective use in cosmeceutical products applied on human skin. One of the strategies that have the potential to resolve the α-T issues is the addition of a phosphate moiety to yield a water-soluble, chemically stable prodrug known as α-tocopherol phosphate (α-TP). However, α-TP is an
amphiphilic molecule that tends to self-assemble in topical formulations forming aggregates, which may influence its penetration into the skin.

Previous studies did not report whether the α-TP aggregates were likely to affect skin penetration (Gavin et al., 2017; Nakayama et al., 2003). In addition, the mechanism by which α-TP permeates into the skin is unknown and the interaction of α-TP with skin lipids has not been documented. Furthermore, the delivery vehicle that was used in previous work by Nakayama et al for α-TP skin permeation studies does not simulate a real topical formulation (Nakayama et al., 2003). Consequently, at present with the available preliminary data it is problematic to translate α-TP into a topical formulation that can be used in humans.

In terms of α-TP’s ability to protect against UVR-induced skin damage, photoprotection studies have not been performed with the long-waveband UVA1 region (375-400 nm) (Kato et al., 2011; Nakayama et al., 2003). This is an important omission in the literature because; UVA1 is a major component of the sunlight spectrum (~75%), unintentionally ignored when estimating the sun protection by a standard solar simulating radiation (SSR) source that has decreasing output at > 365 nm. This region generates reactive oxygen species (ROS), not blocked by traditional UVR filters (Diffey and Osterwalder, 2017).

The primary aim of this thesis was to develop a α-TP cosmeceutical formulation that was suitable for human topical application. To achieve this, the data missing from the literature, in terms of determining α-TP aggregation properties, α-TP interaction with skin lipids, and α-TP photoprotection against UVA1 were sought. Then, this information was used to try and generate an α-TP formulation that could be commercially viable. The first stage towards this aim is to understand the layers
of human skin and routes of permeation, the photochemical basis of skin photobiology, the acute and chronic consequences of UVR, photoageing, photocarcinogenesis, endogenous photoprotection in human skin, and topical (exogenous) photoprotective agents, and how nanomaterial can modulate the skin permeation.

1.2 Skin structure and potential route of drug absorption

The human skin is a multi-lamellar organ that functions as a biochemical and physical barrier to pathogens, chemicals, and solar UVR damaging the internal organs and deeper tissues (Jia and Nash, 2010). The skin is composed of three layers each with different cell types: the epidermis, dermis, and subcutis (see fig.1.1). The epidermis is the outer lamina of the skin. The majority of the cells (95%) are keratinocytes and the rest (5%) are melanocytes, Langerhans cells, and Merkel cells (Lai-Cheong and McGrath, 2017). The epidermis consists of four main layers that differ in the differentiation state of keratinocytes; these are stratum basale, stratum spinosum, stratum granulosum, and SC. The stratum basale, which is also known as basal layer or stratum germinativum, consists of single layer of the actively dividing keratinocytes as well as melanocytes, and Merkel cells (Benson et al., 2012). These keratinocytes proliferate and undergo cell division (mitosis) to give daughter cells. Some remain in the basal layer whilst others undergo terminal differentiation in a 40-day journey that ends in the formation of the SC (McGrath and Uitto, 2016). The melanocytes, which consist of a central body and dendrite tips, are dispersed in the stratum basale. They produce melanin pigments (Hirobe, 2014), that include black to brown eumelanins and the yellow to reddish pheomelanins (Ito and Wakamatsu, 2003). These are packed and stored in
melanosomes which then transfer to neighbouring keratinocytes in the basal layer via dendrites of melanocytes (Seiberg, 2001). Melanin determines the skin colour (Ito and Wakamatsu, 2003) and attenuates the harmful effects of solar UVR (Park et al., 2009). Langerhans cells, distributed throughout the viable epidermis, are bone marrow derived dendritic cells that play a major role in skin’s immune system as antigen-presenting cells (APC). They recognize and bind to foreign antigens and migrate down through the dermis and to the lymphatic ganglia and hence trigger the immune system by activation of T cells (Benson et al., 2012). Merkel cells are linked to sensory nerve endings transmitting signals from skin as to regulate cutaneous sensation (Lai-Cheong and McGrath, 2017; Tachibana and Nawa, 2002).

The *stratum spinosum* (known also as prickle cell layer or squamous cell layer) is composed of several layers (2 to 6) of keratinocytes that migrate upwards changing shape from columnar to polygonal linked via desmosomes known as ‘spines’ or ‘prickles’ giving the structural rigidity throughout this layer (Moss et al., 2015).

The *stratum granulosum*, which composed of 1 to 3 layers of granular keratinocytes, migrate and stratify (flatten) towards the skin surface which indicates a decrease in metabolic activity, characterised by the presence of keratohyalin and membrane-coated granules that act as precursor of intercellular lipid lamellae/domain in the SC (Benson et al., 2012; Moss et al., 2015). Finally, the outermost SC which mainly consists of 70% protein (mostly keratin) and 20% lipids (the remaining 10% is still under elucidation) is composed of 10 to 15 layers of terminally differentiated enucleated keratinocytes known as corneocytes. The SC is the primary barrier of the epidermis restricting the entry of xenobiotics and preventing water loss (Michaels et al., 1975). This is mainly through a highly confluent layer of non-living corneocytes cells rich in protein ‘bricks’ embedded in
a cholesterol, long-chain free fatty acid and a ceramide lipid matrix ‘mortar’ (Elias et al., 1981; Nemes and Steinert, 1999). The epidermis is structurally separated from the dermis by dermal-epidermal junction (DEJ) known as cutaneous basement membrane zone, which is composed of protein and glycoproteins that provide adhesion between collagen fibres in the superficial dermis and keratin filaments of basal keratinocytes in the bottom of epidermis (Lai-Cheong and McGrath, 2017).

The dermis mainly consists of two layers; the papillary and reticular dermis. The papillary dermis is rich in blood supply and sensory nerve terminals and in direct contact with DEJ. The reticular dermis is the main layer of the dermis and in direct contact with hypodermis (McGrath and Uitto, 2016). The dermis consists of interstitial connective tissue with collagen fibres that provide support, and flexible elastic tissue embedded in a mucopolysaccharide gel-like matrix. Within the matrix, several cellular populations are dispersed such as fibroblasts which synthesise the collagen and elastic fibres, mast cells, lymphocytes, dendritic cells, and macrophages. This matrix also includes sensory nerves, blood vessels and lymphatic systems, as well as skin appendages such as hair follicles, sweat and sebaceous glands. The deepest layer is the subcutis (also known as hypodermis) containing adipocytes found as lobules separated from each other by collagen and elastic fibres. These adipocytes produce and store large quantities of fat that acts as energy storage, heat insulator, and a cushion for mechanical shock (Benson et al., 2012; Lai-Cheong and McGrath, 2017).
Fig.1.1: Schematic representation of layers of human skin (Boury-Jamot et al., 2006).

Three main routes have been identified for drug penetration into human skin, the intercellular (paracellular) pathway, transcellular (intracellular) pathway, and the transappendageal pathway i.e., via hair follicles and sweat glands also known as the shunt route (see figure 1.2) (Hadgraft and Lane, 2011). Although the transcellular route appears to be shortest route it requires crossing through densely packed dead corneocytes and successive transfers between corneocytes and lipid bilayers, which slows the penetration of chemicals through this route. The shunt pathway although historically thought not to be the major contributor to drug absorption, as the skin appendages only occupy 0.1% of total human skin, it has recently been shown to be a potential (significant) transport route for a range of compounds ranging from caffeine to nanomaterial to access the skin (Kattou et al., 2017; Nastiti et al., 2017). Most topically applied chemicals permeate across the skin via intercellular (tortuous) route, i.e., via the lipid matrix (Michaels et al., 1975). Several studies have shown that changing the lipid composition of human epidermis dramatically increases water diffusion through human skin (Berenson and Burch, 1951; Sweeney
and Downing, 1970). After the active release from the formulation and partition into SC, it diffuses through the SC via lipid matrix and reaches the SC/viable epidermis interface where it passively undergoes partition from SC lipid into aqueous viable epidermis followed by diffusion through viable epidermis and hydrophilic dermis at the DEJ. Then the active can be uptaken into systemic circulation via dermal capillary network (Barry, 1983; Kalia and Guy, 2001).

![Diagram of drug penetration](image)

**Fig.1.2: Route of drug penetration** (Hadgraft and Lane, 2011).

The simplest method to model the process of skin transport is to assume the skin is a pseudo homogenous membrane through which the drug passively diffuses through as described by Fick’s First Law (Equation 1.1) (Fick, 1855).

\[
J = -D \frac{\partial c}{\partial x}
\]

Equation 1.1
This law states that \( J \), the flux (mass.m\(^{-2}\).s\(^{-1}\)), which is the rate or speed of active transfer per unit area at steady state, is directly proportional to the differential concentration gradient (C) over differential distance (X), and D which is the diffusion coefficient of active in the membrane. In the case of an infinite dose of a drug, the maximum flux, i.e., steady-state permeation is achieved with no depletion of active concentration in the application vehicle. The cumulative amount of the active permeated into the skin versus time results in a steady–state flux (\( J_{ss} \)) under sink condition, which is calculated from the slope of the curve according to integrated form of Fick’s first law of diffusion (Equation 1.2) (Moser et al., 2001; Scheuplein and Blank, 1971).

\[
J_{ss} = \frac{KDc_f}{h} \quad \text{Equation 1.2}
\]

Where \( k \) is partition coefficient of active between membrane and formulation, D is the diffusion coefficient, \( C_f \) represent the concentration of active in the formulation, and \( h \) is thickness of the membrane.

The partition and diffusion of an active both influence the skin permeability (skin penetration) and the permeability coefficient (P) equal \( KD/h \) can be calculated from the steady state flux over concentration (Equation 1.3) (Singh and Singh, 1993):

\[
P = \frac{J_{ss}}{C_f} \quad \text{Equation 1.3}
\]

The permeability coefficient is independent of concentration and hence it can be used to compare different chemicals in terms of their ability to pass into the skin.
As it is important for a photoprotective agent to passively diffuse through the skin to reach the site of action. To help understand how a photoprotective agent benefits the skin, it is important to understand the acute and chronic of UVR, the basis of skin photobiology, photoageing, photocarcinogenesis, and endogenous photoprotection mechanism in human skin. These will be discussed in the subsequent sections.

1.3 Acute and chronic effects of ultraviolet radiation (UVR)

Ultraviolet radiation (UVR) (wavelengths 100-400 nm) is composed of short wave UVC (100-280 nm), medium wave UVB (280-320 nm), and long wave UVA (320-400 nm). UVA is subdivided into UVA2 (320-340 nm) and UVA1 (340-400 nm) (Moseley, 2006). The ozone layer absorbs all UVR below ∼295 nm, and a considerable amount of UVB, thus terrestrial UVR (∼295-400 nm) (Fig. 1.3) typically contains 95% UVA and only about 5% UVB, the former being mostly ∼75% UVA1 (Tewari et al., 2013). UVA penetrates deeper into the skin than UVB and can induce the generation of reactive oxygen species (ROS) (Forestier, 2008; Vile and Tyrrell, 1995). Despite the fact that UVB comprises only about 5% of the total solar UVR, it can cause more intense UVR-induced skin damage than UVA. The intensity of UVB reaches a maximum in mid-day (solar noon when the sun is at its highest) whereas the UVA remains consistent throughout the day. Thus, the ratio of UVB: UVA varies with the time of day, as well as season and latitude. The skin penetration depth is proportional to the wavelength of UVR. The longer the UVA band the deeper the penetration (Lim et al., 2001; Marionnet et al., 2014; Scientific Committee on Emerging and Newly Identified Health Risks, 2012) (Fig 1.4). Moreover, the absorption of UVR photon energy also depends on the
wavelength of solar UVR and this is linked to a certain photobiological outcome through the outcome action spectrum (Young, 1997). The action spectra within the skin vary on the absorption spectra of chromophores and if the action spectra for different outcomes e.g. erythema, DNA damage, and non-melanoma cancer, are similar this implies common chromophores (e.g. DNA chromophore) (Fig. 1.5). Therefore, the absorption of UVR photons by endogenous chromophores in skin layers may consequently results in various acute and chronic clinical or biological effects of UVR (Weatherhead et al., 2013). The acute effects of exposure to UVR are erythema (sunburn reaction), inflammation, oedema, pain, exfoliation, epidermal thickening, and immunosuppression, most of which are a consequence of UVB (Dreher et al., 1998; Matsumura and Ananthaswamy, 2004; Witt et al., 1993; Young, 2006a). Tanning (pigmentation) is caused by both UVA and UVB (Miyamura et al., 2011; Ortonne, 1990), and hypotension is caused by UVA (Johnson et al., 2016; Liu et al., 2014). The long-term (chronic) effects of UVR exposure include photoageing (Rittié and Fisher, 2002), pre-malignant skin lesions (Matsumura and Ananthaswamy, 2004) and skin cancer (Armstrong and Kricker, 2001; Black et al., 1997; Lazovich et al., 2010; Watson et al., 2016). Many studies have used in vitro skin models to investigate the biological effects of UVR using artificial UVA or UVB sources. These have shown evidence for the benefit of photoprotection against cell death, ROS formation, and upregulation of gene expression. Studies can also be performed with solar simulated radiation (SSR), and other UVR spectra, on human skin in vivo but these are much more costly and require ethical approval.
Fig. 1.3: A typical solar spectrum in London U.K. summer at noon (Young et al., 2000).

Fig. 1.4: Penetration depth of ultraviolet, visible and infrared radiation into the skin (Juzeniene et al., 2011; Scientific Committee on Emerging and Newly Identified Health Risks, 2012).
Fig. 1.5: Known action spectra within the skin. Three action spectra are shown: non-melanoma skin cancer (blue), DNA damage (black) and erythema (red). These follow a similar pattern, suggesting that they may have common chromophores (Weatherhead et al., 2013).

1.4 Photochemical bases of skin photobiology

The skin contains many endogenous chromophores such as DNA, trans-urocanic acid, riboflavin, NADPH, porphyrins, quinones and bilirubin (Young, 1997). Chromophores which are inefficient in dissipating the gained energy may act as photosensitizers. They absorb photon energy and can be excited to the single state transferring energy by intersystem crossing to the triplet exited state which then dissipates the energy by interacting with other molecules. This interaction could be with other substrate (type I electron transfer reaction) or with oxygen ($^3$O$_2$) (type II energy transfer reaction) (Li and Grant, 2016; scharffetter-kochanek et al., 1997). The type I reactions form radical ions, which can react with oxygen to produce superoxide radical ion ($O_2^-$) and consequently generate hydrogen peroxide (H$_2$O$_2$) by dismutation, whereas type II reactions generate singlet oxygen ($^1$O$_2$) which reacts with other substrates to form oxidized products such as oxidative damage of DNA repair proteins hence exacerbating the UVR-induced DNA damage (Fig. 1.6).
(Darr and Fridovich, 1994; McAdam et al., 2016). The generated H$_2$O$_2$ can be further reduced to highly reactive oxidant hydroxyl radicals (•OH) catalysed by free iron in Fenton reaction (Equation 1.4). The free iron is released by oxidative stress which damages the iron binding proteins ferritin and transferrin by lysosomal enzymes allowing the free iron to be a catalyst in the production of •OH which can react with other molecules such as lipids, proteins, and DNA causing damage (Bissett et al., 1991)(Fig. 1.6).

$$Fe^{2+} + H_2O_2 \rightarrow OH + OH^- + Fe^{3+} \quad \text{Equation 1.4}$$

The generated ROS by type I and type II reactions is mainly responsible for indirect damage by UVR exposure, whereas the direct damage from UVR is a result of direct absorption of UVR photons by the DNA chromophore (maximum absorbing wavelength is 260 nm, the absorption tail is in UVB and UVA regions) (Sutherland and Griffin, 1981; Young, 1997) may lead to DNA photolesions such as cyclobutane pyrimidine dimer (CPD). The CPD is formed when the 5, 6 double bond of two adjacent pyrimidine DNA bases (thymines and/or cytosines) is split forming 4-carbon cyclobutane ring linking the two pyrimidines (Kim et al., 2013). This CPD photolesion, if not repaired, may be incorrectly recognised by DNA polymerase during cell replication resulting in C to T transition mutations in the key gene p53 tumour suppressor gene and ultimately tumour development (Brash, 2015). Moreover, 6-4 pyrimidone photoproducts (6-4 PP) and Dewar isomers are other DNA photolesions induced by UVR 5 times less frequent that CPD (Eveno et al., 1995; Ichihashi et al., 2003). The formation of 6-4 PP requires the 5:6 double bond to break, rotate, and forms a new bond at C4 and C6 of the two adjacent bases. However, these photoproducts cause severe conformational distortion to DNA
compared to CPD lesions leading to interruption of replication machinery, i.e., triggering apoptosis, if unrepaired (Lo et al., 2005).

Fig. 1.6: A simplified Jablonski diagram illustrating the absorption of photon energy by a chromophore (photosensitizer). It can be excited to singlet state emitting the energy by fluorescence or triplet excited state by a system known as intersystem crossing dissipating the energy by phosphorescence or reacting with other molecules. Photosensitized production of Type I hydroxyl radicals (\(\cdot\)OH) and Type II singlet oxygen (\(1^\text{O}_2\)) from the excited triplet state (\(3^\text{PS}^*\)) of a photosensitizer (PS) (Li and Grant, 2016).

1.5 Photoageing

Human skin ageing is a process that results from both the natural consequence of physiological and structural changes over time (known as intrinsic ageing) and exposure to repeated sub-erythemal doses of sunlight or artificial UVR (known as photo-ageing) leading to progressive loss of structural integrity and physiological function (Bergfeld, 1997; Farage et al., 2008; Naylor et al., 2011). Clinically, photoaged skin appears as very dry and leathery with coarse wrinkles accompanied with yellowish mottled (irregular) pigmentations and telangiectasia, whereas the
intrinsic chronological aged skin is smooth, pale, dry-looking with fine wrinkles (Farage et al., 2008).

The histological changes from prolonged exposure to UVR are seen in the epidermis and dermis. As a result of UVR exposure the epidermis may become atrophic with atypical keratinocytes (dyskeratosis) and irregular melanocytes accompanying a thickened basement membrane whilst intrinsically aged skin is thin (Farage et al., 2008). The dermis of prolonged UVR exposed skin is characterized by hyperactivation of fibroblasts which secrete the elastic fibres, and increase numbers of mast cells (Grimbaldeston et al., 2006, 2003; Rabe et al., 2006). Photoaged skin shows deposition of abnormal amorphous elastic material (known as solar elastosis) in the upper dermis. Other modifications in the upper dermis include a decrease in collagen fibres (Naylor et al., 2011). In contrast, in intrinsically aged skin the dermis becomes thinner with a decrease in fibroblasts, mast cells, elastin and collagen fibres (Rabe et al., 2006).

The rate of UVR induced skin photoaging depends on many factors such as the frequency, duration, and intensity of UVR exposure, and on natural protection by skin pigmentation (Naidoo and Birch-Machin, 2017; Wlaschek et al., 2001). Endogenous antioxidants are produced by the skin to inactivate the UVR-driven ROS and neutralize their harmful effects. However, high levels of ROS generated by UVR exposure may cause imbalance in the endogenous antioxidant enzymatic and non-enzymatic defence systems (known as oxidative stress) (Pinnell, 2003; Sauer et al., 2001). This imbalance, caused by ROS, directly results in lipid peroxidation, protein oxidation and DNA lesions at cellular level as well as promotes the release of pro-inflammatory cytokines and growth factors such as
nuclear factor-kappa B (NF-kB), activation protein-1 (AP-1), and down regulates of transforming growth factor beta (TGF-β). NF-kB increases the interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α). AP-1 causes upregulation of matrix metalloproteinases (MMPs), which degrade the collagen and elastic fibres, whilst TGF-β leads to decrease in synthesis of structural proteins such as collagen whereas increase in elastin expression. Therefore, the UVR-driven ROS production indirectly leading to extracellular matrix (ECM) remodelling (Rittié and Fisher, 2002; Yaar and Gilchrest, 2007) and clinical signs of photoageing (Fig 1.7). The ECM remodelling in severely photoaged skin, is mainly characterized by a loss of collagen VII anchoring fibrils from the DEJ, accumulation of disorganised elastic fibre proteins throughout the dermis, known as ‘‘solar elastosis’’ in later stages, and accumulation of glycosaminoglycans in apparently disorganised aggregates (Naylor et al., 2011).
Fig. 1.7: Role of reactive oxygen species in photoaging (Chen et al., 2012; Yin et al., 2015).

1.6 Lipid peroxidation

Hydrogen peroxide and $O_2^\cdot$ are considered to be among the most important ROS generated by exposure to UVA. These species disrupt biological membranes via iron-mediated oxidative damage in the exposed tissue (Aroun et al., 2012; Vile and Tyrrell, 1995). For example, physiological doses of UVA irradiation have been shown to induce lipid peroxidation (LPO) in membranes of human primary fibroblasts and keratinocytes via pathways involving iron and $O_2^\cdot$ (Morlière et al., 1991; Pelle et al., 2011; Punnonen et al., 1991). This is due to the iron ‘at’ or ‘near’ biological membranes, that can undergo redox cycling by reacting with one electron reductants and oxidants, thus generating harmful oxidants such as $OH^\cdot$ and lipid

1.7 Photocarcinogenesis

UVR is the major risk factor for the development of non-melanoma and melanoma skin cancer. Non-melanoma skin cancers, known as keratinocyte cancer, particularly squamous cell carcinoma (SCC), are caused by cumulative UVR exposure, whereas malignant melanoma (MM) appears to be related to intermittent sun exposure (particularly of childhood) (Andreassi, 2011). Biomolecular studies have demonstrated that UVR-induced photoproducts, are able to provoke UVR-induced mutations in keratinocytes such as mutation in p-53 tumour suppressor gene, which is found in high frequency (~58%) in SCC with 62% being a UVR C to T signature mutations (Brash et al., 1991). This p-53 gene is also known as guardian of the genome which promotes the DNA repair process and eliminates the cells with excessive DNA damage but when mutated, leads to impaired repair mechanism to UVR damage, i.e., inducing resistance to apoptosis and cell death, and thus causing actinic keratosis and SCC (Pacifico et al., 2011). The relation of ROS with skin cancer is not very obvious. However, Halliday suggested that UVR-induced ROS, which lead to oxidative damage to DNA, appear to cause an increase in mutational burden as actinic keratosis progress into SCC in humans (Halliday, 2005). Moreover, the addition of ROS inhibitors and topical antioxidants help to reduce the photo-carcinogenesis in mice skin, suggesting a method to attenuate
carcinogenesis by reducing ROS (Burke et al., 2000; Chen et al., 2012; Gensler and Magdaleno, 1991; Halliday, 2005; Halliday et al., 1999).

1.8 Endogenous UVR photoprotection in human skin
The skin can protect itself from the damaging effect of solar UVR by different mechanisms including endogenous antioxidant enzymes such as catalase, glutathione (GSH) peroxidase, and glutathione reductase, which destroy hydrogen peroxide and lipid hydroperoxides. Moreover, superoxide dismutase (SOD) catalyses the conversion of superoxide into hydrogen peroxide and oxygen (Chen et al., 2012; Pinnell, 2003). The nonenzymatic endogenous antioxidants, which present at concentration higher in the epidermis than dermis, include ascorbic acid, glutathione, tocopherol, and ubiquinol-10. These molecules occupy the aqueous-phase, cytoplasm, lipid-phase, and mitochondria, respectively (Shindo et al., 1994). In addition, another endogenous protective mechanism is due to melanin pigmentation which absorbs solar UVR and act as free radical scavenger, and DNA repair enzymes which remove the DNA photolesions induced by solar UVR (Gilaberte and Gonzalez, 2010; Svobodová and Vostálová, 2010). Chromophores in the SC such as urocanic acid, amino acids such as histidine, tyrosine, tryptophan, and porphyrins could also act as UVR-absorbers (in effect natural sunscreens) (Young, 1997). Moreover, the epidermis can undergo hyperplasia and hyperkeratosis to increase the physical barrier to UVR (D’Orazio et al., 2013). However, all these innate defence systems are susceptible to depletion and they can be overwhelmed by the oxidative stress induced by exposure to UVR. The UVA radiation also disturbs the iron and haeme homeostasis by damaging the haeme-containing proteins (e.g. catalase) releasing the free haeme which immediately
activates haeme-oxygenases (HO) in human skin fibroblasts to maintain a low level of free haeme and prevent cellular damage. However, the catabolism of free haeme result in release of iron, which exacerbates the level of labile iron pool (LIP) released immediately following UVA exposure as a consequence of lysosomal destruction (Pourzand et al., 1999). The increase in LIP acts as pro-oxidant catalysts in Fenton reactions, which convert hydrogen peroxide into more potent hydroxyl radical, amplifying the oxidative stress by direct production of ROS and inducing propagation of the lipid peroxidation chain reaction. The high free iron levels cause a delay in the activation of the iron regulator protein, which induces the iron storage protein ferritin, following UVA exposure, to maintain the iron homeostasis (Vile and Tyrrell, 1993).

In sun care products, topical photoprotective agents are used to prevent skin damage induced by UVR exposure but they differ in mechanisms of action. These will be discussed in the following sections.

1.9 Exogenous (topical) photoprotective agents

Exogenous photoprotection includes the use of preventative strategies and therapeutic interventions to reduce the acute and chronic effects of solar exposure. One behavioural photoprotective approach is to avoid solar exposure during the peak intensity hours, i.e., when UVB is maximal between 10.00 am and 15.00 pm. Photoprotection can also be achieved by wearing protective clothing, hats, sunglasses, and applying a sunscreen prior to UVR exposure (Gonzalez et al., 2008). Although topical sunscreens ingredients have been used since 1928 to prevent solar UVR from reaching the skin, there are also reports of adverse effects of these products (e.g. contact sensitivity, risk of vitamin D deficiency, and
estrogenicity) (Sambandan and Ratner, 2011). Therefore, currently new innovations have been sought to develop topical antioxidants to prevent the undesired effects induced by solar UVR (Hassan et al., 2013).

1.9.1 Topical sunscreens

Topical sunscreens can be applied to the skin as lotions, creams, gels, ointments or sprays products that contain active ingredients that can absorb or reflect UV photons. To enhance consumer compliance and maximize photoprotection, a broad spectrum formulation that attenuates both UVB and UVA, is hypoallergic, non-irritant, cosmetically acceptable, and non-comedogenic is always preferred (Hassan et al., 2013).

The regulation of topical sunscreen varies worldwide. Sunscreens are recognized as over-the-counter drugs (Food and Drug Administration, 2011) and therapeutic goods (Australian Government Therapeutic Goods Administration, 2016) by the United States Food and Drug Administration (FDA) and Australia, respectively. Other regulatory domains such as European Union (EU), China and Japan consider them as cosmetic products (Stiefel and Schwack, 2015; Young et al., 2017). The FDA has rejected many new filters for sunscreen applications due a lack of percutaneous safety and toxicity data (Reisch, 2015). Sunscreen efficacy is mainly based on the sun protection factor (SPF) which indicates the capacity to inhibit erythema. This is calculated from the ratio of the minimum erythema dose (MED) with sunscreen application and the MED without sunscreen application and is measured using SSR and standardised a sunscreen application density of 2 mg/cm² for in vivo testing in human volunteers (Technical Committee ISO/TC 217, 2010). The MED is the lowest dose of SSR that will produce erythema 24 h after exposure.
Sunscreens (table 1.1) are widely classified as inorganic (physical blocker) and organic (chemical absorbers) filters (Food and Drug Administration, 1999; Hexsel et al., 2008; Kullavanijaya and Lim, 2005). Inorganic filters contain opaque particles which reflect, scatter, or absorb solar radiation in the visible and UVR spectral range. Previously, the two commonly used inorganic particles were non-micronized (200-500 nm) zinc oxide (ZnO) and titanium dioxide (TiO$_2$) (Kullavanijaya and Lim, 2005; Sambandan and Ratner, 2011). Because of their opaque nature and ‘whitening effect’, these have become aesthetically unacceptable. This problem has been solved by using micronized or ultrafine particles (10-50 nm), which improves cosmetic acceptability by decreasing visible light scattering. Microfine ZnO is more popular as it offers broader coverage for UVA, particularly UVA1, is photostable and does not react with organic sunscreens (Mitchnick et al., 1999; Pinnell et al., 2000). In contrast, micronized TiO$_2$ provides superior protection against UVB and UVA2 but lacks UVA1 coverage. It has a higher refractive index than ZnO (2.7 vs 2.0, respectively), white (Murphy, 1999), and is more photoreactive and so can lead to the generation of free radicals leading to cytotoxic effects in vitro (Gasparro et al., 1998). In addition to photoreactivity issues, these TiO$_2$ particles tend to aggregate, thereby reducing efficacy. Therefore, they are encapsulated in dimethicone or silica (Mitchnick et al., 1999) or in carnauba wax nanoparticles (Villalobos-Hernandez and Muller-Goymann, 2006) to increase its photostability and achieve stable dispersion.
Table 1.1: List of UV filters used in sunscreen and their peak absorption.

<table>
<thead>
<tr>
<th>Topical sun-screening agents</th>
<th>Peak absorption (nm)</th>
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</thead>
<tbody>
<tr>
<td><strong>Inorganic filters</strong></td>
<td></td>
</tr>
<tr>
<td>1. Zinc oxide</td>
<td>(290-400)*</td>
</tr>
<tr>
<td>2. Titanium dioxide</td>
<td>(290-350)*</td>
</tr>
<tr>
<td><strong>UVB filters</strong></td>
<td></td>
</tr>
<tr>
<td>1. PABA derivatives:</td>
<td></td>
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<tr>
<td>Octyldimethyl PABA (Padimate O)</td>
<td>290-311</td>
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<tr>
<td>2. Cinnamates:</td>
<td></td>
</tr>
<tr>
<td>Octinoxate (ethylhexyl methoxycinnamate, Uvinul® MC80)</td>
<td>311</td>
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<tr>
<td>Cinoxate</td>
<td>289</td>
</tr>
<tr>
<td>3. Salisalates:</td>
<td></td>
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<tr>
<td>Octisalate</td>
<td>307</td>
</tr>
<tr>
<td>Homosalate</td>
<td>306</td>
</tr>
<tr>
<td>Toloramine Salicylate</td>
<td>260-355</td>
</tr>
<tr>
<td>4. Others</td>
<td></td>
</tr>
<tr>
<td>Octocrylene</td>
<td>303-307</td>
</tr>
<tr>
<td>Ensulizole (Phenylenbenzimidazole sulfonic acid)</td>
<td>310</td>
</tr>
<tr>
<td>Ethylhexyl triazone (Uvinil® T 150)</td>
<td>~310</td>
</tr>
<tr>
<td>Diethylhexyl butamido triazone (Uvasorb® HEB)</td>
<td>~310</td>
</tr>
<tr>
<td>Benxyledene malonate polysiloxane (Parso1® SLX)</td>
<td>~310</td>
</tr>
<tr>
<td><strong>UVA filters</strong></td>
<td></td>
</tr>
<tr>
<td>1. Benzophenones:</td>
<td></td>
</tr>
<tr>
<td>Oxybenzone (benzophenone-3)</td>
<td>288,325</td>
</tr>
<tr>
<td>Sulisobenzone (benzophenone-4)</td>
<td>288,366</td>
</tr>
<tr>
<td>Dioxybenzone (benzophenone-8)</td>
<td>288,352</td>
</tr>
<tr>
<td>2. Anthralines:</td>
<td></td>
</tr>
<tr>
<td>Meradimate (menthyl anthranilate)</td>
<td>286,335</td>
</tr>
<tr>
<td>3. Avobenzone (butylmethoxydibenzoylmethane, Parso1® 1789)</td>
<td>360</td>
</tr>
<tr>
<td>4. Ecamsule (terephthalynecenicamphor suphonic acid, Mexoryl SX™)</td>
<td>345</td>
</tr>
<tr>
<td>5. Bisdisulizole disodium (Neo Heliopan® AP)</td>
<td>334</td>
</tr>
<tr>
<td>6. Diethylamino hydroxybenzooyl hexyl benzoate (Uvinul® A Plus)</td>
<td>354</td>
</tr>
<tr>
<td><strong>Broad spectrum filters</strong></td>
<td></td>
</tr>
<tr>
<td>1. Bisoctrizole (Tinosorb® M)</td>
<td>305,360</td>
</tr>
<tr>
<td>2. Bemotrizinol (Tinosorb® S)</td>
<td>310,343</td>
</tr>
<tr>
<td>3. Silatriazole (Diethylhexyl butamido triazone, Mexoryl XI™)</td>
<td>303,341</td>
</tr>
</tbody>
</table>

Modified from (Gonzalez et al., 2008; Latha et al., 2013; Young et al., 2017).

*Peak absorption varied depending on particle size.
Organic filters are active ingredients that primarily act by absorbing specific wavebands of UVR (mostly UVB, but also UVA), thus preventing them from reaching the viable skin layers. The FDA has approved several organic sunscreens. Aminobenzoate including para-aminobenzoic acid (PABA), which was patented in the 1940’s, is an effective organic UVB filter, and was the first commonly used active ingredient in commercially available sunscreens, but its skin staining properties and many reported contact allergies have limited its use (Mackie and Mackie, 1999). It has been replaced by PABA derivatives, such as padimate O that rarely stains the skin and offers better safety, but has less efficacy compared to PABA (Kullavanijaya and Lim, 2005). It is common to combine multiple active ingredients in sunscreen products to achieve the desired photoprotection level. Cinnamates, including octinoxate and cinoxate, are the most commonly used organic UVB filters in the US replacing PABA derivatives because they do not cause any skin staining and rarely cause skin contact allergy or irritation (Kullavanijaya and Lim, 2005; Palm and O’Donoghue, 2007). However, they are less effective than padimate O, photodegrade upon exposure to sunlight (reducing their efficacy), and have poor water resistance hence they require frequent reapplication (Sambandan and Ratner, 2011). Salicylates, including water-insoluble octisalate, and homosalate, and water-soluble trolamine salicylate (found in hair products), are the weakest organic UVB sunscreens. However, they have an excellent safety profile and are photostable and thus can be added to other sunscreen active ingredients in high concentration to augment UVB protection (Kullavanijaya and Lim, 2005; Palm and O’Donoghue, 2007). Octocrylene, which is a weak organic UVB filter with peak absorption at 307 nm, has a favourable safety profile with minimum irritation, and phototoxicity (Nash, 2006) and thus can be combined
with other UVR absorbers to improve the photostability and achieve a higher SPF (Rai et al., 2012). Ensulizole or phenylbenzimidazole sulfonic acid, which is a selective UVB absorber, is water-soluble which offers less oily and lighter consistency in daily moisturizing cosmetic formulations (Sambandan and Ratner, 2011). Other UVB filters are approved for use in the EU and other countries, but not by FDA. These include ethylhexyl triazone (Uvinil® T 150), diethylhexyl butamido triazone (Uvasor® HEB), and benzyledenne malonate polysiloxane (Parsol® SLX) which have maximum absorption at ~310 nm, and are usually combined with others filters to give photostable broad spectrum sunscreens (Kullavanijaya and Lim, 2005; Tuchinda et al., 2006).

Benzophenones are often considered as UVA filters (displayed broad band UVR absorption, i.e., spanning the UVA2 and UVB regions). However, they have the highest incidence of (photo) contact dermatitis among all sunscreens as they are photolabile and become unstable and oxidized rapidly via UVR-induced oxidative reactions and also deplete the endogenous antioxidant systems. The FDA approved 3 benzophenones; oxybenzone (benzophone-3), sulisobenzone (benzophone-4), and dioxybenzone (benzophone-8) (Hexsel et al., 2008). Oxybenzone is the most commonly used, but it has the highest incidence of photoallergic contact dermatitis (Palm and O’Donoghue, 2007; Schallreuter et al., 1996). Meradimate (menthyl anthranilate) absorbs in the UVA (370-400 nm) range and is less widely used (Beeby and Jones, 2000). Avobenzone or butyl methoxydibenzoylmethane (Parsol® 1789), was the first FDA approved organic sunscreen that provides superior photoprotection against UVA, particularly UVA1. Despite its efficacy, it is highly photolabile, 1 h of sunlight exposure results in up to a 90% reduction in its
photoprotective properties (Deflandre and Lang, 1988). To prevent photodegradation, it is usually combined with the other UVR filters (e.g. octocrylene or Tinosorb® S) (Bouillon, 2000; Gaspar and Maia Campos, 2006) or a non-UVR filter such as diethylhexyl 2,6-naphthaline (known as Helioplex™) patented by Neutrogena (Bissonnette, 2008).

The newest UVA organic filter, which blocks UVA transmission more than UVB, is ecamsule (terephthalydenedicamphor suphonic acid, Mexoryl SX™). It was approved by FDA in 2006, it provides protection in the near UVA range. Ecamsule is photostable, water resistant, and has been shown to prevent UVR-induced pigmentation, DNA photolesions, p53 protein accumulation, and photodermatoses (Fourtanier et al., 2008). Bisdisulizole disodium (disodium phenyl dibenzimidazole tetrasulfonate, Neo Heliopan® AP) and diethylamino hydroxybenzoyl hexyl benzoate (Uvinul® A Plus) are UVA filters with maximum absorption at 334 nm, and 354 nm, respectively. They are not available in the US (Hexsel et al., 2008; Tuchinda et al., 2006).

Other broad-spectrum and intrinsically photostable UVB and UVA sunscreen actives, which are not yet available in the US include bisoctrizole (methylene-bis-benzotriazolyl tetramethyl butylphenol, or Tinosorb® M), beotrizinol (bis-ethylhexyloxyphenol methoxyphenyl triazine, or Tinosorb® S), and silatriazole (drometiazole trisiloxane, Diethylhexyl butamido triazine, or Mexoryl XL™) (Kullavanijaya and Lim, 2005; Tuchinda et al., 2006). Bisoctrizole, beotrizinol, and silatriazole have two absorption peaks at 305 and 360 nm, 310 and 343 nm, and 303 and 341 nm, respectively. They are photostable and they lack endocrine-like
adverse effects due to their large molecular weight, which reduces their systemic absorption and toxicity (Hexsel et al., 2008; Tuchinda et al., 2006).

Figure 1.8 shows two absorbance spectra for examples of two broad spectrum sunscreen formulations with SPF 15 and 50+ (Lubrizol, Ohio, USA). Characteristics of each formulation are shown in table 1.2. The formulation with very high protection (SPF 50+) contains a higher concentration of all the UVR filters and contains an additional UVA filter (Tinosorb® S) over the formulation with medium protection (SPF 15). The two products also display differences in their UVA-protection factor (UVA-PF) and UVA: UVB ratio.

Fig.1.8: The simulated absorbance spectra of Lubrizol broad spectrum sunscreen formulations with SPF 15 (blue line) and SPF 50+ (red line).
Table 1.2. Properties and protection factors of Lubrizol sunscreen formulations. The detailed filters contained in each formulation are described, along with their respective SPF, UVA-PF, and UVA/UVB ratios.

<table>
<thead>
<tr>
<th>formulation</th>
<th>Light sun cream SPF 15</th>
<th>Light sun cream SPF 50+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2% Uvinul A Plus (UVA filter)</td>
<td>3% Tinosorb S (UVA filter)</td>
</tr>
<tr>
<td></td>
<td>3% Uvinul MC80 (UVB filter)</td>
<td>10% Uvinul A Plus (UVA filter)</td>
</tr>
<tr>
<td></td>
<td>2% Uvinul T150 (UVB filter)</td>
<td>7.5% Uvinul MC80 (UVB filter)</td>
</tr>
<tr>
<td></td>
<td>1.5% Titanium Dioxide (nano) (UVB filter)</td>
<td>5% Uvinul T150 (UVB filter)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5% Titanium Dioxide (nano) (UVB filter)</td>
</tr>
<tr>
<td>Applied dose</td>
<td>2 mg/cm(^2)</td>
<td>2 mg/cm(^2)</td>
</tr>
<tr>
<td>SPF calculated</td>
<td>19.5</td>
<td>75.5</td>
</tr>
<tr>
<td>SPF rating</td>
<td>15</td>
<td>50+</td>
</tr>
<tr>
<td>SPF category</td>
<td>Medium protection</td>
<td>Very high protection</td>
</tr>
<tr>
<td>UVA-PF</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>Ratio (UVA:UVB)</td>
<td>0.58</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Sunscreens have the potential for adverse effects including allergic contact dermatitis (Goossens, 2004), and long-term use may increase the risk of vitamin D deficiency (Matsuoka et al., 1988; Norval and Wulf, 2009), and estrogenicity which limits their use as photoprotective agents (Schlumpf et al., 2004, 2001). Schlumpf et al found that topically applied sunscreen such as octinoxate and benzophenone-3 (BP-3) induced cell proliferation of MCF-7 breast cancer cell line. Oral administration for 4 days led to dose-dependent increase in uterine weight in Long-Evans rat. These experiments suggested estrogenic activity both in cells and in vivo (Schlumpf et al., 2001). Although after 1 week of topical application (2 mg/cm\(^2\) of 10% w/w) both BP-3 and octinoxate result in systemic absorption of 10-200 ng/ml in females and 20-300 ng/ml in males, no significant change in level in reproductive
hormones were observed (Janjua et al., 2004). It has been suggested that the estrogenic effect of UVR filters may be amplified (by increase in the oestrogen-regulating p-52 gene transcription) in combined sunscreen formulations even when the concentrations of single component used did not yield estrogenic activity when tested separately (Heneweer et al., 2005; Kunz and Fent, 2006). Moreover, high SPF sunscreens use is significantly associated with increased duration of intentionally sun exposure by up to 39%, especially in those misusing sunscreens to acquire either suntan during their holidays in sunny countries as a sign of social wellbeing or using artificial tanning beds that emit UVA/UVB, and thus, increase their risk of cancer (Andreassi, 2011; Autier et al., 2007).

1.9.2 Topical Iron chelators

As the UVR-induced oxidative stress induces the release of free iron ions from iron-binding proteins (ferritin and transferrin), which catalyse the Fenton reaction, iron chelators can act as antioxidants. Iron chelators act as a ligand, which interacts with the free iron through six binding sites forming an iron complex, to suppress the catalytic activity of iron ions and moderate the LIP. This suppresses ROS generation and UVR-induced skin damage.

Bissett and co-workers (1991) were the first group to investigate the *in vivo* efficacy of the topically applied iron chelators, 2,2-dipyridyl-1,10-phenanthroline and 2,2-dipyridylamine (DPA), against photoageing in a hairless mouse model chronically exposed to sub-erythemal UVB. The treatment increased non-haem iron leading to histological and clinical changes of photoageing. Bissett, *et al.*, found that wrinkle formation and epidermal hypertrophy were delayed by the photoprotective iron chelators (Bissett *et al.*, 1991). Another short-term *in vivo* study in humans was
performed by Bissett and colleagues (1994), who showed that by applying 0.4 ml of 5% (dose- 2 mg/cm²) the iron chelator 2-furildioxime (FDO) on dorsal skin for 15 min prior to 3 MED SSR (290-400 nm) suppressed erythema and sunburn cell (SBC) formation, inhibited the induction of inflammatory cells, epidermal hyperplasia and ornithine decarboxylase (ODC) compared to the SSR-treated vehicle (Bissett et al., 1994). Bissett and MacBride (1996) also investigated the in vivo synergistic photoprotective effect by the combination of FDO with padimate O sunscreen during short and long-term studies. For short-term studies, the SPF was determined in guinea pigs by applying 0.4 ml of 5% of FDO, 5% padimate O, or combination of both on the dorsal skin for 15-20 min followed by exposure to varying doses of SSR. For long term studies, wrinkles and tumours were assessed in hairless mice and 0.1 ml of the test formulations (5% of FDO, 5% padimate O, or 5% FDO and 5% padimate O) were applied on the dorsal skin for 2 h before irradiation. Mice were irradiated three times weekly with 30 mJ/cm² UVB radiation per exposure (0.5 mice MED). Topically applied FDO iron chelator combined with SPF 4 sunscreen provided synergistic protection (SPF ~32) compared to FDO alone. In hairless mice exposed to long term UVR, the combination of FDO and sunscreen synergistically delayed tumour onset and reduced the skin wrinkling (Bissett and McBride, 1996).

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone), which is widely used in Japan as a skin whitening agent, has been proven to have iron chelator activity. A chronic study by Mitani and co-workers investigated topical application of 0.1 ml of 5% Kojic acid on the hairless mice dorsal skin when applied 90 min before each SSR exposure (a dose of 10.8 J/cm² was given five times weekly for 20 weeks yielding
a total dose of 1080 J/cm$^2$). At the end of the irradiation period, the kojic acid showed anti-wrinkle properties, suppressed epidermal hyperplasia, suppressed fibrosis and reduced the dermal deposition of glycosaminoglycan (Mitani et al., 2001).

Several studies have linked the prolonged exposure to potent iron chelators with systemic depletion of iron, including iron-containing enzymes and the induction of hypoxia-inducing factor-1 (HIF-1). To counter a new strategy based on light-activated caged-iron chelators pro-drugs have been developed, which after physiologically relevant UVA exposure i.e. stimulus, release the active moiety (free iron chelator) in a dose-dependent manner. Yiakouvaki, et al (2006) have shown that a light-activated prodrug, derived from the potent iron chelators salicylaldehyde isonicotinoyl hydrazone (SIH) and pyridoxal isonicotinoyl hydrazone (PIH), presented with the 1-(2-nitrophenyl)ethyl (2-NPE) caging group, has no cytotoxic effects and significantly suppresses the intercellular LIP and consequently the necrotic cell apoptosis in human fibroblasts after exposure to physiologically relevant UVA (Yiakouvaki et al., 2006).

1.9.3 Topical antioxidants

Modern topical sunscreens combine the use of primary photoprotective agents (UVR filters) that absorb or reflect UVR and secondary photoprotective agents (antioxidants) that can reduce the photo-oxidative damage that results from UVR-induced ROS generation, providing photoprotection with no possibility of interfering with vitamin D synthesis in the skin. Topical antioxidants include plant (botanical) antioxidants and the classic antioxidants (vitamin C, and E).
Polyphenols are phytochemicals present in plants, subdivided into hydrolysable tannins and phenylpropanoids. Flavonoids, which belong to phenylpropanoids, are natural pigments that act as photoprotective agents by absorbing UVR, possessing antioxidant properties and modulate signalling pathways (Gilaberte and Gonzalez, 2010). Silymarin, which is an extract from the seeds of the milk thistle, is a mixture of three flavonoids, silybin (silibinin), silidianin, and silicristine. The main biologically active component (70%-80%) is silybin. Topical silymarin dosing studies of 9 mg per application on hairless mice before acute exposure to 900 mJ/cm² dose of UVB have shown to statistically significant inhibition of sunburn (by 77%, $p < 0.001$) and apoptotic SBC (by 100%), skin oedema (by 44%, $p < 0.001$), depletion of catalase activity (by 28%), and ODC activities (by 64%, $p < 0.001$) which are well-established biomarkers for tumour promotion. Chronic photocarcinogenesis studies have shown to have a remarkable antitumor effect of silymarin with 75% reduction in tumour incidence and a 92% reduction in tumour multiplicity and highly significant 97% reduction in tumour volume (each has $p < 0.0001$) (Katiyar et al., 1997).

Genistein is soya bean isoflavone, which is a potent antioxidant, which presents naturally as a glycoside that masks its estrogenic activity and thus can be applied topically on skin. Wie, and colleagues investigated photoprotection by topically applied genistein (5 μmol) on hairless mice and found that it inhibits UVB-induced acute and chronic cutaneous photodamage. In the acute sunburn study, mice were irradiated with daily UVB (1.8 kJ/m²) for 10 days. Topical applied genistein for 60 min before each exposure completely prevented UVB-induced acute skin burns. In the chronic UVB exposure study, mice were irradiated with UVB twice weekly (0.3
kJ/m²) for 4 wk. The prior and post-UVB treatment with genistein alleviated photodamage that was characterised by epidermal hyperplasia with nuclear atypia, skin thickness and elastosis. However, a stronger effect was noticed by the pre-UVB treatment. In the same study, genistein was shown to inhibit UVB-induced skin carcinogenesis in mice. The possible mechanisms of the anticarcinogenic action include scavenging of ROS, blocking of oxidative and photodamage to DNA, inhibition of tyrosine protein kinase, downregulation of EGF-receptor phosphorylation and MAPK activation, and suppression of oncoprotein expression in UVB-irradiated cells and mouse skin (Wei et al., 2003). In human skin (Skin type II, IV), the application of 5 μmol genistein/cm² to dorsal skin either 60 min before or 5 min after UVB (up to 100 mJ/cm²) exposure, substantially blocked erythema whereas post-UVB application showed very little protection.

Resveratrol is a polyphenolic phytoalexin extracted from grapes, peanuts, and walnuts. A study conducted by Afaq et al., (2003) used a single topical application of resveratrol (25 μmol/0.2 ml acetone per mouse) to hairless albino mice and showed a significant inhibition of UVB (180 mJ/cm²) induced increase (by 2-fold) in skin thickness and oedema, induction of cyclooxygenase, lipid peroxidation (a marker of oxidative stress), and ODC activities (Afaq et al., 2003).

Ferulic and caffeic acids are two hydroxycinnamic acids found in olives and olive oil. In vivo experiments were performed on 6 healthy volunteers of skin types II and III. Skin sites were exposed to 2 MED dose of UVB irradiation and then 0.2 ml of caffeic or ferulic acid saturated solutions was immediately applied to the irradiated sites for 3 h. The percentage inhibition of erythema of caffeic and ferulic acid was
statistically higher than the vehicle control 26.31% \( p < 0.05 \) and 47.85% \( p < 0.001 \), respectively (Saija et al., 2000).

Green tea is widely known for its antioxidant and cancer prevention properties. White tea is the least processed tea and may retain high level of polyphenols. Camouse and co-workers performed an in vivo study on 90 healthy volunteers (skin types I-III) and showed that topical application of white and green tea at a concentration of 2.5 mg/cm\(^2\) for 15 min prior to 2 MED SSR offered significant reduction in the depletion of Langerhans cells (by 35% and 22% \( p < 0.01 \), respectively) and a reduction in oxidative DNA damage which was measured by levels of 8-hydroxy-2’-deoxyguanosine (OhdG) in skin biopsies \( p < 0.01 \) and \( p < 0.001 \), respectively) compared to the vehicle-treated skin (Camouse et al., 2009).

Topical 10% (w/v) L-ascorbic acid (L-AA) pre-treatment prior irradiation with 2-3 MED UVB has been shown significantly elevate the level of this vitamin in porcine skin compared to the vehicle control (543 vs 20 µg/g wet weight, \( p < 0.005 \)) and to protect from UVB-induced skin damage, by reducing erythema (measured by skin blood flow) and number of SBC formation to one half and to one sixth, respectively compared to the vehicle control (1.4 vs 2.91 cutaneous blood flow \( p < 0.001 \), and 20 vs 33 SBC/mm biopsy \( p<0.005 \), respectively) (Darr et al., 1992).

The photoprotective properties of L-AA derivative, such as sodium-L-ascorbyl-2 phosphate (L-A-2P) were investigated by Nayama, et al, (1999), using cultured mouse skin, against photodamage induced by a single dose of UVB (290-320 nm, \( \lambda_{\text{max}} \) 312 nm). The cutaneous level of L-AA in irradiated skin (20 kJ/m\(^2\)) was depleted by half (after 3 h) of that in the unirradiated control skin. Tissue treated with 2, 20 and 100 mM antioxidant lead to 1.03-, 2.17- and 6.27-fold increase in
the level of L-AA, respectively compared to the vehicle control. The level of L-AA in skin pre-treated with 20 mM L-A-2P was maintained within normal limits, even after 24 h. Pre-treatment with 20 mM L-A-2P significantly prevented such SBC, DNA fragmentation and lipid peroxidation mainly due to the maintenance of a normal level of L-AA by converting of L-A-2P back to L-AA in the skin (Nayama et al., 1999).

The in vitro photoprotection of natural α-T was investigated by Wu, et al (2008) using keratinocytes which were treated for 24 h with 10^6-10^{12} M of α-T prior to UVA irradiation (10-20 J/cm^2). The results showed a significant suppression of NADPH oxidase (NOX-1) activity, and inhibition of the formation of malondialdehyde-thiobarbituric acid (which indicates lipid peroxidation), and also prevented the upregulation of IL-8 and the activation of AP-1 compared to the vehicle control (Wu et al., 2008). A recent study by Wu and co-workers using keratinocytes treated with 2.9-14.7 IU/ml of α-T in serum free medium for 24 h prior to UVA irradiation (8 J/cm^2) resulted in a statistically significant decrease in cell viability reduction, lipid peroxidation, ROS formation, and increased glutathione (GSH) compared to the irradiated vehicle control (Wu et al., 2014). In vivo photoprotection studies have been done using topical α-T (0.1 ml/cm^2 skin) applied to the skin of albino hairless mice exposed to UVR (280-365 nm). Treatment 60 min before and 2 min post-irradiation, significantly inhibited the erythemal response (MED increased by ca. 50%) whereas α-tocopherol acetate (α-TA) had no effect (Roshchupkin et al., 1979). Another study by Lopez-Torres and colleagues, who treated hairless mice with 5 mg/cm^2 topical α-T 24 h before a single exposure of 10 MED. They found a significant increase in dermal SOD activity by
30% ($p < 0.01$), protection of epidermal glutathione peroxidase and SOD depletion and an increase in epidermal total reduced glutathione and dermal ascorbate levels by 50% ($p < 0.05$) and 40% ($p < 0.01$), respectively. Moreover, topical treatment increased α-T levels both in the epidermis and the dermis (62-fold and 22-fold, respectively ($p < 0.001$)). Furthermore, α-T treatment significantly reduced the formation of epidermal lipid hydrogen peroxides after UVR irradiation ($p < 0.05$) mainly due to the up-regulation of a network of enzymatic and non-enzymatic antioxidants (Lopez-Torres et al., 1998). Jurkiewicz, et al (1995), have evaluated the effect of the 0.1 ml topically applied tocopherol sorbate, α-T, and α-TA on UVR-induced free-radical generation on hairless albino mice. Only tocopherol sorbate provided a significant reduction in UVR-induced ascorbate free radical formation by 50% ($p < 0.05$). In studies on a chronically exposed mouse model, tocopherol sorbate was also found to be significantly more protective than α-T and α-TA against skin photoageing (Jurkiewicz et al., 1995). Burke, and colleagues (2000) have investigated the effects of 0.1 ml of 5% lotion of d-α-tocopherol succinate. Dorsal topical application was thrice weekly for 24 weeks to hairless pigmented mice 30 min before UVR exposure (265-440-nm, peak 310 nm). The cumulative dose was ~15 J/cm$^2$ that induced pigmentation and skin cancers. α-tocopherol succinate was less effective than α-T in protecting against UVR-induced blistering, tanning, and skin cancer (Burke et al., 2000).

Premi et al, 2015, have evaluated the effect of prior and post UVA irradiation treatment of murine fibroblast and melanocytes with α-T. They found that in melanin containing melanocytes, CPDs DNA photolesions are generated for more than 3h after UVA exposure known as dark CPD that initiate UVR C to T signature
mutations. These dark CPDs induced by ROS and nitrogen species generated by UVA exposure combined to form peroxynitrite which excites an electron in melanin to triplet state that has high energy of UVA photon and induce CPDs by energy transfer to DNA. These dark CPDs are abolished by α-T treatment which act as novel triplet quencher and act as evening-after sunscreen and have potential to prevent carcinogenesis process in skin occurs hours after the UV exposure ends (Premi et al., 2015).

Another study by Delinasios et al., (2018), recently reported that pre-irradiation treatment with synthetic α-T for 24 h showed significant protection against DNA damage (oxidized purines and “dark” CPD) induced by UVA (320-400 nm, doses of 5 and 10 J/cm²) in human keratinocytes. This effect on both was more evident at 5 J/cm² (66% and 60% decrease, respectively, p < 0.001). In the same study, post-UVA1 treatment with α-T for 2.5 h significantly reduced the formation of oxidized purines, compared to non-treated cells (70% and 32% decreases at 5 and 10 J/cm², with p < 0.001 and p < 0.01, respectively). “Dark” CPD were also decreased at both UVA1 doses tested (52% and 44% decrease at 5 and 10 J/cm², with p < 0.05 and p < 0.01, respectively). These study results highlighted that inclusion of α-T in topical sunscreen or after sun application appeared to mitigate the continuous DNA photoproduct formation even hours after the sun exposure ends (Delinasios et al., 2018).

1.10 Does exogenous antioxidant add benefit to sunscreen?

Several studies have provided evidence (see table 1.3) that photoprotection is enhanced by the addition of a single or a mixture of antioxidants (AOx) to a broad-spectrum sunscreen (SS) as these active ingredients have different mechanism of
actions mainly boosting the suppression of MMP induction, erythema, and tanning compared to AOx or SS alone.
Table 1.3: The list of studies that combined single or mixture of antioxidants to a broad-spectrum sunscreen and their added benefits.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Product tested</th>
<th>UV dose</th>
<th>Human subjects</th>
<th>Findings</th>
</tr>
</thead>
</table>
| (Matsui et al., 2009) | 1) SS: benzophenone, avobenzone, octyl methoxycinnamate, (SPF 25)  
                       | 2) AOx: vitamin E, vitamin C (aminopropyl ascorbic phosphate), caffeine, botanical tea extract (echinacea pallida extract, gorgonian extract, chamomile extract)  
                       | 3) SS+ AOx (SPF 25) | 2 MED SSR (1MED=52 mJ/cm²) | 5 volunteers (Age 10-40 y, skin type I-III)  
                       | Dose: 2mg/cm² for 15 min prior to irradiation | SS alone: ↓ depletion of CD1a⁺ Langerhans cells (LC) numbers, ↓ MMP-1 by 43%.  
                       | SS + AOx: ↓ depletion of CD1a⁺ Langerhans cells numbers, ↓ MMP-1 by 60%.      |                                                                                                                                                  |
| (Wu et al., 2011)   | 1) SS: benzophenone (2%), Parsol 1789 (3%), avobenzone (3%), methoxycinnamate (6%), (SPF 25)  
                       | 2) AOx: Tocopherol acetate (0.5%), ascorbic phosphate (0.1%), Echinacea pallida extract (0.01%), chamomile extract (0.12%), and caffeine (0.18%)  
                       | 3) SS+ AOx (SPF 25) | 1.5 MED SSR | 40 volunteers (skin type III or IV)  
                       | Dose: 2mg/cm² for 30 min prior to irradiation | SS alone: ↓ erythema by 45%, ↓ tanning by 60%, ↓ epidermal thickness by 20% (ns), ↓ depletion of LC.  
                       | AOx alone: ↓ tanning by 30%, and ↓ epidermal thickness by 40% ($p < 0.05$), ↓ depletion of LC.  
                       | SS + AOx: ↓↓ erythema by 55%, ↓↓ tanning by 70%, ↓ epidermal thickness, ↓ MMP-9 induction.  |                                                                                                                                                  |
| (Young et al., 2018) | SS+AOx: Tinosorb S®, Uvasorb®HEB, Parsol®1789, Tinosorb M®, tocopherol glucoside (pre-tocopherol®) (UVA/PF 25.3, SPF 50⁺). | 15-30 SED SSR | 16 volunteers (skin type I and II)  
                       | Dose: 0.75, 1.3, and 2mg/cm² | SS+AOx significantly reduced the CPD DNA photolesions measured by immune-staining and HPLC-MS/MS for 1.3 and 2 mg/cm² applied dose. |
1.11 Vitamin E

Vitamin E, a fat-soluble vitamin, acts to stabilize biological membranes and prevent their damage by limiting the chain reaction between lipid peroxide with polyunsaturated fatty acids, and also act to scavenge ROS suppressing the chain initiation and propagation by donating its phenolic proton to the oxygen radicals (Burton et al., 1982).

1.11.1 Chemical structure

Vitamin E is a generic name for eight geometric isomers, four tocopherols and four tocotrienols. The four isomers of tocopherols include alpha, beta, delta, and gamma tocopherol (α-, β-, δ-, and γ-T, respectively) which differ with respect to their aromatic methyl group. Similar structural variants are observed in the four corresponding tocotrienols (figure 1.9). Both the tocopherols and tocotrienols structure contain a chroman head and phytyl side chain. The tocotrienols differ in the three double bonds in their phytyl chains (isoprenoid) with no chiral carbons. The four analogues of tocopherol contain three asymmetric carbons i.e. chiral carbons, at carbon 2 on the chroman ring and 4′ and 8′ on the phytyl side chain occur as RRR-side-chain stereoisomers (Jean-Marc, 2007; Jiang, 2014; Wang and Quinn, 1999). Vitamin E is an endogenous antioxidant that is found in all skin layers; i.e., the dermis, the epidermis, and the SC. The human epidermis contains 87% α-T, 9% γ-T, 3% γ-tocotrienol and 1% α-tocotrienol (Fuchs et al., 2003). Synthetic α-T consists of mixtures of all eight stereoisomers (RRR, RSR, RRS, RSS, SRR, SSR, SRS, and SSS) and named as all-racemic-α-tocopherol (all-rac-α-T, or dl-α-T). The natural form of vitamin E, is RRR-α-tocopherol (RRR,α-T, or d-
α-T) was initially discovered by Evans and Bishop in 1992, and is mainly found in vegetable oils, such as corn, rapeseed, soya bean, and sunflower oil whole wheat flour, margarine, and dairy products (Baumann, 2009; Gliszczyńska-Świgło et al., 2007; Zingg et al., 2010) and is the most active stereoisomer (Brigelius-Flohé and Traber, 1999). Vitamin E biological activities are given as international units (IU) or α-tocopherol equivalent (α-TE). The relationship is that 1 mg of RRR-α-T has an activity of 1 α-TE and is equal to 1.49 IU, whereas RRRα-tocopherol acetate (RRR-α-TA) and all-rac-α-T have activity of 1.36 and 1.1 IU/mg, respectively (Cheeke and Dierenfeld Sue, 2010). The α-T isomer acts to control smooth muscle proliferation, decrease protein kinase C activity by direct interaction after integration into cellular membrane or indirectly by inhibition of generation of membrane-derived diacylglycerol, increase phosphoprotein phosphatase 2A activity, and modulate the gene expression of α-tropomyosin whereas other isomers of vitamin E, which have similar antioxidant activity, are not associated with these effects. α-T also down regulates the vascular and intercellular cell adhesion proteins induced by oxidised-LDL on endothelial cells, thus reducing the adhesion of white blood cells to endothelium. In contrast, γ-T protects by acting as a nucleophile which attacks electrophilic mutagenic ‘peroxynitrite’, i.e., scavenging the reactive nitrogen species such as nitrogen dioxide (NO₂) and thus protects lipids, DNA, and proteins against peroxynitrite-induced damage (Brigelius-Flohé and Traber, 1999). Vitamin E is a very potent antioxidant that protects against skin changes and damage induced by exposure to UVR such as sunburn, photocarcinogenesis, photoimmunosuppression. It delays wrinkle formation and also promotes wound healing (Baumann, 2009). Vitamin E also elicits anti-inflammatory properties by suppression of NF-kB activity, which leads to an inhibition of pro-inflammatory
cytokine generation and reduction in IL-1β, IL-6 and TNF-α levels (Tahan et al., 2011). The beneficial effects of topically applied vitamin E require its delivery into the viable skin layers. However, this may be limited by its poor chemical stability due to its photodegradation by UVR exposure and it is readily oxidized by atmospheric oxygen. Furthermore, vitamin E is too hydrophobic (table 1.4) to easily pass through the SC which hinders its delivery to the viable skin layers (Cichewicz et al., 2013). For these reasons, numerous attempts have been made to develop an efficient vitamin E topical product by improving its skin delivery, chemical stability, and water solubility.
Fig. 1.9: Eight naturally occurring isomers of vitamin E (Brigelius-Flohé and Traber, 1999).
Fig. 1.10: Stereoisomers of dl-α-tocopherol (all-rac-α-tocopherol) (Brigelius-Flohé and Traber, 1999).
Table 1.4: Chemical structure of vitamin E derivatives and their molecular weight and their calculated log P. The chemical structures were drawn and the corresponding MW and Log P were calculated using ChemAxon (MarvinSketch)

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Chemical structure</th>
<th>Molecular weight (MW)(g/mole)</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tocopherol</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>430.71</td>
<td>10.98</td>
</tr>
<tr>
<td>α-tocopherol acetate</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>472.73</td>
<td>10.89</td>
</tr>
<tr>
<td>Compound</td>
<td>Structure</td>
<td>MW</td>
<td>Purity</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------</td>
<td>-----</td>
<td>--------</td>
</tr>
<tr>
<td>(\alpha)-tocopherol phosphate</td>
<td><img src="image" alt="Structure" /></td>
<td>510.70</td>
<td>10.33</td>
</tr>
<tr>
<td>(\alpha)-tocopherol glycine</td>
<td><img src="image" alt="Structure" /></td>
<td>487.82</td>
<td>9.97</td>
</tr>
<tr>
<td>(\alpha)-tocopherol alanine</td>
<td><img src="image" alt="Structure" /></td>
<td>502.93</td>
<td>10.54</td>
</tr>
<tr>
<td>α-tocopherol pyroglutamate</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>541.84</td>
<td>10.22</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------------------------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>α-tocopherol oleate</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>695.17</td>
<td>17.90</td>
</tr>
<tr>
<td>α-tocopherol linoleate</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>693.15</td>
<td>17.54</td>
</tr>
<tr>
<td>α-tocopherol linolenate</td>
<td><img src="https://via.placeholder.com/150" alt="Structure" /></td>
<td>691.14</td>
<td>17.17</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------------------------------------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>α-tocopherol stearate</td>
<td><img src="https://via.placeholder.com/150" alt="Structure" /></td>
<td>697.19</td>
<td>18.26</td>
</tr>
<tr>
<td>α-tocopherol palmitate</td>
<td><img src="https://via.placeholder.com/150" alt="Structure" /></td>
<td>669.13</td>
<td>17.37</td>
</tr>
<tr>
<td>δ-tocopherol</td>
<td><img src="image" alt="δ-tocopherol structure" /></td>
<td>402.66</td>
<td>9.95</td>
</tr>
<tr>
<td>δ-tocopherol glycoside</td>
<td><img src="image" alt="δ-tocopherol glycoside structure" /></td>
<td>564.80</td>
<td>7.69</td>
</tr>
</tbody>
</table>
1.11.2 Strategies to improve the delivery of vitamin E into the skin

Several strategies have shown potential to improve the delivery of vitamin E into various viable skin layers, these include, chemical permeation enhancers (CPE), nanocolloids, hydrogel linked with an antioxidant, and pro-drugs (Table 1.4, 1.5, and 1.6). CPE may have a detrimental effect on skin integrity with long-term use, and hydrogel may add an additional release step of vitamin E from the gel network which may delay the onset of action. Both nanocolloids and pro-drugs showed higher skin permeation to epidermal and dermal layers, but the pro-drug requires metabolism to vitamin E in the viable skin layers.

1.11.2.1 Chemical enhancers

Coadministration of α-T with formulation excipients such as CPE is one of the passive enhancement strategies that can manipulate the skin barrier properties by forming micropores within SC lipids or by disturbing the lipid packing. In 2003, Rangarajan & Zatz examined various α-T formulations containing CPE (o/w emulsion containing isopropyl myristate (IPM), simple IPM, alcoholic and hydroalcoholic gels) using a porcine skin model which resembled human skin. They showed that an o/w emulsion containing IPM was the most desirable formulation to deliver α-T into skin. (Rangarajan and Zatz, 2003). Nada, et al (2014) demonstrated synergistic permeation enhancement for dimethyl sulfoxide (DMSO) when combined with tocopherol polyethylene glycols (TPGs) in monophasic liquid formulations, which resulted in 10-fold higher permeation of α-T through rat epidermis (Nada et al., 2014).
1.11.2.2 Nano-colloids

Nano-colloids include lipid nanoparticles, liposomes (deformable or rigid), micro- and nano-emulsions, and vesicular carriers. Lipid nanoparticles, which often consist of solid lipids dispersed in water, can improve the chemical and photostability of encapsulated α-T and they can have an occlusive effect leading to improved skin hydration, thus enhancing the encapsulated α-T penetration into the skin. In 1999, Kim and Lee prepared lipid nanospheres (nanoparticles) of Soya bean lecithin which was further stabilized by the co-surfactant (tween 80), to generate 147-205 nm carriers. *In vitro* permeation experiments through rat skin showed a 2.6-fold greater permeation of α-T from the lipid nanospheres compared to the free α-T. When the composition and the size of nanospheres were varied, it was found that a higher weight ratio of the lecithin (surfactant) to oil, produced a smaller size and thus a higher the permeation coefficient (P) (Kim and Lee, 1999). Dingler *et al* (1999) prepared solid lipid nanoparticles (SLN) loaded with α-T in a cream formulation to evaluate its physical stability and penetration into SC by tape stripping on three human subjects after applying a 20 μL dose of test formulation and control containing 5% α-T (0.25 mg/cm²). They found that SLN could protect α-T from chemical degradation and offer a 2-fold higher SC deposition over the free α-T (control), probably as a result of an occlusive effect, which improved the dermatological efficacy (Dingler *et al.*, 1999).

Vesicular systems (liposomes or niosome), has been used in topical products since 1963. They showed similar bilayer structure to a membrane and can alter the fluidity of the cell membrane. In the skin they may fuse with the SC bilayer lipids and hence they can enhance the penetration of antioxidants into the skin. Vesicles can be rigid
or elastic deformable nanostructures. Deformable liposomes can enhance α-T deposition in the skin layers. Gallarate et al (2006), prepared elastic deformable liposomes consisting of hydrogenated phosphatidylcholine (HPC) from soya bean lecithin (Phospholipon®) and a surfactant, either sodium cholate (SC), tween 80 (T80) or sucrose mono-palmitate (SMP). When the liposomes were loaded with 0.17% α-T, they maintained their deformity, i.e., they easily permeate through a filter with pore size smaller than their own diameter, unlike the HPC alone and the HPC with the dipotassium glycyrrhizinate (DPG) which showed no deformity, i.e., they were unable to pass through the porous membrane. The porcine skin deposition of α-T at 17 h from the deformable liposomes was 8-10-fold greater than the non-deformable liposomes. However, increasing the DPG in the HPC-DPG (2:1) liposomes caused a 5.8-fold greater skin deposition compared to HPC-DPG (6.25:1), which could have been due to the fusion of liposomes loaded with α-T with SC lipids. In 2013, Tavano and co-workers prepared niosomes from tween 60 loaded with α-T with or without curcumin (T60-T, T60-T-C) that displayed a particle size of 531 nm and PDI of 0.19. They showed that the amount of α-T that permeated across full-thickness rabbit skin at 12 h from niosomes was 3.8-4.3-fold greater than free α-T (Gallarate et al., 2006).

Microemulsions (ME) are transparent, thermodynamically stable dispersions of water and oil stabilized by an interfacial film of surfactant molecules. The systems which form spontaneously, i.e., they have a low interfacial energy with a mean particle size < 200 nm and PDI of up to 0.10. Rozman et al, (2009) combined vitamin E and C in a gel-like ME, o/w ME, and o/w ME carbomer and showed that the in vitro dermal deposition in porcine skin for both vitamins from an o/w ME
was 1.2-1.3-fold higher compared to a gel-like ME and o/w ME carbomer. Release studies at skin temperature showed that the vitamins release from the gel-like ME was comparable to the o/w ME and faster than the o/w ME thickened by a carbomer polymer (Rozman et al., 2009). Cichewicz, et al., (2013) developed a ME from water and oil (mono-caprylin and IPM) stabilized by a surfactant (decylglucoside) and a co-surfactant (propylene glycol) to promote the delivery of α-T and lipoic acid (a scavenger for ROS and metal chelator). They investigated the influence of a cationic additive (phytosphingosine addition on ME) on the penetration into the skin, which possesses a negative surface charge. Three MEs were prepared containing surfactant: oil: water (s: o: w) at 60: 30: 10 (ME-O) and 46:23:31 (ME-W), as well as a cationic form of ME-W containing 1% phytosphingosine (ME-Wphy). All MEs showed 3-13-fold greater SC deposition over the free α-T. Moreover, increasing the water content of ME-W enhanced the SC and epidermal deposition of α-T by 3.5-fold compared to ME-O. The addition of phytosphingosine increased the zeta potential up to +29 mV, i.e., generated a surface positive charge, and enhanced the penetration of α-T into the viable skin by 2-fold compared to ME-W (Cichewicz et al., 2013).

Nanoemulsions (NE) are thermodynamically unstable dispersion i.e. leading to phase separation over time by flocculation, coalescence, and creaming, that are prepared by high or low energy methods. However, it remains stable under physical stresses such as temperature change or dilution (Nastiti et al., 2017). Kong et al (2011) prepared hyaluronic acid (HA) based NE (L6) from HA-glyceryl monostearate (GMS) solution, methylene oxide (oil), and tween 80-span 20 as surfactant, loaded with 0.1% α-T with particle size of ca. 57 nm offered higher flux
at steady state of encapsulated α-T over free α-T (14.7 µg/cm² vs. not detected, respectively) across SC prepared from dorsal rat skin (Kong et al., 2011).

Abla and Banga, (2014), prepared two formulations; a nano-lipid carrier (NLC) and emulsion. The NLC consisted of an aqueous phase (0.5% Pluronic F68, and 0.1% SDS in distilled water), and a lipid phase (1.4% tripalmitin, 0.6% oleic acid, 1.2% Tween 80, and 0.1% α-T) which generated NLC’s with a particle size of 67 nm, a PDI of 0.2, and a zeta of -32 mV. The emulsion contained 1% pluronic F68 in water loaded with 0.1% α-T (particle size of 586.5 nm, PDI of 0.7, and zeta of -10 mV). The in vitro permeation across full-thickness human skin revealed epidermal skin deposition of α-T from NLC was 4.2-fold greater than from the emulsion, and 4.8-7.6-fold higher than the control (p < 0.05). The in vitro release across dialysis cassettes (2000 kDa) of α-T from NLC at 2 h was 7.5-fold quicker than emulsion (Abla and Banga, 2014).

1.11.2.3 Hydrogel linked with antioxidant

Hydrogels are three-dimensional (3D) hydrophilic polymer networks, which can swell in water. The polymer can be linked with potent antioxidant moieties to display synergistic effects and to protect α-T from UVR-induced photodegradation. Cassano et al (2009) prepared a novel dextran-methacrylate and aminoethyl methacrylate hydrogel linking trans-ferulic acid loaded with 22 mg/mL α-T. The ferulate hydrogel showed control-release pattern of α-T across full thickness rabbit skin compared to the free α-T and the cumulative amount of α-T deposited at 24 h was 65% (due to preserving it as intact form during its release and deposition) compared to that of analogous hydrogel without ferulic groups (Cassano et al., 2009).
1.11.2.4 Pro-drugs

The prodrug strategy is the most favourable method to improve the stability and permeation of α-T into skin layers. It involves the esterification of α-T with a moiety such as a glucoside, a fatty acid, an amino acid, an acetate group, or a phosphate group. The main limiting step is the hydrolysis by esterase, which is required to release the free α-T upon reaching the viable epidermis.

The prodrug α-TA is currently incorporated into several cosmetics and skin care formulations, but several studies showed varying extents of metabolism due to differences in the skin models and formulation types. α-TA is more hydrophobic compared with other new pro-vitamins, which can be used in aqueous formulations for skin application. Dingler, et al, (1999) performed a tape stripping experiments on human volunteers after applying a finite dose (0.25 mg/cm²) of both α-TA and α-T in the same vehicle and demonstrated that both have comparable deposition in the SC (Dingler et al., 1999).

Rangarajan and Zatz (2000), have investigated the permeation and metabolism of α-TA after applying a finite dose (5 µl) from various formulations including a simple IPM solution, an o/w emulsion, a ME, and alcoholic and hydroalcoholic gels, on viable porcine skin. They showed a greater extent of metabolism was obtained from a simple IPM solution, a ME containing IPM and a hydroalcoholic gel. The metabolism in porcine skin was 15-20% of the total α-TA that permeated into viable skin (depending on the formulation composition) but no metabolism was observed in SC (Rangarajan and L Zatz, 2000).
Nabi, et al., 2001 studied the bioconversion of α-TA to free α-T in human skin explants, and two organotypic models, living skin equivalent (LSE) and EpiDerm™ skin, after topical application of a 4 mg/cm² dose of a test lotion containing 1% α-TA, and showed a maximum conversation value of 0.5 nmole/cm² at 6 h in LSE and at 10 h in the human explants (Nabi et al., 2001).

Another *ex vivo* study in viable human skin was performed by Baschong and co-worker (2001) in which they studied the effects of various formulations of α-TA in Mygliol oil, surfactant-solubilized in water, liposomes, or nanotopes on the bioconversion to free α-T. They found that skin deposition of α-TA in Mygliol oil was limited to the skin surface and SC, whereas other formulations showed deposition in the deeper skin layers. The highest skin deposition was from a nanotopes formulation followed by a liposome formulation and a surfactant-solubilized formulation of α-TA with up to 50% bioconversion to free α-T and no conversion was detected in surface or SC (Baschong et al., 2001).

Mavon et al (2004) prepared a pro-vitamin E glucoside conjugate, δ-tocopherol glucoside (δ-TG) which acted as a reservoir to release the free δ-T into the SC upon cleavage of the glycosidic bond by the β-glucocerebrosidase enzyme. The topical application of an infinite dose (100 µL/cm²) containing 0.1% δ-TG or TA and a finite dose (10 µL/cm²) containing 0.05% δ-TG or TA (both dissolved in myritol solution) in a reconstructed human epidermis (RHE) skin model resulted in 3-fold less δ-TG in the SC and epidermis than TA. Between 45-90% of δ-TG metabolised at 2-18 h, respectively. In viable human skin, the finite dose also resulted in 2-5-fold less δ-TG in various skin layers (SC, epidermis, and dermis) than TA but no metabolism into free T was detected for TA. The bioconversion of δ-TG to free δ-
T was mainly in the SC and epidermis, in these layers it was 5-fold greater than dermis. However, and the extent of metabolism of δ-TG at 2-18 h was 5-19% in viable human skin which was less compared to RHE model (Mavon et al., 2004).

Ostacolo et al. (2004), synthesised a new pro-vitamin of α-T using amino acid conjugates including glycine, alanine, and pyroglutamate, which should be converted into α-T by esterase in the viable skin. The release of free α-T and active amino acid were proposed to combine both the antioxidant and the natural moisturising factor effects upon topical application. Ostacolo and co-workers investigated the permeation of an infinite dose (0.7 mg/cm²) of α-T, provitamin α-TA (both dissolved in 1% IPM) and their amino acid conjugates (dissolved in 50% ethanol, 10% propylene glycol, 40% water) on full-thickness rabbit skin. The study showed a 3.8-fold greater α-TA (15 nmole/cm²) epidermal deposition at 4 h over α-T (4 nmole/cm²). The alanine, glucine, and pyroglutamic acid conjugate showed a 15-fold (60 nmole/cm²), 5-fold (20 nmole/cm²), and 2.5-fold (10 nmole/cm²), greater epidermal deposition at 4 h over α-T (4 nmole/cm²), respectively. All the amino acid conjugates of α-T showed the same accumulation trend in the dermis. The total levels of free α-T from all amino acid conjugates are comparable or higher compared to α-TA or α-T, respectively. However, pyroglutamic acid derivative was comparable to α-TA and has been shown to have the highest extent of metabolism (up to 40%) in both epidermis and dermis (Ostacolo et al., 2004).

Marra et al. (2009), prepared pro-vitamins of α-T from the amino-acids conjugates, including L-proline, L-serine, L-tyrosine, L-asparagine and L-citrulline. They used the same experimental conditions and the rabbit skin model that was used by Ostacolo et al. (2004). The in vitro skin deposition and metabolism of the five pro-
vitamins demonstrated that they all accumulated into the skin in significant amounts and were able to generate free α-T. The extent of metabolism found for the asparagine and tyrosine derivatives were ca. 40%, comparable to the result previously obtained with α-TA. These new derivatives are more hydrophilic and more water-soluble than the currently used acetate derivative; hence it was proposed that they could be incorporated in hydrophilic vehicles, thus facilitating the formation of gel formulations (Marra et al., 2009).

Ben-shabat et al. (2013), developed six new pro-vitamins, α-T fatty acid ester conjugates including oleate, linoleate, α/γ-linolenate, stearate, and palmitate. This group investigated their skin permeation and bioconversion to free α-T in vivo using Sprague-Dawley rats upon topical application of 50 μl of 4.25 mM of α-T fatty acid conjugate and compared it with α-TA (both dissolved in IPM). The skin deposition of α-TA was comparable to oleate and α-linolenate forms (p > 0.05). The total level of free α-T was similar for all the α-T fatty acid conjugates and not statistically different from α-TA. There were significant differences between the level of total α-T and endogenous α-T after application of α-TA, oleate, linoleate, linolenate and palmitate conjugates (p < 0.05) indicating hydrolysis to free α-T by each of the prodrugs (Ben-Shabat et al., 2013).

Another water-soluble derivative of α-T is α-TP. Only couple of studies has investigated its skin penetration. One study by Nakayama and colleagues (2003), used an ex vivo culture system of fresh dorsal skin derived from hairless mice rather an in vivo approach to assess cutaneous permeation, photoprotection and ester hydrolysis of α-TP. They used a commercially available molecule that was topically applied 50 μL of 9 mM or 0.5% (w/v) α-TP dissolved in culture medium. They
found that at 3 h, 2% (188 nmole/g skin) was absorbed, 10% (21 nmole/g skin) of which was converted into α-T. In the same study, they also showed that a 2 h incubation of 1 mM α-TP in mouse skin homogenates resulted in a 26-fold increase in α-T concentration, while α-TA showed only 3-fold increase compared to untreated skin (Nakayama et al., 2003). However, in a three-dimensional (3D) human skin model, treated with 0.5% (9.4 mM) α-TP containing medium for 6 h, only 0.2% (ca. 18 nmole/g skin) of the applied dose was absorbed into skin of which 4% (0.65 nmole/g skin) was converted to α-T (Nakayama et al., 2003). There results indicated that α-TP may permeate into human skin but the the use of culture medium in permeation study does not simulate the real topical formulation. In conclusion, there are missing data in the literature on the delivery of α-TP into skin layers to achieve the skin deposition, i.e. maximum benefit from a topical formulation.
Table 1.5: The list of studies which showed to enhance the skin permeation of Vitamin E by using chemical enhancers.

<table>
<thead>
<tr>
<th>Author</th>
<th>Key enhancers</th>
<th>Methodology</th>
<th>Control vehicle</th>
<th>Skin or membrane permeation evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rangarajan and Zatz, 2003</td>
<td>Isopropyl myristate (o/w emulsion) or isocetyl alcohol, PEG-15 (Hydro-alcoholic Gel)</td>
<td>250-300 µM thick layer porcine skin (ear)</td>
<td>α-T in IPM or in alcoholic Gel</td>
<td>1.3-1.8-fold higher than IPM solution, 1.4-1.7-fold higher than hydro-alcoholic gel, and 1.9-2.6-fold higher than alcoholic gel. Hydroalcoholic gel 1.3-1.5-fold higher alcoholic gel</td>
</tr>
<tr>
<td>Nada, et al 2014</td>
<td>Dimethyl sulfoxide (DMSO), tocopherol polyethylene glycols (TPGs) (Monophasic liquid formulations)</td>
<td>Neonatal rat epidermis (dorsal side)</td>
<td>α-T in gel or in emulsion</td>
<td>10-fold higher than emulsion, 50-fold higher than gel Synergistic enhancing permeation of DMSO and TPGS result in enhanced T permeation.</td>
</tr>
</tbody>
</table>
Table 1.6: The list of studies which showed to enhance the skin permeation of Vitamin E using nanocarriers.

<table>
<thead>
<tr>
<th>Author</th>
<th>Nano-system type and composition</th>
<th>Size and charge</th>
<th>Methodology</th>
<th>Control vehicle</th>
<th>Skin or membrane permeation evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kim and Lee, 1999</td>
<td>Lipid nanospheres:</td>
<td>Size 147-205 nm</td>
<td>Rat skin including subcutaneous tissue in two chambers diffusion cells.</td>
<td>1.25% Free vitamin E</td>
<td>2.6-fold greater permeation. The higher the weight ratio (S:O) the smaller the size and thus the higher the permeation coefficient (P).</td>
</tr>
<tr>
<td>Dingler, et al, 1999</td>
<td>SLN&lt;sup&gt;TM&lt;/sup&gt;/Lipopearls&lt;sup&gt;TM&lt;/sup&gt;: Cetylpalmitate (15%), Tego care 450 (polyglycerol methylglucose disteate) (1.8%) as emulsifier, 5% dl-α-T</td>
<td>size of 280 nm at 4°C, PDI of 0.04</td>
<td>SC tape stripping on forearm of three human volunteers aged 20-30. Applied dose 20 µl of α-T (0.25 mg/cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>5% α-T in isopropanol</td>
<td>SC deposition: ca. 2-fold using SLN due to occlusive effect.</td>
</tr>
<tr>
<td>Gallarate, et al, 2006</td>
<td>Deformable liposomes: Hydrogenated soya lecithin (HPC) phospholipon 90H, sucrose monopalmitate (SMP), di-potassium glycyrrhizinate (DPG), Tween 80 (T80), Sodium cholate (SC). HPC-SC, HPC-T80, HPC-SMP (6.25:1)</td>
<td>α-T loaded: 0.17% Size 117-236 nm</td>
<td>Full thickness porcine skin (ear)</td>
<td>un-deformable liposome, HPC-DPG (6:1, 2:1, 6.25:1).</td>
<td>Skin deposition at 17 h: 8 -10-fold. HPC-DPG (2:1) un-deformable liposomes: 5.8-fold skin deposition compared to HPC-DPG (6.25:1) (fusion with SC lipid)</td>
</tr>
<tr>
<td><strong>Rozman et al., 2009</strong></td>
<td>o/w ME</td>
<td>Size was 1.9-2.4 nm, PDI 0.21-0.28</td>
<td>Full-thickness skin porcine skin (ear)</td>
<td>Gel-like ME o/w ME carbomer</td>
<td>1.2-1.3-fold in dermis (p &gt; 0.05).</td>
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<tr>
<td><strong>Kong et al., 2011</strong></td>
<td>Hyaluronic acid-based NE (L6): Methylene oxide (O), Tween 80-Span 20 (S), HA-GMS solution (W) (2:3:95)</td>
<td>Drug load: 0.1%</td>
<td>Size of α-T loaded NE droplet is ~57.3 nm</td>
<td>Stratum corneum prepared from full-thickness dorsal skin of rat</td>
<td>Flux Jss in 24 h for L6 NE: 14.68±4.13 µg/cm²·h. Control: not detected</td>
</tr>
<tr>
<td><strong>Cichewicz, et al., 2013</strong></td>
<td>PE ME: monocaprylin and IPM (O), decylglucoside (S), water (W) with or without 1% phytosphingosine. ME-O (30:60:10) (w/o), ME-W (23:46:31) (o/w), ME-Wphy (22.7:45.3:31:1) (o/w)</td>
<td>Size 47-53 nm, zeta (-4 to+29mV)</td>
<td>Full thickness porcine skin (ear)</td>
<td>α-T in PG</td>
<td>Whole skin (SC+ED): 30-fold, the SC: 3-13-fold, (p &lt; 0.01). ME-Wphy &gt; ME-W (2-fold) ME-W &gt; ME-O (3.5-fold) ME-Wphy &gt; ME-W &gt; ME-O.</td>
</tr>
<tr>
<td><strong>Tavano et al. 2013</strong></td>
<td>Niosomal vesicular carrier: 1x 10⁻⁴ mol Tween 60, 1x 10⁻⁶ mol α-T, with or with no 1.03 x10⁻⁶ curcumin (T60-T or T60-T-C)</td>
<td>T60-T (α-T loaded: 8.4x10⁻⁷mol, size 544 nm, PDI 0.156), T60-T-C (α-T loaded: 5.56x10⁻⁷ mol, size 531 nm, PDI 0.188)</td>
<td>Full thickness rabbit skin (ear)</td>
<td>Free α-T solution</td>
<td>Amount permeated at 12 h: 3.8-4.3-fold.</td>
</tr>
<tr>
<td><strong>Abla and Banga, 2014</strong></td>
<td>NLC: Lipid phase (Tripalmitin 1.4%, Oleic acid 0.6%, Tween 80 1.2%, and α-T 0.1%), aqueous phase (Pluronic F68 0.5%, SDS 0.1%, in distilled water). NE: Pluronic F68 1% in distilled water and dl-α-T 0.1% (1mg/ml).</td>
<td>NLC (size 67 nm, PDI 0.2, zeta -32 mV). NE (size 586.5 nm, PDI 0.7, zeta -10 mV).</td>
<td>Human full-thickness skin and dialysis cassettes (2000 kDa)</td>
<td>Endogenous α-T in blank skin and α-T in mineral oil</td>
<td>NLC: 4.2-fold greater than NE, and 4.8-7.6-fold higher than control (p &lt; 0.05). Dermal amount NS. Release from NLC at 2 h: 7.5-fold quicker than NE.</td>
</tr>
</tbody>
</table>
Table 1.7: The list of studies which showed to enhance the skin permeation of Vitamin E using prodrug.

<table>
<thead>
<tr>
<th>Author</th>
<th>Type of prodrug</th>
<th>Methodology</th>
<th>Control vehicle</th>
<th>Skin or membrane permeation evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dingler, et al, 1999</td>
<td>5% dl-α-TA in isopropanol</td>
<td>SC tape stripping on forearm of three human volunteers aged 20-30.</td>
<td>5% α-T in isopropanol</td>
<td>The stripping test showed a slightly higher penetration of α-TA into SC over α-T but the difference is not statistically significant.</td>
</tr>
<tr>
<td>Mavon et al, 2004</td>
<td>0.1% and 0.05% δ-T-glucoside in myritol solution</td>
<td>Reconstructed human epidermis (RHE) and viable full-thickness human skin.</td>
<td>0.1% and 0.05% α-TA in myritol solution</td>
<td>In RHE, 3-fold less δ-TG in SC and ED than TA. The extent of TG metabolism was 45-90% at 2-18 h. In human skin, 2-5-fold less δ-TG in SC, ED and D than TA. The highest conversion of δ-TG to δ-T was in SC and ED representing 15% and 30% of the metabolite. The extent of metabolism in viable human skin was 5-19%, at 2-18 h.</td>
</tr>
<tr>
<td>Ostacolo, et al, 2004</td>
<td>Amino-acid-pro-VE (glycine, alanine, pyroglutamate) 0.7 mg/ml in 50% ethanol/10% propylene glycol /40% water. (insoluble in IPM)</td>
<td>In vitro skin permeation and metabolism using full thickness rabbit skin (ear) in Franz cell diffusion cell</td>
<td>1% α-T and α-TA in IPM due to low water solubility</td>
<td>2.5-4-fold greater α-TA at 2-6 h in ED over α-T. 1.3-3-fold and 6-15-fold greater alanine conjugate higher over glycine and pyroglutamic acid conjugates, respectively. Pro-vitamins showed the same accumulation trend in the dermis. The amount deposited are comparable or higher compared to α-TA or α-T. pyroglutamic acid derivative and α-TA have shown to have the highest extent of metabolism (up to 40%).</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Fatty Acids Tested</td>
<td>In Vivo/Cutaneous Permeability and Metabolism</td>
<td>Concentration</td>
<td>Remarks</td>
</tr>
<tr>
<td>-----------</td>
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<td>---------------------------------------------</td>
<td>---------------</td>
<td>---------</td>
</tr>
<tr>
<td>Ben-Shabat et al, 2013</td>
<td>Fatty acid pro-VE (e.g. oleate, linoleate, α/γ-linolenate, stearate, palmitate) at 4.25 mM in IPM.</td>
<td>In vivo cutaneous permeability and metabolism: Male Sprague-Dawley rat (300-400 g; Harlan Laboratories)</td>
<td>4.25 mM α-TA in IPM</td>
<td>The skin deposition of α-TA comparable extent of deposition to oleate, and α-linolenate forms ($p &gt; 0.05$). The total level of free α-T was similar for all the α-T fatty acid conjugates and not statistically different from α-TA. There were significant differences between the level of total α-T and endogenous α-T after application of α-TA, oleate, linoleate, linolenate and palmitate ($p &lt; 0.05$) indicating hydrolysis to free α-T.</td>
</tr>
</tbody>
</table>
| Nakayama, et al, 2003 | α-TP in culture medium at 0.5% (9 mM) | Cultured skin derived from hairless mice rather than *in vivo* (treated for 3-6 h) 
3D human skin model (treated for 6 h) | α-TA | Limited amount of α-TP absorbed at 3-6 h (18-188 nmol/g skin) and converted into α-T (0.65-21 nmol/g skin) in cultured mouse skin. 
1 mM TP increased the α-T concentration by 26-fold in contrast α-TA increased it by 3-fold. 
Limited amount of α-TP (18 nmole/g skin) absorbed into the skin and only 0.65 nmole/g skin converted into α-T |
1.12 Vitamin E phosphate

The phosphorylation of α-T, at its oxygen-sensitive 6-hydroxyl position, on its chroman ring by phosphoric acid yields a water soluble and chemically stable compound (α-TP) which can be incorporated in cosmeceutical formulations and has greater biological activity. α-TP was first synthetized by Karrer and Bussmann in 1940 (Karrer and Bussmann, 1940). However, it is also found naturally as an endogenous compound in small amounts in human hepatic and adipose tissue, as well as in primary coronary cell cultures (Ghayour-Mobarhan et al., 2014; Negis et al., 2005).

In term of photoprotection, Nakayama and colleagues used a culture system of fresh dorsal skin derived from hairless mice rather in vivo approach to assess the UVB photoprotective effect of α-TP. They showed that pretreatment with 50 μL of 0.5% (w/v) α-TP on the fresh cultured (dorsal) skin for 3 h followed by UVB irradiation (10-40 kJ/m², 63% UVB 291-320 nm, 37% UVA 321-380 nm) provided significant protection against UVR-induced skin damage by a reduction of lipid peroxidation by 0.64 (p < 0.01), SBC by half (p < 0.01), and epidermal DNA degradation compared to an irradiated vehicle control.

Another study, conducted by Kato and co-workers, assessed application of 0.1 mL of 2% α-TP onto the skin surface of a 3D human skin model and measured the intradermal conversion to α-T. The concentration of α-T was found to increase in a time-dependent manner (40, 90, and 185 nmole/g skin after 4, 16, and 24 h incubation with provitamin E, respectively). In post-irradiation treatment with 2% α-TP for 2 h was following UVB irradiation (80 mJ/cm²). The α-TP significantly reduced the number of UVB-damaged epidermal cells and PGE-2 secretion.
compared to the irradiated vehicle-treated skin (16 vs 26 damaged cells/0.5 mm epidermis ($p < 0.001$) and 600 vs 1000 pg/ml PGE-2 ($p < 0.05$), respectively).

In normal human epidermal keratinocytes (NHEK), pre-irradiation treatment with $\alpha$-TP at a concentration of 0.5 $\mu$M for 24 h significantly suppressed PGE2 synthesis stimulated by different agents including UVB (60 mJ/cm$^2$), IL-1B (10 ng/mL), 0.5 mM tert-butyl hydroperoxide (tBHP), and 1 mM H$_2$O$_2$ for 0.5 h by 56%, 58%, 87%, and 50%, respectively. Moreover, pre-irradiation treatment with 0.5 $\mu$M $\alpha$-TP also inhibited the induction of cyclooxygenase 2 (COX-2) mRNA expression, which promotes inflammation and PGE-2 synthesis (by 33% for UVB, 51% for IL-1B, 23% for tBHP, but not for H$_2$O$_2$). It also reduced UVB-induced MAPK P38 protein phosphorylation (which may be related to UVB-induced elevation of COX-2 promotor activity) comparable to MAPK P38 inhibitor (by 55% vs 62%, respectively) (Kato et al., 2011).

$\alpha$-TP was proven to prevent dry rough skin, by promoting ceramide synthesis thus increases the water-holding capacity of the SC and the barrier function of the skin. In vivo studies conducted in hairless mice showed that daily application of 1% and 2% $\alpha$-TP for 4-weeks duration improved the hygroscopicity and moisture-retaining ability of SC by 1.5-fold with an additional 1.7-fold higher ceramide compared to placebo-treated skin. Moreover, in vitro studies on NHEK cells treated with 50 $\mu$M $\alpha$-TP for 24 h showed that 147.8 nmole/mg protein of $\alpha$-TP was incorporated in the keratinocytes and 3.1% is slowly converted to $\alpha$-T (4.7 nmole/mg protein). There was also significantly induced upregulation of ceramide synthetases (serine palmito-transferases, SPTCL1 and SPTCL2) by 1.2 and 2.2-fold, respectively, two-fold stimulation in the mRNA expression of several differentiation enzymes such as transglutaminase (TGM1), cytokeratin 10 (KRT 10), involucrin (IVL), and
loricrin, and 1.4-fold enhancement in calcium uptake that induces differentiation of keratinocytes enhancing ceramide synthesis (Kato and Takahashi, 2012).

α-TP traffics across the cell membrane is thought to be via a specific carrier protein belonging to the organic anion transporters (OAT) family, which can be inhibited by glibenclamide and probenecid. In vitro α-TP can act as a strong antioxidant by an extraordinary mechanism due to chemical structure that possesses three domains, which is a hydrophilic phosphate head group (functional domain), a signaling domain which accounts for signaling activity, and phytyl tail (hydrophobic domain) which allow α-TP to integrate into cell membrane and protect biological membranes to stop the propagation of lipid peroxidation caused by ROS. Unlike α-T, α-TP has been shown to act as detergent which has a membrane destabilizing activity and induce haemolysis in a concentration dependent manner (Rezk et al., 2007, 2004).

Gavin et al (2017), demonstrated that α-TP at a concentration of 1% w/v in hydro-alcoholic solution (30% ethanol: 70% deionized water) can self-assemble forming unilamellar vesicles, together with tubular micellar vesicles in smaller quantities evident by cryo-TEM imaging. Dynamic light scattering showed a mean of particle size and polydispersity index (PDI) of these aggregates were shown to be 100 nm and 0.35, respectively (Gavin et al., 2017).

A recent study by Harper et al (2018) showed that α-TP self-assembled at a critical aggregate concentration (CAC) of ca. 5.5 µM, and at a concentration of 0.1 mM (0.005% w/v > CAC) in 20% ethanol, 80% water at pH 7.4 ± 0.2 (25 mM Trizma®). The hydrodynamic size of the aggregates and PDI were 175 nm and 0.36, respectively. The α-TP aggregates showed a slight negative charge (-14.9 mV)
whereas the α-T formed spherical liposomes with a 3-fold higher diameter (563 nm) and smaller negative charge (-10.5 mV). Atomic force microscopy tapping height images of 0.15% α-TP and α-T dispersed in 20% ethanol, 80% water at pH 7.4 with and without 150 mM Trizma showed that the (+) α-TP pure stereoisomer molecules form cylindrical micelles in hydroalcoholic vehicle (height was 22 nm) and with the addition of Trizma® they transformed into planar bilayer island (height was 4.6 nm). This change in shape was thought due to Trizma®-ion pairing with negatively charged phosphate group of (+) α-TP pure stereoisomer and thus reducing the repulsion between polar heads in the aggregates. The addition of Trizma® to the vehicle did not alter the structure of natural α-T (spherical liposomes with diameter of 551 nm and height of 86 nm). A nanoaggregates of (+) α-TP pure stereoisomer showed an enhanced antimicrobial effect on oral biofilms over α-T (38 vs. 58 µM, \( p < 0.05 \)) (Harper et al., 2018). However, the cutaneous penetration of α-TP nanoaggregate and whether it protects from UVA1 cell viability reduction and ROS generation has not been investigated yet.

### 1.13 Nanomaterial in cosmeceuticals

Several active pharmaceutical ingredients have self-aggregation properties; these include tetracaine, phthalocyanine derivative photosensitizers and dexamethasone phosphate. This aggregation makes it difficult for them to penetrate the skin, i.e., the larger the size of the aggregate the less the penetration into various skin layers, hence reducing the local pharmacological efficacy. Reducing the aggregate size to the nanoscale by optimizing the formulation composition may result in enhanced skin penetration. For instance, nanoemulsions show greater skin delivery and efficacy compared to corresponding emulsion.
The self-assembled lipid or surfactant or amphiphilic-active based nanomaterial have been shown to have self-enhanced skin permeation by different mechanisms such as fusion with skin lipids as shown in non-deformable vesicles, squeezing through the tortuous lipid route as shown in deformable vesicles, follicular targeting, furrow deposition, and through the eccrine glands, which is still not well studied (Fig 1.1) (Nastiti et al., 2017).

The efficacy of topical vitamin E in the final cosmeceutical product requires stability in the formulation and SC penetration and maintaining adequate concentrations in epidermis and dermis. These properties may be achieved using nanomaterial, i.e. nanoaggregates of pro-vitamin E to maximize the photoprotective benefits after applying the topical active to the skin.
Fig.1.11: Mechanism of nanomaterial skin absorption and permeation through potential route of penetration depending on their properties (Nastiti et al., 2017)
1.14 Aims of thesis

α-TP has been shown to be a promising active to protect the skin from UVB-induced damage. However, previous studies did not report if α-TP aggregates were likely to affect skin penetration. Furthermore, how α-TP interacts with SC lipids and permeates into the skin has not been documented. In addition, the delivery vehicle used in previous work did not mirror a real topical formulation. These missing data prevent its translation into a commercial cosmeceutical formulation. The potential photoprotective properties of α-TP have not been performed in the long-waveband UVA1 region. This is an important omission from the literature because UVA1 is a major component of solar UVR, unintentionally ignored when estimating sun protection by SSR sources that have decreasing output at > 365 nm. This waveband also generates ROS, not blocked by traditional UVR filters. The primary aim of this thesis was to develop a cosmeceutical formulation of α-TP that is suitable for human topical application to protect from skin damage induced by UVA1.

In order to achieve this aim the following objectives were set:

1. To investigate α-TP aggregation in a vehicle and evaluate the influence of the aggregates on the percutaneous deposition (Chapter 2).

2. To employ a Langmuir SC-mimicking lipid monolayer model that could be used to explore the interaction between α-TP and SC lipids (Chapter 3).

3. To assess the cytotoxicity of α-TP in human keratinocytes (Chapter 4).

4. To examine the protective effects of α-TP against cell viability reduction and ROS generation induced by UVA1 (Chapter 4).
5. To investigate the formulation of α-TP with nanoparticles, i.e., with a physical sunscreen, to evaluate if a commercial formulation for use in humans could be generated (Chapter 5).
Chapter 2. Characterising the \textit{in vitro} transport of α-tocopherol phosphate through membrane

\textit{Chapter summary:}

In this Chapter, the maximum solubility of pro-vitamin E (α-TP) was determined in a hydroalcoholic vehicle at different pHs. The α-TP was shown to form naosized aggregates. The critical aggregation concentration of α-TP was determined in a selected formulation at pH 7.4 and compared it with α-T. Transport studies were performed to understand the penetration of α-TP through the skin. The amount of α-TP deposited in each skin layer from different formulations was determined and compared with α-T. The short-term chemical and chemical stability of α-TP nanomaterial at pH 7.4 (32 °C) were also reported.
2.1 Introduction

α-T is a very useful antioxidant agent that can protect against damage induced in the skin by ultraviolet radiation (UVR) and reduce oxidative stress. A recent study has shown that α-T abolishes UVA-induced DNA photolesions even if applied after the exposure (Premi et al., 2015). However, to obtain an effective photoprotection, a significant amount of topically applied α-T needs to reach the viable epidermis.

α-T (C_{29}H_{50}O_{2}, MW of 431.7 g/mole, log P 10) has two major limitations, firstly, it has poor chemical stability, due to sensitivity to light and oxygen which reduces its shelf life and thus efficacy. Secondly, α-T is very lipophilic, i.e., practically water insoluble (calculated log P= 12, and pK_s above 10), which limits the concentrations that penetrate into the viable skin layers (Cichewicz et al., 2013). These limitations hinder the use of α-T in the skin.

Generating a vitamin E derivative that is metabolised in the skin epidermis to form vitamin (akin to a prodrug system) is one of the approaches that have been used to overcome the issues of administering this agent into the skin. The derivative should increase vitamin E stability and/or aqueous solubility, thus improve its skin deposition. There are several pro-vitamin E esters, such as α-tocopherol acetate, α-tocopherol alanine, and α-TP that have been suggested as suitable for use in the skin.

α-TP (C_{29}H_{49}O_3P, MW 508.7 Da, log P 10.78, log D 3.75) is one of dl-α-T derivative that is substantially soluble in water. It has an amphiphilic nature, and it can be easily incorporated in a variety of aqueous cosmeceutical formulations,
which are preferred by users (Nakayama et al., 2003), without the need of surfactant.

α-TP was proven to prevent dry rough skin by enhancing ceramide synthesis, and can act as an antioxidant (Kato and Takahashi, 2012). Moreover, α-TP has also a proven efficacy for prevention of inflammation (Kato et al., 2011), skin protection against UVB (Kato et al., 2011; Nakayama et al., 2003), and synergism inhibitory effect with vitamin C derivative on prostaglandin E₂ (PGE₂) caused by UVB irradiation (Kato et al., 2011). Therefore, this derivative of vitamin E could be a promising cosmeceutical active for the skin.

The addition of phosphate to α-T generates an amphiphilic molecule which self-assembles at concentration above a CAC ~ 5.5 µM to form nano-aggregates. In 20% ethanol, 80% water mixture at pH 7.4 (25 mM tris.Hcl buffer) these aggregates of α-TP are liposomes, but their properties depend upon the vehicle that they are dispersed in (Harper et al., 2018). Whether these nano-aggregate affect the skin permeation of α-TP to the skin has not been studied yet.

The phosphate functionality in the α-TP derivative increases the polar surface area of the molecule in a similar manner the phosphate group into phospholipids, hence α-TP may have superior interaction with SC intercellular lipid lamellae and this may increase the ability to penetrate into the skin paracellularly through lipid bilayer. However, the charged phosphate may reduce skin partitioning and drug-drug interactions may negatively affect the delivery of topically applied α-TP into the skin. For instance, when topically applied tetracaine, acyclovir, diclofenac diethylamine, and dexamethasone phosphate form aggregates in aqueous solutions, their permeation was reduced into the skin (Cai et al., 2016; Inacio et al., 2016;
Shah et al., 2009). Therefore, further study is required to understand how the nanosized drug aggregates of α-TP should be presented to the skin to facilitate their optimal deposition into the tissue.

The aim of this chapter was to investigate the saturated solubility and tendency of α-TP to form aggregates in an aqueous vehicle at different pHs and how these aggregates influence its transport into the skin. A 20% ethanol, 20% propylene glycol, 60% water mixture at pH 7.4 and 9 (100 mM Tris.HCl buffer) was selected as a vehicle for α-TP as it was able to solubilize the same amount of the active as the commercial formulations (Kato et al., 2011; Nakayama et al., 2003). The artificial regenerated cellulose and silicone synthetic membranes were used in order to understand the mechanism by which the different aggregates influenced the transport processes into the skin. The porcine skin was then used to study the skin deposition of topically applied α-TP from different formulations and compare it with α-T. Porcine skin is an acceptable model of human skin for chemical deposition studies (Abd et al., 2016; Barbero and Frasch, 2009).

2.2 Materials

α-TP (all-racemic mixture of 8 isomers or dl-α-tocopherol phosphate, purity: 92.7%, cosmetic grade) was a gift from Shawa Denko (Tokyo, Japan). α-T (type VI, purity: 70%, 695 mg d-α-tocopherol per g, 1036 IU/g), α-tocopherol acetate (α-TA) (Activity: 1306 IU/g, semisynthetic d-α-tocopherol acetate, synthesized from natural α-tocopherol), propylene glycol, and trifluoroacetic acid were purchased from Sigma Aldrich (Dorset, UK). Hydrochloric acid was from Fluka Chemicals (Gillingham, UK). High performance liquid chromatography (HPLC) grade water, isopropanol, sodium hydroxide, and tris base were sourced from Fisher Scientific
Absolute ethanol was from VWR Chemicals (Leicestershire, UK). Miglyol 810 was from Cremer Oleo (Hamburg, Germany). Unless otherwise noted all the chemicals were analytical grade. Spectra/Por® cellulose ester (CE) dialysis membranes with a molecular weight cut off (MWCO) of 0.5-1, 20, 1000 KD were sourced from Spectrum Labs (Breda, The Netherlands, Europe). Chemicals were reagent grade unless otherwise specified.

2.3 Methods

2.3.1 Solubility studies

Saturated solutions of α-TP were used to determine the α-TP solubility in a co-solvent composed of 20% ethanol, 20% propylene glycol, and 60% Tris. HCl buffer at pH 3, 5.5, 7.4, and 9. Three suspensions were prepared at each pH by adding an excess amount of α-TP to the co-solvent and stirring in a water bath at 32˚C for 24 h to reach equilibrium. At equilibrium, the suspensions were transferred to 1.5 ml micro-centrifuge tubes, which were centrifuged at 32˚C for 15 min at 13,000 g. The supernatant was recovered from the samples and diluted with co-solvent prior to analysis using HPLC (method described in a subsequent section).

For both α-T and α-TA an excess amount of each was dissolved in the same co-solvent at pH 7.4 until turbid solutions were produced (note α-T and α-TA are both oils). These liquids were stirred in a water bath at 32˚C for 24-h to achieve saturated equilibrium. After 24-h, an aliquot of the solution was taken from below the excess oil droplet which was at the surface of the sample. The α-TA samples were diluted 1:4 in the HPLC mobile phase and the samples of α-T were not diluted prior to analysis by HPLC.
2.3.2 Photon correlation spectroscopy (PCS)

The unattenuated derived count rate per second (KCps), which provides an indication of the amount of light scattered by the sample, was used to analyse the extent of \( \alpha \)-TP aggregation in aqueous solutions at both pH 7.4 and pH 9 (method previously described in Cai et al, 2016, Inacio et al, 2016, and Harper et al, 2018). Briefly, a 2% (20 mg/ml or 35 mM) stock solution of \( \alpha \)-TP was used to prepare a series of standard \( \alpha \)-TP solutions (32.5, 30, 27.5, 25, 22.5, 20, 17.5, 15, 12.5, 10, 7.5, 5, 2.5, 0.5, and 0.125 mM) in a 20% ethanol, 20% propylene glycol, 60% water mixture (100 mM Tris.HCl buffer). These solutions were equilibrated at 32°C and adjusted to pH 7.4 or 9. The solutions were then incubated for 24 h prior to the PCS measurements being performed. The solutions were analysed using a Zetasizer Nano-ZS (Malvern Instruments, UK) fitted with 633 nm He-Ne laser beam. Detection of the light scattering signal was achieved at 137° backscattering angle with sample equilibrated at 32°C using 20:20:60 % (v/v/v) (ethanol/propylene glycol/Tris.HCl buffer) as the dispersant (additives were propylene glycol, weight%: 22.89 and ethanol, weight%: 16.36, refractive index 1.365, viscosity 1.6122 mPa.s). For both \( \alpha \)-T and \( \alpha \)-TA, 427 μg/ml or 1 mM of \( \alpha \)-T, and 440.8 μg/ml or 0.93 mM of \( \alpha \)-TA stock solutions from which a series of standard solutions (for \( \alpha \)-T - 427.0, 170.8, 68.2, 42.7, 21.35, 8.54, 4.27, 2.13, 0.85, 0.43, 0.17, 0.085, 0.043, and 0.02 μg/ml, for \( \alpha \)-TA - 440.8, 220.4, 110.2, 55.1, 27.55, 11.02, 5.51, 2.76, 1.10, 0.55, 0.27 μg/ml) were prepared via serial dilution in the ethanol: tris.HCl buffer (20:80, v/v) co-solvent at pH 7.4 (viscosity of the solvent mixture was 1.6603 cP, and the refractive index was 1.3411 at temperature 32°C). Each measurement comprised 10-14 runs. Triplicates of each sample were assessed. The resultant KCps values were plotted against log drug concentration and the CAC was
determined by intersection of two linear models applied to data (method as per Cai et al, 2016). The α-TP nanoaggregate size was analysed at a concentration of 35 mM (above CAC) using the same method. The cuvette material refractive index was set at 1.59, the material absorbance at 0.01, the dispersant refractive index at 1.365. The duration of experiments ranged from 60 - 100 s during which the measurement position and attenuation was automatically optimised by the machine. The α-T concentrations in the PCS studies were not corrected to account for the 70% purity of this natural extract, because the impurities, i.e, lipids would be active and form liposomes and hence may affect the overall dynamic light scattering and CAC determination of α-T.

The aggregate size and polydispersity index of the α-TP was monitored to assess the short-term aggregate physical stability after incubation of 2% w/v α-TP for 0, 1, and 7 days in a water bath at 32° C.

2.3.3 Chemical stability

The chemical degradation rates of α-TP (10, 20, and 100 μg/mL) when dispersed in a 20% ethanol, 20% propylene glycol, 80% water at pH 7.4 (Tris buffer, 100 mM) vehicle was calculated over a week (7 days). Samples were stored at 37° C in clear HPLC snap neck vials (1 mL) and the concentration was assessed at 0, 1, 2, 3, and 7 days. The HPLC system was equilibrated (0.1 mL/ min flow rate) overnight and compared to α-TP calibrants at 5, 10, 20, 50 and 100 μg/mL. The HPLC method used to generate each chromatogram was as described in Section 2.3.5. Peak area was used to calculate the sample concentration with the linear gradient of the data time points being used to calculate the percent remained of α-TP at each time part compared to the original concentration at t=0.
2.3.4 Atomic force microscopy

Height images of the untreated mica, the vehicle (20% propylene glycol, 20% ethanol, 80% water, 100 mM tris at pH 7.4), the (+) α-T (3.5 mM) dispersed in the vehicle and the α-TP (0.9 mM) dispersed in the vehicle were obtained using an atomic force microscope (AFM; Bruker icon dimension, UK). Mica was chosen as a solid substrate on to which 2-3 drops of the test samples were placed; the samples were dried with nitrogen and then imaged. All images were obtained in tapping mode using high resonance frequency (HRF = 320 kHz) pyramidal cantilevers with uncoated Si₃N₄ tips having force constants of 46 N/m in air. Scan speeds were set at 0.9 Hz. Measurements were recorded using the NanoScope 1.50 AFM image analysis software (Bruker, USA) and were analysed using Gwyddion 2.45 software (freeware version; Czech Metrology Institute, Brno, Czech Republic). The α-T concentration in the AFM imaging studies was not corrected to account for the impurities of this natural product because the impurities, i.e., lipids would be active and form liposomes and hence may affect the overall size and shape of α-T aggregates.

2.3.5 Commercial tocopherol phosphate formulation

The simplest lotion formulation was based on the commercial α-TP product (Shawa Denko). It was prepared by weighing out 0.67 g of α-TP, 1.33 g of glycerine, and 1.0 g of propylene glycol and transferring them into a glass Biju bottle. Water (30 mL) was added to the mix and then it was sonicated for 30 min until the α-TP was completely dispersed. Methylparaben (0.07 g) was added as a preservative to get a final formulation (F1) with total weight of 33 g and pH of 8.5. F2 and F3 was prepared in the same way but replacing 1 mL or 2.33 mL water with 1% citric acid
in the formulation to reduce the pH to 7.9 and 6.8, respectively. F3 with pH 6.8 was chosen as a control in skin deposition study (see Section 2.3.7).

### 2.3.6 HPLC method development

The quantification of α-TP was performed using reversed phase high performance liquid chromatography (HPLC) consisting of 717 plus auto-sampler (Waters, Elstree, UK), a photodiode array ultra violet (UV) detector and a quaternary pump (Spectra Physics, UK). The detection wavelength for α-TP, α-T and α-TA were 287, 290, and 284 nm, respectively. The mobile phase comprised of isopropanol: water (85:15 v/v) and 0.1 % trifluoroacetic acid (TFA) set at a flow rate of 1 mL min\(^{-1}\) for α-TP and 1.2 mL min\(^{-1}\) for α-T and α-TA to optimise the retention time. The concentration of α-T in the samples, which were analysed in the solubility study, artificial membrane transport studies, and the skin deposition studies sections (section 2.4.1, 2.4.6, and 2.4.9, respectively), was determined using HPLC hence the concentration of the natural α-T was corrected based on its 70% purity because this analysis method separated and analysed only the pure α-T.

α-TP was separated using C18 stationary phase (Phenomenex 5 μm, 250 x 4.60 mm) at room temperature with a 50 μL injection volume. For the silicone membrane transport studies Miglyol oil was employed hence the ratio of the mobile phase was changed to isopropanol: water (90:10, v/v) to enhance the solubility of Miglyol oil in the mobile phase. In addition, a flow rate was set at 1 mL min\(^{-1}\) to optimise the compound retention. All the other parameters were kept the same. The peak area versus concentration (μg/mL) calibration curves were linear in the range of 10-100 μg/mL. System suitability was evaluated for α-TP using the data from three calibration runs performed on a single day (intraday) with the same set of standards,
followed by two further calibration runs completed on two separate days (inter-day) with a fresh set of standards on each day. The LOD and LOQ were calculated from the following equations 1 and 2, respectively:

\[
LOD = \frac{3.3 \sigma}{s} \quad \text{Equation 1.1}
\]
\[
LOQ = \frac{10 \sigma}{s} \quad \text{Equation 1.2}
\]

Where \( \sigma \) = the standard deviation of the response, \( S \) = the slope of the calibration curve. The \( S \) was determined from the calibration curve of \( \alpha \)-TP and the standard error of \( y \)-intercept was calculated using the LINEST function in Microsoft Excel. The chemical stability of one set of standard solutions (10, 20, 40, 50, 79, 80, and 100 \( \mu \)g/ml) of \( \alpha \)-TP dissolved in the mobile phase was assessed upon the storage in the fridge (< 8°C), at day 1 and day 40. The obtained peak area from the HPLC-UV was used to calculate sample concentration and used to calculate the percent remained of \( \alpha \)-TP.

**2.3.7 Artificial membrane transport studies**

Two types of barriers were employed for the transport studies a silicone membrane (0.12mm, GBUK healthcare, UK) and a cellulose ester (CE) dialysis membrane of three different specifications (MWCO 0.5-1, 20, 1000 KDa, Spectrum labs, Breda, The Netherlands, Europe). All the artificial membrane transport studies were carried out using static upright individually calibrated Franz diffusion cells (Fig 2.1), with average surface area of 2.22 ± 0.11 cm\(^2\) and average receptor compartment volume of 8.93 ± 0.35 mL. The barrier was cut, mounted and then sealed with parafilm between two chambers of the Franz glass diffusion cells, which
were loaded with a 13 mm magnetic stirrer in the receptor chamber to avoid the build-up of drug at the lower membrane interface. The Franz cell receptor chamber temperature was maintained at 37˚C by means of a water bath and this resulted in membrane surface temperature of 32˚C (in order to mimic the surface temperature of human skin). After equilibration, the Franz cells were inverted to check the membrane integrity and any leaking cells were resealed (previously published method in Cai et al., 2016). For the silicone membrane studies, the topically applied dose of each of α-T, α-TA, or α-TP was not matched in term of applied concentration, but matched in term of thermodynamic activity (a). A volume of 1 mL, i.e., an infinite dose of α-T (the pure oil, a=1), α-TA (the pure oil, a=1), and α-TP (1.7 mg/mL saturated solution in Miglyol fluid, a=1) was applied to the apical surface of the membrane fitted between the donor and the receiver compartment of the Franz diffusion cells. The applied dose of α-T was not corrected because the impurities may affect the thermodynamic activity of α-T product and thus may affect the transport of α-T across the artificial membrane. Miglyol oil (Cremer Oleo, Hamburg, Germany) was loaded into the receiver chamber and the cells were allowed to equilibrate at 37 ºC for 60 min. The donor chamber was covered with aluminium foil to protect from light and parafilm to prevent evaporation of the solution during the study. Aliquots of 1 mL were withdrawn using a plastic syringe, from the sampling arm of the Franz cell receiver compartment at 0.5, 1, 2, 3, 4, 5, and 24 h and replaced immediately with equal volume of the pre-warmed receiver fluid to keep the liquid volume in the receiver compartment constant. The samples were dissolved in the mobile phase then transferred to HPLC vials in order to assay the α-TA, α-T, and α-TP content at each time point. For cellulose ester membrane studies, the α-TP diffusion from 1 mL of 90% saturated solution across the
membrane was investigated using the same set of static upright Franz diffusion cells. Like the silicone studies, the cellulose ester membrane was fitted between the donor and the receiver compartment of the Franz diffusion cells and secured in place by parafilm. The receiver compartment was filled with ethanol/propylene glycol/Tris.HCl buffer (20:20:60, v/v/v) vehicle at 37 °C. The α-TP was dissolved in the same vehicle to prevent effects of solvent back diffusion. The Franz cell receiver compartment was sampled at 1, 2, 3, 4, 5, 6 and 24 h. Three MWCO, i.e., 0.5-1 kD, 20 kD, and 1000 kD were compared at pH 7.4 and 9, when modifying the pH of the donor solution, the pH of the receiver was also changed to minimise back diffusion. A total of 5 replicates of each experiment were performed.

For both the silicone and cellulose membrane studies, the cumulative amounts of drug (µg) penetrating per unit surface area of the membrane (cm²) were corrected for sample removal and plotted against time (h). The steady-state flux (Jss) was calculated from the slope of the linear portion of the curve (R²> 0.99), using at least 4 points with values above the assay limit of quantification (LOQ). All data was checked to ensure sink conditions, i.e., sample donor solution never fall < 90% of its initial concentration and the receiver solution never showed > 10% of its permeant drug concentration. All the reported data obeyed these sink conditions.
2.3.8 Skin deposition studies

The porcine skin studies used white adult porcine ears sourced from local abattoir (Ginger pig, UK). The pig ears were cleaned with deionized water prior to use. Any residual water on skin surface was removed by wiping with a clean tissue, visible residual hair was trimmed carefully, and skin was fresh or stored at -20°C for a maximum of 3 days prior to use. If the samples were frozen they were thawed carefully before use. The skin tissue was prepared from porcine ears that had been carefully pulled from the ear cartilage tissue. The subcutaneous fat was removed from the skin using forceps and a scalpel. The thickness of the tissue was 1.67±0.72 mm measured by a Starrett micrometer (The L. S. Starrett Company Ltd., Jedburgh, Scotland). The porcine skin was sealed between the donor and receiver chamber of the Franz cells and the cell sealing confirmed by cell inversion and monitoring solvent back diffusion. After 1 h equilibration at 37°C in the water bath an infinite dose of the test systems (matched in term of thermodynamic activity (a=1) and
Topically applied dose was used for the α-T pure oil, α-TP saturated solution. The volume of applied test systems were varied to give a dose of 8.7 ± 0.9 (range 7.5-9.2) mg/cm² when topically applied to the skin. The applied dose of α-T did not account for the impurities found in the natural α-T product because the impurities may affect the thermodynamic activity of α-T and hence may influence skin deposition of α-T. Transport was allowed to proceed for 3 h and samples were taken from the receiver fluid. At the end of the transport studies, the Franz cells were dismantled, and the donor compartment was washed with methanol. The skin was removed and cleaned with cotton buds (later extracted with methanol and retained for analysis) and mounted on a cork board. The SC of the skin was removed by tape stripping (ca. 22 strips until the skin was translucent) using adhesive tape (Scotch 845 book tape, 3M, Bracknell, UK), as reported by Primo et al., (2008), and the first two strips were considered as part of the applied formulation and hence its removal was part of the formulation wash off (Primo et al., 2008; Sheth et al., 1987). The tape strips were applied sequentially by pressing the adhesive tape onto the skin by applying defined pressure (500 mg weight) over a constant and reproducible time (10 s). Once the strips were removed they were collected together and active was extracted from the adhesive tape by immersing it in 100% MeOH (extraction was validated in preliminary work). The adhesive tape was removed from the solvent and discarded. The test agents were also extracted from the epidermis and dermis. The epidermis was separated from the dermis using a scalpel as previously reported in the literature (Diembeck et al., 1999). Both the epidermis and dermis were homogenized using 100% MeOH and left in contact with the extraction solution for 24 h. Samples were then centrifuged at 17000 rpm (Biofuge, Heraeus, Germany) for 15 min. The skin surface wash off solution, the SC
extraction fluid, the epidermis supernatant and dermis supernatant were analysed using HPLC. The total recovery efficacy was 98.15 ± 2.17%.

2.3.9 Statistical analysis

All the values were expressed as their mean ± standard deviation (SD). The homogeneity of variance (Levene’s test) and the normality (Shapiro-Wilk test) of all sample groups data were assessed prior to statistical analysis. The critical aggregate concentration (CAC) analysis was performed using OriginPro software (OriginPro version 8.6, OriginLab corporation, US). The statistical analysis was performed using statistical package of social sciences, SPSS version 17 (IBM Corp., USA) with a significance level of 0.05. Two-group comparison of mean was performed using Student’s independent t-test for data with equal variance or Mann-Witney’s test for non-equal variance. One-way analysis of variance (ANOVA) test for normally distributed data more than two groups’ comparison and non-parametric Kruskal-Wallis tests for non-normally distributed data. Post hoc comparisons of the means of individual groups were performed when appropriate using Tukey’s test or Dunnett’s test for equal variance normally distributed data, whereas or Games Howell test for unequal variance non-Gaussian distributed data. Data were presented using Prism software (GraphPad Prism, version 5.02, December 2008).

2.4 Results

2.4.1 Solubility studies

The solubility of α-TP in the co-solvent at 32 °C, was 0.21 ± 0.01 mg/mL, 0.48 ± 0.1 mg/mL, 20.56 ± 1.39 mg/mL and 36.45 ± 2.27 mg/mL at pH 3, 5.5, 7.4 and 9, respectively. The solubility significantly increased when increasing the pH above 6 (pka2) (p < 0.001 at pH 7.4 and p < 0.01 at pH 9, Games-Howell test, multiple
comparison one-way ANOVA, fig. 2.2). The α-T and α-TA oil showed minimal solubility 0.017 ± 0.01 mg/mL and 0.37 ± 0.02 mg/mL, respectively) compared to α-TP (p < 0.001, Games-Howell test, multiple comparison one-way ANOVA) in the tris/ethanol (80:20, v/v) co-solvent at pH 7.4 at 32°C.

### 2.4.2 Tocopherol phosphate aggregation

α-TP formed aggregates at a critical aggregate concentration (CAC) of 4.19 mM in the 20:20:60 % (v/v/v) (ethanol: propylene glycol: Tris buffer (100 mM)) vehicle at pH 7.4 (Fig 2.3a). No trend in the light scattering data was observed when the dispersion vehicle was at pH 9.0, which suggested that no aggregation occurred over the tested concentration range (Fig 2.3.b). The aggregation of α-T could not be measured accurately in 20:20:60 % (v/v/v) (ethanol: propylene glycol: Tris buffer (100 mM)) vehicle due to its low solubility in the vehicle which meant the aggregates were very few. The CAC of 1 mM α-T and 0.93 mM α-TA in the ethanol/Tris buffer (20:80, v/v) co-solvent at pH 7.4 (32°C) was ~ 11.38 µM and 10.60 µM, respectively (Fig 2.4).
Fig. 2: The aqueous solubility and di-anionic form percentage of α-TP in 20:20:60 % v/v/v ethanol: propylene glycol: Tris.HCl buffer at different pHs at 32 °C. Data represent the mean ± standard deviation (n=3). Image shows the ionisation of α-tocopherol phosphate.
Fig. 2.3: Plot of light scattering derived count rate vs log concentration of α-TP in the aqueous vehicle at pH 7.4 (a) and 9 (b) (32°C). CAC refers to critical aggregation concentration, note that at pH 9 there was no aggregation. Data represent the mean ± standard deviation (n=3).
Fig.2.4: Plot of light scattering of derived count rate versus log concentration of α-T (a), and α-TA (b) in ethanol: tris buffer (20:80, v/v) at pH 7.4 (32°C). CAC refers to critical aggregation concentration. Data represent the mean ± standard deviation (n=3).
2.4.3 Tocopherol phosphate aggregate size and physical stability

The size of the α-TP aggregates using a concentration of 2% (35 mM) α-TP in a 20% ethanol, 20% propylene glycol, 60% water mixture at pH 7.4 (100 mM Tris.HCl buffer) was 9.36 ± 1.59 nm using DLS (n=3) (Fig. 2.5a). The AFM images showed α-TP at 0.9 mM concentration formed spherical vesicles/liposomes with diameters of 51.67 ± 4.51 nm and height of 17 ± 0.1 nm (n=3) (Fig. 2.6 a,b) and at higher concentration (6.3 mM) it formed rods with diameters 306.7 ± 35.1 nm to 571.3 ± 28.9 nm and height of 10 nm (Fig. 2.6 c,d). The size of the α-T aggregates using a concentration of 1 mM α-T in a 20% ethanol, 20% propylene glycol, 60% water mixture at pH 7.4 (100 mM Tris.HCl buffer) was 205.15 ± 21.28 nm using DLS (n=3) (Fig. 2.5 b) and diameters of 373.30 ± 28.87 (n=3) and height of 60 nm using AFM (Fig. 2.7a, b). The aggregates appeared to be spherical for both α-TP and α-T. The α-TP liposomes characteristics, the hydrodynamic size and PDI, remained fairly unchanged (table 2.1), after a short-term (7 days) storage at 32°C.

Fig.2.5: Hydrodynamic size of 35 mM α-TP (a) and 1 mM α-T (b) in the in 20% ethanol, 20% propylene glycol, 60% water at pH 7.4 (100 mM tris.HCl) using dynamic light scattering. Data represent mean ± standard deviation (n=3).
Fig. 2.6: Atomic force microscopy tapping height images (left), cross-section profile (right) of α-TP at concentrations of 0.9 mM (a,b), 6.3 mM (c,d,e, f) in tris formulation at pH 7.4.
Fig. 2.7: Atomic force microscopy tapping height images (left), cross-section profile (right) of 3.5 mM α-T in tris formulation at pH 7.4.

Table 2.1: The short-term stability of tocopherol phosphate aggregate size at 35 mM concentration in 20% ethanol, 20% propylene glycol, 60% water at pH 7.4 (32°C), at day 0, day 1, and day 7.

<table>
<thead>
<tr>
<th>Duration</th>
<th>Size (nm)</th>
<th>Change in the size</th>
<th>PDI</th>
<th>Change in the PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>9.36±1.59</td>
<td>-</td>
<td>0.27 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td>Day 1</td>
<td>12.66±2.57</td>
<td>3.30 nm</td>
<td>0.20 ± 0.05</td>
<td>-0.07</td>
</tr>
<tr>
<td>Day 7</td>
<td>12.95±0.77</td>
<td>3.60 nm</td>
<td>0.34 ± 0.03</td>
<td>0.07</td>
</tr>
</tbody>
</table>

2.4.4 HPLC method verification

The HPLC quantitative method for the vitamin E derivatives was verified as ‘fit-for-purpose’ based on LOD and LOQ, assay precision (< 2.18%), and the calibration linearity (> 0.9999), prior to conducting the transport study. The maximum UV absorbance for α-TP in the mobile phase was 287 nm. Using this wavelength, the α-TP peak was eluted in the HPLC chromatogram (Fig. 2.8 a) with a retention time 7.94 ± 0.06 min (n=42). The LOD and LOQ were calculated statistically from the data derived from five calibration curves (Fig. 2.9) and found to be 1.13 ± 0.32 and 3.42 ± 0.97 µg/ml, respectively. Three calibration curves were
run on a single day to determine the HPLC assay’s intraday precision (Table 2.2, 2.3, and 2.4). Two additional calibration curves obtained from two separate days were employed to determine the inter-day precision (Table 2.5 and 2.6). A summary of this intra and inter-day variability (Table 2.7) demonstrate that the coefficient of variance (CV%) for both data sets was < 4%. The chemical stability of standard solutions of α-TP, which were prepared and stored in the fridge for up to 40 days showed that α-TP in mobile phase is highly stable with minimum degradation and after 40 days i.e. α-TP retained 96.6-100% of its initial concentration for the 10-100 μg/mL solutions indicating standard solutions can be stored in the fridge without influencing the stability of α-TP thus not influencing the HPLC quantitative experiments results (table 2.8). Figure 2.8 b and c show the retention time for α-T (ca. 8.5 min) and α-TA (ca. 11 min), respectively. The Linearity, limit of detection, limit of quantitation, of α-TP, α-T and α-TA are shown in table 2.9, 2.10 and 2.11, respectively.
Fig. 2.8: Sample chromatogram of 100 µg/mL  α-TP (a), α-T (b), α-TA (c). The flow rate was 1, 1.2 mL.min\(^{-1}\), respectively, using 80% isopropanol, 20% water, and 1% trifluoroacetic acid mobile phase.
Fig. 2.9: Summary of the five calibration curves for α-TP. Data represent the mean ± standard deviation (n=5).

Table 2.2: Day 1 (intra-day) α-TP calibration curve 1. The peak area data represents mean ± standard deviation (n=6), CV refers to coefficient of variance.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Retention time (min)</th>
<th>Peak area (µV.S)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.57</td>
<td>1045402 ± 14659</td>
<td>1.40</td>
</tr>
<tr>
<td>80</td>
<td>7.57</td>
<td>827046 ± 2396</td>
<td>0.29</td>
</tr>
<tr>
<td>70</td>
<td>7.55</td>
<td>708347 ± 64956</td>
<td>0.92</td>
</tr>
<tr>
<td>50</td>
<td>7.58</td>
<td>511078 ± 10602</td>
<td>2.07</td>
</tr>
<tr>
<td>40</td>
<td>7.59</td>
<td>405832 ± 3859</td>
<td>0.95</td>
</tr>
<tr>
<td>20</td>
<td>7.59</td>
<td>202546 ± 941</td>
<td>0.46</td>
</tr>
<tr>
<td>10</td>
<td>7.60</td>
<td>107904 ± 2630</td>
<td>2.44</td>
</tr>
</tbody>
</table>
Table 2.3: Day 1 (intra-day) α-TP calibration curve 2. The peak area data represents mean ± standard deviation (n=6), CV refers to coefficient of variance.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Retention time (min)</th>
<th>Peak area (µV.S)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.60</td>
<td>1047804 ± 18902</td>
<td>1.80</td>
</tr>
<tr>
<td>80</td>
<td>7.62</td>
<td>812548 ± 6947</td>
<td>0.85</td>
</tr>
<tr>
<td>70</td>
<td>7.62</td>
<td>723200 ± 6536</td>
<td>0.90</td>
</tr>
<tr>
<td>50</td>
<td>7.60</td>
<td>510599 ± 5344</td>
<td>1.05</td>
</tr>
<tr>
<td>40</td>
<td>7.60</td>
<td>406110 ± 3600</td>
<td>0.89</td>
</tr>
<tr>
<td>20</td>
<td>7.62</td>
<td>203262 ± 2987</td>
<td>1.47</td>
</tr>
<tr>
<td>10</td>
<td>7.63</td>
<td>108385 ± 2050</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Table 2.4: Day 1 (intra-day) α-TP calibration curve 3. The peak area data represents mean ± standard deviation (n=6), CV refers to coefficient of variance.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Retention time (min)</th>
<th>Peak area (µV.S)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.56</td>
<td>1050757 ± 6791</td>
<td>0.65</td>
</tr>
<tr>
<td>80</td>
<td>7.56</td>
<td>825875 ± 10988</td>
<td>1.33</td>
</tr>
<tr>
<td>70</td>
<td>7.56</td>
<td>725122 ± 9978</td>
<td>1.38</td>
</tr>
<tr>
<td>50</td>
<td>7.54</td>
<td>515182 ± 3530</td>
<td>0.69</td>
</tr>
<tr>
<td>40</td>
<td>7.56</td>
<td>407794 ± 4516</td>
<td>1.11</td>
</tr>
<tr>
<td>20</td>
<td>7.55</td>
<td>203151 ± 3813</td>
<td>1.88</td>
</tr>
<tr>
<td>10</td>
<td>7.56</td>
<td>107439 ± 2677</td>
<td>2.49</td>
</tr>
</tbody>
</table>
Table 2.5: Day 2 (inter-day) α-TP calibration curve 4. The peak area data represents mean ± standard deviation (n=6), CV refers to coefficient of variance.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Retention time (min)</th>
<th>Peak area (µV.S)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.80</td>
<td>1006141 ± 18864</td>
<td>1.87</td>
</tr>
<tr>
<td>80</td>
<td>7.73</td>
<td>781962 ± 11136</td>
<td>1.42</td>
</tr>
<tr>
<td>70</td>
<td>7.72</td>
<td>694709 ± 6733</td>
<td>0.97</td>
</tr>
<tr>
<td>50</td>
<td>7.75</td>
<td>499028 ± 7612</td>
<td>1.53</td>
</tr>
<tr>
<td>40</td>
<td>7.73</td>
<td>397736 ± 5939</td>
<td>1.49</td>
</tr>
<tr>
<td>20</td>
<td>7.75</td>
<td>193692 ± 1276</td>
<td>0.66</td>
</tr>
<tr>
<td>10</td>
<td>7.59</td>
<td>101879 ± 1473</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Table 2.6: Day 3 (inter-day) α-TP calibration curve 5. The peak area data represents mean ± standard deviation (n=6), CV refers to coefficient of variance.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Retention time (min)</th>
<th>Area (µV.S)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.71</td>
<td>1015961 ± 3979</td>
<td>0.39</td>
</tr>
<tr>
<td>80</td>
<td>7.70</td>
<td>805466 ± 3954</td>
<td>0.49</td>
</tr>
<tr>
<td>70</td>
<td>7.71</td>
<td>699907 ± 5625</td>
<td>0.80</td>
</tr>
<tr>
<td>50</td>
<td>7.70</td>
<td>492246 ± 4008</td>
<td>0.81</td>
</tr>
<tr>
<td>40</td>
<td>7.74</td>
<td>405494 ± 3451</td>
<td>0.85</td>
</tr>
<tr>
<td>20</td>
<td>7.79</td>
<td>191123 ± 3441</td>
<td>1.80</td>
</tr>
<tr>
<td>10</td>
<td>7.85</td>
<td>102462 ± 2348</td>
<td>2.29</td>
</tr>
</tbody>
</table>
Table 2.7: Intra-day and inter-day variability for α-TP. CV refers to coefficient of variance. The data represents mean ± standard deviation (n=18).

<table>
<thead>
<tr>
<th>Concentrations (µg/ml)</th>
<th>Intra-day assay CV% (n=18)</th>
<th>Inter-day assay CV% (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.30</td>
<td>2.11</td>
</tr>
<tr>
<td>80</td>
<td>1.20</td>
<td>2.49</td>
</tr>
<tr>
<td>70</td>
<td>1.48</td>
<td>1.18</td>
</tr>
<tr>
<td>50</td>
<td>1.37</td>
<td>2.18</td>
</tr>
<tr>
<td>40</td>
<td>0.95</td>
<td>1.43</td>
</tr>
<tr>
<td>20</td>
<td>1.33</td>
<td>2.78</td>
</tr>
<tr>
<td>10</td>
<td>2.18</td>
<td>3.34</td>
</tr>
</tbody>
</table>

Table 2.8: Stability of α-TP standards in mobile phase, assessed by comparison of average peak area at day 1 and day 40, when stored at < 8°C.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Concentration at day 1 (n=18)</th>
<th>Concentration at day 40 (n=2)</th>
<th>% Remained</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>11.22±0.85</td>
<td>11.23 ± 0.94</td>
<td>100%</td>
</tr>
<tr>
<td>20</td>
<td>20.57±0.88</td>
<td>19.87 ± 0.80</td>
<td>96.60%</td>
</tr>
<tr>
<td>40</td>
<td>40.58±0.90</td>
<td>40.07 ± 0.68</td>
<td>98.75%</td>
</tr>
<tr>
<td>50</td>
<td>50.98±1.31</td>
<td>50.20 ± 1.53</td>
<td>98.48%</td>
</tr>
<tr>
<td>70</td>
<td>71.28±1.67</td>
<td>69.02 ± 1.11</td>
<td>96.83%</td>
</tr>
<tr>
<td>80</td>
<td>81.40±1.59</td>
<td>80.74 ± 0.82</td>
<td>99.19%</td>
</tr>
<tr>
<td>100</td>
<td>103.63±1.96</td>
<td>101.61 ± 0.86</td>
<td>98.04%</td>
</tr>
</tbody>
</table>
Table 2.9: Linearity, limit of detection, limit of quantitation, and inter-day and intraday variability of α-TP. The data represents mean ± standard deviation (n=5).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>α-TP</th>
<th>ICH recommended value (ICH 1995)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity ($r^2$)</td>
<td>$r^2 &gt; 0.999$</td>
<td>$r^2 &gt; 0.99$</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>1.13 ± 0.32</td>
<td>-</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>3.42 ± 0.97</td>
<td>-</td>
</tr>
<tr>
<td>Intra-day variability</td>
<td>1.40 %</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Inter-day variability</td>
<td>2.22 %</td>
<td>&lt; 5%</td>
</tr>
</tbody>
</table>

Table 2.10: Linearity, limit of detection, limit of quantitation, of α-T.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>α-T</th>
<th>ICH recommended value (ICH 1995)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity ($r^2$)</td>
<td>$r^2 &gt; 0.996$</td>
<td>$r^2 &gt; 0.99$</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>5.73</td>
<td>-</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>17.36</td>
<td>-</td>
</tr>
<tr>
<td>Intra-day variability</td>
<td>--</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Inter-day variability</td>
<td>--</td>
<td>&lt; 5%</td>
</tr>
</tbody>
</table>
Table 2.11: Linearity, limit of detection, limit of quantitation, of α-TA.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>α-TA</th>
<th>ICH recommended value (ICH 1995)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity ($r^2$)</td>
<td>$r^2 &gt; 0.999$</td>
<td>$r^2 &gt; 0.99$</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>1.06</td>
<td>-</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>3.21</td>
<td>-</td>
</tr>
<tr>
<td>Intra-day variability</td>
<td>--</td>
<td>&lt; 1%</td>
</tr>
<tr>
<td>Inter-day variability</td>
<td>--</td>
<td>&lt; 5%</td>
</tr>
</tbody>
</table>

2.4.5 Chemical stability

The short-term chemical study (Table 2.12) demonstrated that α-TP in the aqueous vehicle (20% ethanol, 20% propylene glycol, 60% water at pH 7.4 (100 mM tris.Hcl buffer) at 32° C, is at concentration 10, 20, and 100 µg/mL was degraded by 0.83, 1.46, and 2.26 µg/mL over a week, *i.e.*, retained 87.2 %, 92.4%, and 97.7% of initial concentration, respectively. The chemical degradation did not influence the transport or skin deposition studies’ results as the studies were running for maximum of 2 days (48 h), but the chemical stability was probably important for subsequent formulation development.
Table 2.12: Chemical stability study of 10, 20, and 100 µg/mL α-TP in the co-solvent at 32°C, at day 0, 1, 2, 3, and 7 days. The data represents mean ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>Duration</th>
<th>Change in the concentration (µg/mL)</th>
<th>% Remained</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/mL</td>
<td>Day 0: 10.71 ± 0.53</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Day 1: 10.56 ± 0.07</td>
<td>98.60%</td>
</tr>
<tr>
<td></td>
<td>Day 2: 10.33 ± 0.07</td>
<td>96.50%</td>
</tr>
<tr>
<td></td>
<td>Day 3: 10.17 ± 0.08</td>
<td>94.96%</td>
</tr>
<tr>
<td></td>
<td>Day 7: 9.34 ± 0.01</td>
<td>87.20%</td>
</tr>
<tr>
<td>20 µg/mL</td>
<td>Day 0: 19.24±0.06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Day 1: 18.63±0.03</td>
<td>96.68%</td>
</tr>
<tr>
<td></td>
<td>Day 2: 18.63±0.01</td>
<td>96.68%</td>
</tr>
<tr>
<td></td>
<td>Day 3: 18.50±0.07</td>
<td>96.15%</td>
</tr>
<tr>
<td></td>
<td>Day 7: 17.78±0.001</td>
<td>92.41%</td>
</tr>
<tr>
<td>100 µg/mL</td>
<td>Day 0: 98.19 ± 0.65</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Day 1: 98.44 ± 0.15</td>
<td>100.25%</td>
</tr>
<tr>
<td></td>
<td>Day 2: 98.42 ± 0.07</td>
<td>100.20%</td>
</tr>
<tr>
<td></td>
<td>Day 3: 98.80 ± 0.15</td>
<td>100.60%</td>
</tr>
<tr>
<td></td>
<td>Day 7: 95.93 ± 0.04</td>
<td>97.70%</td>
</tr>
</tbody>
</table>

2.4.6 Silicone membrane transport studies

The flux of α-TA through silicone membrane was statistically higher than α-T (3,278.87 ± 223.48 vs 1,513.03 ± 97.14 µg.cm⁻².h⁻¹, p < 0.01, Mann-Witney’s test) (Fig 2.10 a, b). The 24 h cumulative amount of α-TA that penetrated the membrane was significantly higher than α-T (43,465.27 ± 8,170.76 vs 20,857.16 ± 2,703.65
μg.cm$^{-2}$, $p < 0.01$, Mann-Witney’s test). α-TP could not be detected in the receiver fluid of the Franz cells, which suggested that it could not pass through the silicone membrane.

![Graph showing permeation](image)

**Table 2.4.7:** Permeation of α-TA, α-T and α-TP across a silicone membrane (a). Flux and the 24 h cumulative amount calculated from the permeation profile (b). Data represent mean ± standard deviation (n=5).

<table>
<thead>
<tr>
<th>Infinite dose</th>
<th>Flux (μg.cm$^{-2}$.h$^{-1}$)</th>
<th>24 h cumulative amount (μg.cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-TA oil</td>
<td>3,278.87 ± 223.48**</td>
<td>43,465.27 ± 8,170.76*</td>
</tr>
<tr>
<td>α-T oil</td>
<td>1,513.03 ± 97.14</td>
<td>20,857.16 ± 2,703.65</td>
</tr>
<tr>
<td>α-TP in Miglyol oil</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

**Fig.2.10:** Permeation of α-TA, α-T and α-TP across a silicone membrane (a). Flux and the 24 h cumulative amount calculated from the permeation profile (b). Data represent mean ± standard deviation (n=5).

### 2.4.7 Cellulose ester membrane permeation

The transport of α-TP at pH 9 through the cellulose ester membrane was faster compared to that at pH 7.4 for each of the three different MWCO grades (Fig. 2.11.
a,b,c) (0.5-1 kDa- $p < 0.01$, Mann-Whitney nonparametric test, 20 kDa- $p < 0.001$, 1000 kDa- $p < 0.001$, Student’s independent t-test) (Table 2.13). The 1000 kDa MWCO provided the fastest release and the best reproducibility whereas the 0.5-1 kDa MWCO did not let the α-TP at pH 7.4. The flux lag time ($t_{lag}$) of α-TP across 20 kDa MWCO cellulose membrane was significantly reduced at pH 9 by 4-fold compared to that at pH 7.4 (table 2.14). This indicated that α-TP at pH 9 is less aggregated and have less restriction to passively diffuse through the 20 kDa MWCO cellulose membrane compared to pH 7.4.
Fig.2.11: Permeation of 90% saturated solution of α-TP across three cellulose ester membranes a) 0.5-1 kDa, b) 20 kDa, and c) 1000 kDa. Data represent the mean ± standard deviation (n=5).
Table 2.13: Flux at steady state of 90% saturated solution α-TP at pH 7.4 and 9 across three cellulose ester membranes (mean of n=5 ± standard deviation).
b) The change in flux upon increasing the pH was statistically significant for the cellulose ester membranes (0.5-1 kDa- \( p < 0.01 \), Mann-Whitney’s test, 20 kDa- \( p\)-value < 0.001, 1000 kDa- \( p < 0.001 \), Student’s independent t-test). Data represent the mean ± standard deviation (n=5).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cellulose ester membrane MWCO (kDa)</th>
<th>Flux (( \mu g/cm^2.h ))</th>
<th>0.5 – 1 kDa</th>
<th>20 kDa</th>
<th>1000 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-TP at pH 7.4</td>
<td>Not detected</td>
<td>6.20 ± 7.50</td>
<td>329.19 ± 62.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-TP at pH 9</td>
<td>8.80 ± 13.74</td>
<td>37.27 ± 1.16</td>
<td>1577.98 ± 327.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( p)-value</td>
<td>( p &lt; 0.01 )</td>
<td>( p &lt; 0.001 )</td>
<td>( p &lt; 0.001 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.14: Flux lag time (\( t_{lag} \)) of 90% saturated solution α-TP at pH 7.4 and 9 across three cellulose ester membranes (mean of n=5 ± standard deviation).
The change in flux lag time (\( t_{lag} \)) upon increasing pH was statistically significant for cellulose ester membranes with MWCO 20 kDa (\( p < 0.05 \), Mann-Whitney’s test) but not with not 0.5-1kDa or 1000 kDa (\( p > 0.05 \) Mann-Whitney’s test). Data represent the mean ± standard deviation (n=5).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cellulose ester membrane MWCO (kDa)</th>
<th>Flux ( t_{lag} ) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-TP at pH 7.4</td>
<td>Not detected</td>
<td>36.20 ± 27.20</td>
</tr>
<tr>
<td>α-TP at pH 9</td>
<td>59.84 ± 47.01</td>
<td>9.32 ± 9.98</td>
</tr>
<tr>
<td>( p)-value</td>
<td>( N/A )</td>
<td>( p &lt; 0.05 )</td>
</tr>
</tbody>
</table>

2.4.8 Commercial α-tocopherol phosphate

The commercial lotions of α-TP F1/F2/F3 were prepared and modified from the manufacturer formulation Lotion VEL 09-001/09-003/09-004 (Shawa Denko) at pH 8.5, 7.9, and 6.8, respectively (see fig. 2.12). These formulations only differed in the content of citric acid, which reduces the pH and causes the turbid appearance.

The lotion (F3) with pH 6.8 was used as a control in the skin deposition study.
Fig. 2.12: a) The commercial lotions of α-TP F1/F2/F3 at pH 8.5, 7.9, and 6.8, respectively. The turbidity increases when reducing the pH below 8.5 at room temperature. b) The list of ingredient percentage for each formulation which differ in the content of citric acid.

### Skin deposition studies

Skin deposition studies demonstrated that for α-TP at pH 7.4, 92% of the applied formulation remained on skin surface, 3.8% deposited in SC, 1.2% deposited in epidermis, 0.34% deposited in dermis. The amounts deposited in the SC and epidermis was significantly higher compared to α-T (505.98 ± 295.93 μg vs 41.46 ± 3.97 μg, p < 0.05 and 170.10 ± 28.67 μg vs 26.87 ± 3.09 μg, p < 0.001, post-hoc turkey’s test, one-way ANOVA, respectively). The α-TP at pH 7.4 and α-T deposition in the dermis were not statistically different (Fig. 2.13.b). α-TP deposition was comparable in the epidermis and dermis when administered in pH 7.4 (aggregated form) and 9 (non-aggregated form) (pH 7.4), but both formulations
(pH 7.4 and 9) showed a higher deposition in the epidermis compared to commercial formulation (pH=6.8) \( p < 0.001 \), post-hoc turkey’s test, one-way ANOVA) (Fig. 2.13.a). \( \alpha \)-TP at pH 7.4 showed higher deposition in SC than pH 9 and the commercial formulation \( p < 0.05 \), post-hoc turkey’s test, one-way ANOVA).
2.5 Discussion

α-TP was used in this work in its phosphate disodium salt form (MW: 554.65, pka₁: 1.8 and pka₂: 6.74), but it was assumed that this amphiphilic molecule can rapidly dissociate in water, from the sodium counter ions to produce either the non-ionic, monoionic or dianionic forms, depending on the pH. The solubility of α-TP in the 20% ethanol, 20% propylene glycol, 60% tris.HCl buffer (v/v/v) at 32° C dramatically increased when the pH rose above 6.7 (pKa₂), presumably due to an increase in the percentage of the ionised di-anionic form of the α-TP molecule, which was the most water soluble species. When the α-TP was exclusively (~100%) in the dianionic form, there appeared to be no molecular aggregation at the concentrations tested in this work, which was probably due to the extensive interactions between α-TP and the solvent. When the α-TP was partially (~15%) in its mono-anionic form (i.e., pH 7.4), the aggregation occurred in the millimolar concentration (4.19 mM). This finding was higher, compared to a previously published value for (+) α-TP (CAC- 5.5 ± 0.2 μM) in 20% ethanol, 80% water at pH 7.4 (25 mM Trizma®) at 20 °C (Harper et al., 2018), but similar to dexamethasone phosphate, which was previously found to have a CAC of 3.4 mM at 25°C in aqueous solution (Shah et al., 2009). This data demonstrated that the physical attributes of this α-TP were very sensitive to the dispersion vehicle properties and conditions, such as temperature. The propylene glycol addition at low concentration (20%) seemed to cause a minor change to the vehicle, but this additive induced superstructures to form (Benaouda et al., 2016). The α-TP would be more soluble in the water-ethanol solvent after adding the propylene glycol, and hence this would increase the CAC value. The α-T and α-TA were found to aggregate at lower concentrations (CAC- 11.38 and 10.6 μM, respectively).
presumably due to their higher lipophilicity. Since the concentration of α-TP that is topically applied to the skin is approximately 36 mM (20 mg/ml), it is likely that the aggregation reported in this work is consequential to its use in pharmaceutical and cosmetic products (Kato et al., 2011; Nakayama et al., 2003).

Introducing a phosphate group to α-T when forming α-TP, generated a 3-fold larger ionisable polar surface area (91°A) compared to α-T, which has a smaller unionized polar surface (29.5°A). This change in the polar surface area was probably, in part, responsible for the manner α-TP self-assembled in aqueous solution. The negatively charged phosphate group increased the repulsion between α-TP aggregate and hence reduced the size of aggregate compared to α-T. It was found by DLS that α-TP in 20% ethanol, 20% propylene glycol, 60% water at pH 7.4 (100 mM Tris.HCl buffer) formed small nanostructures at higher concentrations than α-T. The AFM images obtained by tapping mode confirmed that α-TP aggregates in Tris buffer were also smaller than α-T aggregates, and both were spherical in shape. The size of the α-TP aggregates, obtained from the AFM appeared to be greater than those obtained by DLS, due to the assembly of a couple of micelles and the absence of propylene glycol from the vehicle, which causes the increase in α-TP aggregate size. A previous study reported that 1% w/w α-TP in 30% ethanol, and 80% water had a hydrodynamic size of 100 nm, and when imaged by cryo-TEM, formed predominantly unilamellar vesicles with small proportion of tubular micelle structures (Gavin et al., 2017).

The α-TP liposomes were found to be physically stable over 7 days, the size and PDI increased by 3 nm and 0.07, respectively. In a previous study, which assessed the short-term physical stability of an o/w microemulsion containing 1% phytosphingosine (ME-Wphy) and 1.1% w/w α-T, found that after 7 days, the initial
hydrodynamic size and PDI increased by 2 nm and 0.02, respectively (Cichewicz et al., 2013). In another study conducted by Harper et al., it was also found that (+)α-TP at 1.5 mM concentration, was physically stable over 8 days, where the size and PDI remained relatively consistent (Harper et al., 2018).

The HPLC short-term chemical stability showed that 100 µg/mL of α-TP, dissolved in aqueous vehicle, degraded by 2.26 µg/mL over a week, i.e., retained most of its initial concentration. However, at 20 µg/mL and 10 µg/mL, it lost 7.6% and 12.8% of its initial concentration. This meant that the 100 µg/ml α-TP was considered chemically stable since the remaining concentration was within 97.7-100.6% of the initial concentration. In a study conducted by Ang and co-workers, it was found that 10 mg/mL of apomorphine, retained ca. 100% of initial concentration while at a low concentration (50 µg/mL), when present in the buffer or without the buffer it lost 80% and 40%, respectively of its initial concentration (Ang et al., 2016). This also agreed with the findings of Harper and colleagues, who showed that 20 µg/mL α-TP stored in 37°C, exhibited a degradation rate of 1.2 µg/mL/week and agreed with the industrial chemical degradation tolerance (10% degradation of a 500 µg/mL solution over 12 week at 40° C) (Harper, 2017; Harper et al., 2018).

The porous synthetic cellulose ester membrane was used in this work as an artificial skin model which eliminates the complexity of human skin. It is recommended by the FDA for the evaluation of topical aqueous formulations. This model was employed to understand the mechanistic of passive transport of α-TP across a membrane. The diffusion of a 90% saturated solution of α-TP nanostructures across the 20 KDa and 1000 KDa cellulose ester membrane at pH 9 (which has no molecular aggregation) was 6-fold and 5-fold higher than pH 7.4, respectively. This may indicate that the membrane penetration of α-TP in the aggregated form was
substantially slower than in the non-aggregated form. This is mainly due to the superstructures of α-TP aggregates formed at pH 7.4. Their extensive intermolecular bonding was thought to have a greater restriction on diffusion than α-TP free molecules. This is a consequence of increased size which results in difficulty in passing through and leaving the barrier. This agrees with Inacio et al who showed that the larger tetracaine aggregates delayed membrane penetration compared to non-aggregated tetracaine (Inacio et al., 2016).

The silicone membrane was thought to mimic SC and was used previously as a simple artificial skin model, but it lacks the lipid lamellae that are found in the SC, which represent a major route for many cosmetic actives. Silicone membrane allows the permeation of lipophilic compounds, but this model may act as a barrier for other amphiphilic, relatively large compounds, limiting their permeation. α-TP did not permeate the non-porous silicone membrane, and this suggested that α-TP had a poor affinity to hydrophobic barriers. It is accepted that the silicone membrane studies used Miglyol oil as the delivery vehicle, and this could have helped the α-T and α-TA pass this barrier (Moddaresi et al., 2010). However, it was apparent from the visual examination that the synthetic barrier, that it remained intact during the time course of the silicone membrane permeation studies; no unusual thickening of the barrier or back diffusion of Miglyol oil into the donor compartment was observed. Hence, if Miglyol oil had an influence on the transport, it was minor. α-TP did show an applicable solubility in the Miglyol (1.7 mg/mL) and the general unfavorability of the α-TP to the silicone was thought to be valid.

The *ex vivo* skin permeation studies were performed using porcine skin, which histologically resembles human skin. Porcine skin shows a comparable SC thickness of 21-26 µM to human skin, an average of hair follicle density is 20/cm².
close to 14-32/cm² in human forehead skin, and they contain the same classes of SC lipids with similar lateral packing. The porcine skin is widely used for skin-permeation studies and it was used in this work because it was easy to obtain, whereas human skin requires ethical approval and donor consent forms (Abd et al., 2016; Barbero and Frasch, 2009). The skin deposition studies demonstrated that the total skin penetration of α-TP at pH 7.4 was 5-fold higher than α-T, i.e., 5.34% vs 1.17% of the applied dose that permeated the whole skin. The amount of α-TP deposited in the SC and the epidermis was found to be 12.2-fold and 6.3-fold higher than α-T (505 μg vs 41 μg and 170 μg vs 27 μg, respectively). These results with α-TP mirror previous work, which used the hydrophilic pro-vitamin E with amino acid conjugates dissolved in 50% ethanol, 10% propylene glycol, 40% water. The control treatment of α-TA or α-T in 1% isopropyl myristate, topically applied as an infinite dose (0.7 mg/mL), resulted in a 3.8-fold greater α-TA epidermal deposition than α-T at 4 h (15 vs 4 nmole/cm²). Moreover, the alanine, glycine and pyroglytamic acid conjugates showed 15-fold (60 nmole/cm²), 5-fold (20 nmole/cm²) and 2.5-fold (10 nmole/cm²) higher epidermal depositions over α-T (4 nmole/cm²) at 4 h but all the conjugates had similar accumulation in the dermis (Ostacolo et al., 2004). Mavon et al, showed that topical application of an infinite dose (100 μg/cm²) of tocopherol glycoside in myritol solution on human skin at 18 h resulted in 0.8, 0.35, and 0.16 μg/cm² deposition in SC, epidermis and dermis, respectively, i.e, 0.8%, 0.4%, 0.2% of the applied dose deposited in each of the layers, respectively (total skin deposition in whole skin was 1.4%) (Mavon et al., 2004). They also showed that α-TA was deposited in the SC, epidermis and dermis (3, 0.8, 0.8 μg/cm², respectively). Hence, other pro-drugs of α-T were less effective than α-TP in term of skin deposition (table 2.14). This superior skin deposition of
α-TP indicated that the phosphate group present resulted in nanostructure forms of α-TP and provides a more effective delivery into the deeper skin layers, compared with other α-T conjugates.

Table 2.15: Average amount deposition of α-TP in skin layers compared to other prodrug in the literature.

<table>
<thead>
<tr>
<th>Type of prodrug/control</th>
<th>Average amount deposited in skin layer (μg/cm²)</th>
<th>Skin source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC</td>
<td>Epidermis</td>
<td>Dermis</td>
</tr>
<tr>
<td>α-TP</td>
<td>252.2</td>
<td>85.1</td>
<td>20.29</td>
</tr>
<tr>
<td>α-T</td>
<td>20.7</td>
<td>13.4</td>
<td>1.95</td>
</tr>
<tr>
<td>δ-TG</td>
<td>0.8</td>
<td>0.35</td>
<td>0.16</td>
</tr>
<tr>
<td>α-T Alanine</td>
<td>-</td>
<td>29.27</td>
<td>9.76</td>
</tr>
<tr>
<td>α-T glycine</td>
<td>-</td>
<td>10.06</td>
<td>10.06</td>
</tr>
<tr>
<td>α-T pyroglytamic acid</td>
<td>-</td>
<td>5.62</td>
<td>2.71</td>
</tr>
<tr>
<td>α-TA</td>
<td>-</td>
<td>7.56</td>
<td>6.62</td>
</tr>
</tbody>
</table>

The epidermal and dermal skin deposition from α-TP, when topically applying a dose of 8.7 ± 0.9 mg/cm² at pH 7.4 (to generate the α-TP in the aggregated form) and α-TP at pH 9 (α-TP in a non-aggregated form), showed no differences in skin deposition. These results indicated that the α-TP aggregates and the non-aggregated material contributed to the skin penetration. However, the cellulose ester membrane transport studies results indicated that the α-TP aggregation (at pH 7.4) did slow down the diffusion of the molecule compared to pH 9. Therefore, if α-TP followed the same route into the skin at pH 7.4 and 9, perhaps one would expect the pH 9 formulation to show more extensive skin deposition, but this was not the case. The reason for this is probably related to transport into the skin, which may be different at the two different pHs. In the presence of the aggregates at pH 7.4, the drug may
have passed into hair follicles. The hair follicles are not thought to be very size selective, most probably because of the double-barrier of SC and the tight junctions in the infundibulum (upper) region of the hair follicle, and the continuous intact tight junctions bidirectional barrier that extend from the infundibulum down to suprabulbar (lower) region of porcine hair follicles, which likely provide a rate limiting barrier to transport of small, intermediate, and large molecules into the skin (Bäsler et al., 2016). Moreover, it is possible that α-TP nano-sized aggregated (size ~10 nm, PDI 0.22) may possess some deformability to squeeze between corneocytes, to enhance its penetration. There is little evidence in literature about the mechanism by which α-TP penetrates the skin. Only one study by Gavin and co-workers hypothesised that α-TP nanomaterials are deformable and may act by disruption of the lipid packing in SC, by investigating the extrudability through using 0.1 µm filter, that showed increasing the content of α-TP in nanomaterial increased the passage through filter easily with little resistant in syringe compared to the absence of α-TP from nanomaterial. They also found that the addition of α-TP to phospholipids altered the expected liposomal structures and produced a long flexible sheets of membranes or structures using cryo-transition electron microscope (cryo-TEM) (Gavin et al., 2017). However, this evidence is still lacking as human SC lipids consists of sphingolipids (ceramide) and indicates the absence of phospholipids. It was important to further understand if phosphorylation of vitamin E could provide enhanced interactions with SC intercellular lipids monolayer model and to investigate if α-TP acts to disturb the SC lipid by acting as a self-enhancer, which was hard to see using simple homogeneous artificial skin models such as silicone membranes. Those aspects of α-TP formed the focus of the work described in Chapter 3.
2.6 Conclusion

The solubility of α-TP was found to increase dramatically when pH rose and this correlates with percent increase in di-anionic form. α-TP was found to aggregate at pH 7.4 in millimolar concentration due to the presence of its mono-anionic form. α-TP structure has showed to predispose the molecule to self-association in nanosized liposomes or micelles by adjusting the formulation composition and pH. These α-TP nanomaterials were appeared to be physically and chemically stable for short-term use. These nanomaterials found to be small enough to pass into skin via follicular route and/or through intercellular route. α-TP may be a good alternative to α-T in topical formulations as it showed greater epidermal deposition over α-T.

The α-T and α-TP has different physiochemical properties and thus permeate the skin through different pathway, i.e., hair follicle route is one of the permeation routes for ionised α-TP while unionized α-T limited to the SC route. In addition, α-TP showed superior epidermal deposition compared to other α-T pro-vitamins such as α-TA and amino acid conjugates. It was considered important to further investigate if the α-TP can interact and diffuse through SC lipid. Thus, the next key step in the following Chapter was to validate a SC-mimicking lipid Langmuir monolayer model and employed that model to predict if α-TP has potential to overcome the barrier function of SC.
Chapter 3. Alpha tocopherol phosphate skin lipid monolayer interaction

*Chapter summary*

In this Chapter, a SC-mimicking lipid monolayer model was employed to study the molecular-level interaction of α-TP, α-T, and α-TA with lipid monolayer. The monolayer subphase injection studies determined the penetration kinetics of α-TP, α-T, and α-TA from subphase into lipid monolayer. The compression isotherm analysis monitored the change of lipid phase behaviour over a range of lipid-active mixtures composition and surface pressure. This data demonstrated that the α-TP aggregates had a favourable interaction with SC lipids which allowed them to partition into or diffuse through intercellular lipid and fluidize it. These findings agreed with the efficient epidermal deposition of α-TP nanoaggregates observed in Chapter 2.
3.1 Introduction

In chapter 2, self-assembled nanoaggregates of α-TP were shown to permeate the full thickness of porcine skin. It was hypothesised from the permeation studies that α-TP passed into the skin as consequence of penetration through intercellular lipids. This hypothesis did not accord to or agree with the general principles of passive diffusion which suggest that charged molecules should not pass a confluent lipid barrier. Therefore, further data on the α-TP lipid interactions was required. In order to evaluate α-TP-SC interaction model systems can be employed. However, these models must mimic closely the SC. Although, the SC consists of enucleated corneocytes dispersed in a lipid matrix which is thought to composed of cholesterol, long-chain free fatty acid (FFA) and ceramides, its exact composition is still unknown (Masukawa et al., 2008; Neubert et al., 2011; Ponec et al., 2003; Weerheim and Ponec, 2001). Previous work by Elias (Elias, 1995), identified the individual lipids present in the SC and the composition of human SC lipids, which indicates the virtual absence of phospholipids and the enrichment of free fatty acids (25%), ceramide sphingolipids (35%), and cholesterol (20%) which exist together with small quantities of cholesterol sulphate, triglycerides, sterol esters, and hydrocarbons (Elias, 2005; Lampe et al., 1983; Norlén et al., 2007). These SC intracellular matrix lipids restrict transport through the skin, with the barrier function being influenced by the SC lipid packing and rigidity (Groen et al., 2011b). The human and pig SC lipids are arranged as lamellar, bilayer structures as shown by small-angle x-ray diffraction measurements. For instance, in the porcine SC Bouwstra et al (1995) found two phases, with repeat distances of 6.0 nm and 13.2 nm. In the human SC, the repeat distances of lamellar phases were found to be 6.4 nm and 13.4 nm and identified as the short periodicity phase (SPP) and long
periodicity phase (LPP), respectively (Bouwstra et al., 1995, 1991) (Fig. 3.1). Parrott and Turner, (1993) determined the lipid organisation of intact SC by preparing a model system from a mixture of cholesterol and bovine ceramide III (ceramide 2 family). An equimolar molar ratio (1:1) of cholesterol and ceramide (Chol/Cer) was used to perform x-ray diffraction measurements, which showed two lipid phases, one with a periodicity 4.5 nm and second with a periodicity of 10.4 nm (Parrott and Turner, 1993). Groen, et al (2009), performed small-angle x-ray diffraction (SAXD) using various lipid mixtures mimicking the lipid composition in the SC (pig Cer/Chol/FFA 1:1:1), by measuring the LLP and they found lamellar phase with a repeat distance of 12.8 nm (Groen et al., 2009). Groen, et al (2011), also performed neutron diffraction studies to measure the SPP and found that lamellae phase with a repeat distance of 5.4 nm (Groen et al., 2011a). These studies demonstrate that lipid mixtures can serve as an excellent model for the lipid organization in the SC. Previous studies also showed that mixtures prepared with either synthetic ceramide or extracted ceramide mixed with cholesterol and with fatty acids mimic the SC lipid organization very closely (Bouwstra et al., 2001; de Jager et al., 2006; Groen et al., 2008). Although ceramide and cholesterol play an essential role in the formation of the two lamellar phases, the addition of a FFA is crucial for the formation of the densely packed orthorhombic crystalline structure (Bouwstra et al., 2001; Chen et al., 2013).
Fig. 3.1: Structure of the multiple lamellae. The stratum corneum is formed by corneocytes (bricks) and lipids (mortar). The lipid layer is packed with intercellular spaces and multiple intercellular lamellae. The multiple lamellae have both lateral and lamellar levels of organization. The lateral organization is categorized as orthorhombic crystalline, hexagonal gel, and liquid lamellar, according to the temperature. The lamellar organization is categorized as long periodicity phase (LPP) and short periodicity phase (SPP), according to the type of ceramide (Cha et al., 2016).

One mean by which α-TP could permeate through the SC or overcome the SC barrier function is to disrupt the lipid packing, and thus causing self-enhancement for its own permeation and/or enhancement of the permeation of other co-administered actives. However, the skin lipid lamellae present within the SC are relatively inaccessible to direct experiments being present in small amounts between compressed corneocytes. Therefore, Langmuir air-liquid interface monolayer-oriented techniques have successfully proved to be a useful tool to study the interaction of potential skin penetrants with SC lipid models composed of equimolar ceramide, cholesterol and palmitic acid. Two major techniques have been used in characterizing penetrant-lipid monolayer interactions, firstly the
compression isotherm analysis, which studies the interfacial properties of spread SC lipids/test compound monolayers. Secondly, the constant area assay, which studies the penetration of test compounds into the SC-mimicking monolayers. The most widely used technique to characterise a monolayer is a compression isotherm analysis, which results in the surface pressure-molecular area ($\Pi$-A) isotherm (Eeman et al., 2009; Maget-Dana, 1999). As a monolayer is compressed, there is a change in the molecular packing and the monolayer undergoes several phase transitions (Davies and Rideal, 1981). These phase changes can be identified by monitoring surface pressure as a function of the area occupied by the film (see fig 3.2).

![Figure 3.2: Pressure–area isotherm of an amphiphilic molecule](Matharu et al., 2012).
The constant area assay measures the kinetics of adsorption or penetration into a SC lipid monolayer of substances injected and dissolved in the subphase. The lipid film area is constant and changes in surface pressure are monitored over time as a test compound (penetrant) is added to the buffer subphase. An increase in surface pressure resulting from injection of a test compound into the subphase indicates an interaction between the penetrant and the lipid monolayer/film. Therefore, both techniques, the compression isotherm analysis and constant area assay, are important to understand the molecular-level interactions through the kinetics of partitioning/penetration and change of the lipid phase behaviour over wide range of composition and surface pressures. These techniques indicate if the penetrant merges with and disrupts the packing of SC lipids, and increases fluidity, thus overcoming the SC lipid barrier. The air-liquid interface Langmuir monolayer was used widely in previous studies to investigate the influence of permeation enhancers, such as lipophilic azole, and oleic acid, addition on the compressional behaviour of monolayer films of equimolar ratio of three major lipids (ceramide N-acyl-sphingosine/Chol/FFA) mixture, which corresponding to that of human SC. The result was changing the monolayer from a condensed liquid film to liquid expanded film and hence increased its fluidity (Mao et al., 2013; Schlickler and Lee, 1991). Both the penetration kinetics and Π-A isotherm have been reported for a lipopeptide fengycin A (C16), using a SC model consisting of three major lipids (C24-ceramide 2 (N-lignoceroyl-D-sphingosine)/Cholesterol/Lignoceric acid (C24-fatty acid)) (C24Cer2, Chol, and C24FA) that constitute the human SC. The Π-A isotherm of the pure fengycin exhibited a sigmoidal shape and this increase in surface pressure is followed by a final plateau of constant surface pressure with no sharp increase in surface pressure is observed even at very low areas per molecule,
indicating that the fengycin monolayer cannot adopt a liquid-condensed state even under high compression. Whatever the composition and the lipid proportion of SC-mimicking monolayers, the Π-A isotherms obtained in the presence of fengycin showed the existence of two states, a fluid-like and a solid-like, as well as a phase transition between these two states, this meant that fengycin molecules are thus progressively squeezed out of the mixed monolayers when the surface pressure is increased, without carrying lipid molecules out of the monolayer thus the remained lipid caused the solid-like state. Penetration experiments of fengycin from the subphase into SC-mimicking monolayers indicated that the lipopeptide insertion at the lipid interface was enhanced in the presence of cholesterol, but they did not indicate that fengycin was able to diffuse through the extracellular lipid matrix of the SC (Eeman et al., 2009). The fengycin example shows the utility of SC monolayer as a model system to study the interaction between test compounds and chosen lipids indicating their potential to diffuse through lipids and overcome the barrier function of the SC.

The aim of this chapter was to use the air/liquid monolayer techniques with a lipid model composed of an equimolar mixture of ceramide, cholesterol, and palmitic acid (Cer/Chol/PA), mimicking SC lipid lamellae to understand the interaction of α-TP, α-T and α-TA with the SC. The intention was to correlate this with skin deposition data in chapter 2, thus to understand how α-TP permeates into the skin. Therefore, we investigated the penetration kinetics and interaction between α-TP and SC lipids over different composition ratios (low, medium, and high), which govern the ability of the penetrant to diffuse into the uppermost layer of the skin. Hence, air/liquid interface monolayer interaction techniques are a key step in the determination of the potential of α-TP to overcome the physical barrier of the SC.
The ceramide that was used in this experiment belonged to the NS (nonhydroxy sphingosine) ceramide family with non-hydroxy fatty acid chains composed mainly of stearic acid (C18:0) and nervonic acid (C24:1) attached to sphingosine headgroup through an amide bond (Mao et al., 2013).

In all the subphase injection studies and the compression isotherm analysis, the correction of α-T concentrations was not needed to exclude the effect of impurities (vegetable oil components, other tocopherol, fatty acids, and sterols, which characterized by European Food Safety Authority (European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed, 2010)) on the lipid monolayer interactions of α-T. This is because the impurities may have activity in these assays. Hence the concentrations of α-T noted in this chapter represent the concentration for the product of α-T as a whole.

3.2 Materials

α-TP (all-racemic mixture of 8 isomers or dl-α-tocopherol phosphate, purity: 92.7%, cosmetic grade) was a gift from Shawa Denko (Tokyo, Japan). α-T (type VI, purity: 70%, 695 mg d-α-tocopherol per g, 1036 IU/g), α-tocopherol acetate α-TA (Activity: 1306 IU/g, semisynthetic d-α-tocopherol acetate, synthesized from natural α-tocopherol), bovine brain ceramide, cholesterol, palmitic acid, sodium chloride, dimethyl sulfoxide, and propylene glycol were purchased from Sigma Aldrich (Dorset, UK). Chloroform and tris base were sourced from Fisher Scientific (Loughborough, UK). Absolute ethanol was obtained from VWR Chemicals (Leicestershire, UK).
3.3 Method

3.3.1 Monolayer subphase injection studies (constant area assay)

A 50 mm diameter perfluoroalkoxy alkane (PFA) Petri dish (Saint-Gobain, Poestenkill, NY, USA) with a maximum volume of 25 mL was placed on a stirring plate (Whatman stirrer, WC-303), filled with tris-HCl buffer (0.1 M, at pH 7.4) and equilibrated until a stable surface pressure was obtained (the maximum drift in surface pressure, \( \leq 0.2 \text{ mN/m per 2 min} \) using Nima TR516 software) (see fig. 3.3). This PFA petri dish was employed to perform a constant area assay using a skin SC lipid monolayer model, which consisted of equimolar mixtures (1:1:1) of ceramide, cholesterol, and palmitic acid (Cer/Chol/PA). This mixture was used to mimic SC intercellular lipid at the air/liquid interface, as described previously in the literature (Mao et al., 2013). The SC lipid monolayer film was produced using 5.1 mg ceramide, 4 mg cholesterol, and 3 mg palmitic acid dissolved in 5 mL chloroform (a 2.4 mg/mL lipid mixture, dissolved in chloroform). In this assay, two drops of lipid solution were spread at the air/liquid interface until a 30 mN/m pressure was reached on the buffered subphase, and approximately 15 min was allowed for solvent evaporation and for the lipid monolayer to equilibrate at room temperature. A 100 \( \mu \text{L} \) aliquot of \( \alpha \)-TP (concentration of 1, 2, and 12 mM) in the vehicle (20% ethanol, 20% propylene glycol, 60% water at pH 7.4 (100 mM Tris.HCl buffer)) was injected into the subphase below the SC lipid monolayer film and the pressure was monitored over time at a constant surface area. The experiment was repeated using 2.3 mM (1 mg/mL) \( \alpha \)-T in the same vehicle at pH 7.4 or DMSO and using 2.1 mM (1 mg/mL) \( \alpha \)-TA in DMSO. The test compound-lipid interactions were measured by a change in surface pressure over time. The adsorption profiles (the increase in the surface pressure at the time of drug injection) obtained were
fitted using OriginPro software by a nonlinear curve fit (Hill) model according to equation 3.1 to estimate the three parameters, the maximum obtained surface pressure \( V_{max} \), the time to reach 50% of maximum surface pressure (K), and the Hill slope (n).

\[
y = V_{max} X \ X^n \ (K^n + X^n)
\]

Equation 3.1

**Fig.3.3:** The Langmuir trough set-up for monolayer subphase injection studies.

**3.3.2 Compression isotherm analysis**

A Langmuir trough (Model 312D, NIMA Langmuir-Blodgett (LB) NIMA technology equipment, Ltd, Coventry, England) (area 500 cm\(^2\)) was used to acquire surface pressure-area (\( \Pi - A \)) isotherms. A 300 mL subphase of 150 mM NaCl, was used to deposit the 1 mg/mL of SC lipid monolayers from a solution in chloroform. A 40 μL aliquot of SC lipid solution or SC lipid-active mixtures containing 10%, 30%, or 60% (v/v) α-TP was spread at the air/liquid interface and left for 15 min
for solvent evaporation. Following evaporation, compression measurements were performed. Monolayers were compressed with a constant slow barrier speed of 6 cm².min⁻¹ until the monolayer collapsed. Films composed of 100% α-T, 100% α-T, and 100% α-TA were used as controls.

3.3.3 Mean molecular area per molecule analysis (miscibility of SC lipids and α-tocopherol phosphate)

The interaction between the SC lipids and the α-TP was further evaluated with mean molecular area per molecule (MMA) plots. The MMA of five different SC lipid/α-TP mixtures were plotted as a function of α-TP percentage at nine surfaces pressures to provide a measure of miscibility between the SC lipids and the α-TP. The symbols connected with solid lines are experimental MMAs from the averaged isotherms. The dashed lines represent the calculated MMAs according to the additivity rule, as shown in equation 3.2.

\[ A_{12} = X_1A_1 + X_2A_2 \]  
Equation 3.2

Were \( A_1 \) and \( A_2 \) represent MMAs for the pure monolayers of component 1 (SC lipids) and component 2 (α-TP), respectively, under the same surface pressures while \( X_1 \) and \( X_2 \) are the fractions of each component in the mixture. In this study, the SC lipids were an equimolar mixture of three SC lipids and were treated as a single component to evaluate the overall interactions with α-TP. If two components are ideally mixed or phase-separated, the MMA of their mixtures follow the additivity rule and lie on the dashed line. However, most of lipid mixtures studied in the literature show a non-ideal behaviour and deviate from the ideal mixing
dashed line. Repulsive or attractive interactions in binary lipid mixtures can be assessed respectively by positive or negative deviation from the additivity rule.

### 3.3.4 Monolayer compressibility

The compressibility of a monolayer can be used to characterise the phase state of a monolayer (Maget-Dana, 1999; Alsina et al., 2006; Davies, and Rideal 1963). The compressibility ($C_s$) of a monolayer at any area, $A$, is defined according to equation 3.3. However, for a more precise measurement, the reciprocal of $C_s$ is used to characterise the properties of the surface film and is called the compressibility modulus ($k_s$, or $C_s^{-1}$), which is defined according to equation 3.4. The compressibility properties can be determined from the slope of a $\pi$-$A$ isotherm at specific surface pressures (Dennison et al., 2010).

\[
C_s = -\frac{1}{A} \left( \frac{\delta A}{\delta \pi} \right)_T \quad \text{Equation 3.3}
\]

\[
K_s = -A \left( \frac{\delta \pi}{\delta A} \right)_T \quad \text{Equation 3.4}
\]

Where $\delta A$ refer to a change in area ($\text{Å}^2$/molecule), $\delta \pi$ refers to change in interfacial surface pressure value (mN m$^{-1}$), and $T$ indicates this is true for a specific temperature. The phase state of monolayer are classified by $K_s$ ranges into liquid expanded phase (12.5-50 mN/m), intermediate phase between liquid expanded to liquid condensed (higher than 50 below 100 mN/m) liquid condensed phase (100-250 mN/m), and solid state (1000-2000 mN/m) (Maget-Dana, 1999).

### 3.3.5 Statistical analysis

All the data were expressed as their mean $\pm$ standard deviation (SD). The homogeneity of variance (Levene’s test) and the normality (Shapiro-Wilk test) of
all sample groups data were assessed prior to statistical analysis. The statistical analysis was performed using statistical package of social sciences, SPSS version 17 (IBM Corp., USA) with a significance level of 0.05. Two-group comparison of mean was performed using Student’s independent t-test for data with equal variance or Mann-Witney’s test for non-equal variance. One-way analysis of variance (ANOVA) test for normally distributed data more than two groups’ comparison and non-parametric kruskal-Walis tests for non-normally distributed data. Post hoc comparisons of the means of individual groups were performed when appropriate using Tukey’s test or Dunnet’s test for equal variance normally distributed data, whereas or Games Howell test for unequal variance non-Gaussian distributed data. Data were presented using Prism software (Graphpad Prism, version 5.02, December 2008).

3.4 Results

3.4.1 Monolayer subphase injection studies (constant area assay)

The injection of α-TP in the subphase of SC lipid monolayer, induced a surface pressure change of ~10 mN/m whereas, α-T and α-TA induced a surface pressure change of less than 3 mN/m (Fig. 3.4). This lower effect on surface pressure may be due to a consequence of low solubility of α-T and α-TA in the polar subphase. α-TA precipitated upon injection as the carrier solvent was diluted in subphase and was thereby sequestered from interacting with monolayer interface. The injection of an increased concentration of α-TP (1, 2, and 12 mM) in the subphase led to a significant increase in the maximum obtained surface pressure (V_{max}) compared to the vehicle control (1 mM- p < 0.001, 2 mM- p < 0.01, 12 mM- p < 0.01, One-way ANOVA with Dunnett’s multiple comparisons test) (see fig. 3.5, table 3.1). The time to reach 50% of maximum surface pressure (K) decreased significantly (1 mM-
$p = 0.001$, 2 mM- $p = 0.001$, 12 mM- $p < 0.001$, One-way ANOVA with Dunnett’s multiple comparisons test) for all the three concentrations compared to the vehicle control. The hill slope (n) significantly increased only for the injection of the 12.6 mM of α-TP ($p < 0.01$, One-way ANOVA with Dunnett’s multiple comparisons test). This indicated that the interaction with SC lipids occurred faster. However, at high concentration 12.6 mM > CAC, the $V_{\text{max}}$ slightly decreased compared to low concentrations, below the CAC, the difference was not statistically significant ($p > 0.05$, One-way ANOVA with Dunnett’s multiple comparisons test).

**Fig. 3.4:** The adsorption kinetics of α-TP, α-T, α-TA following its injection into a SC lipid monolayer subphase. The change in surface pressure versus the change in time for a) 2 mM of α-TP in the vehicle (red line) 2.3 mM of α-T in vehicle or DMSO (green lines), 2.1 mM of α-TA in DMSO (blue line), the vehicle alone (yellow line), and vehicle at pH 7.4 (grey line) at the time of injection. α-TP induced a surface pressure change of up to 10 mN/m whereas, α-T and α-TA induced a surface pressure change of less than 3 mN/m. Data represents the mean (n=3). Subphase: Tris buffer at (25 °C/pH 7.4).
Fig. 3.5: The adsorption kinetics of increased concentration of α-TP following its injection into a SC lipid monolayer subphase. The change in the surface pressure with time for 0, 1, 2, 12.6 mM of α-TP in vehicle pH 7.4 at the time injection. α-TP showed faster interaction with SC lipid monolayer in a concentration dependent manner. Data represents the mean (n=3). Subphase: Tris buffer at (25 °C/pH 7.4).

Table 3.1: The adsorption kinetics of α-TP to SC lipid monolayer indicated by three parameters, the maximum surface pressure $V_{\text{max}}$, the time to reach 50% of maximum surface pressure (K), and the Hill slope (n). Penetration kinetic profiles of α-TP (0, 1.0, 2.0, and 12.6 mM) in the vehicle (pH 7.4) following its injection in the subphase were fitted by a nonlinear curve fit (Hill) model to estimate the three parameters ($V_{\text{max}}$, K, and n). Data represent the mean ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>α-TP concentration (mM)</th>
<th>$V_{\text{max}}$</th>
<th>K</th>
<th>n (hill slope)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.8 ± 2.5</td>
<td>2025.0 ± 709.0</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>1</td>
<td>22.9 ± 2.4***</td>
<td>120.4 ± 33.8**</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>18.4 ± 5.4**</td>
<td>210.1 ± 124.1**</td>
<td>1.7 ± 0.0</td>
</tr>
<tr>
<td>12.6</td>
<td>17.7 ± 2.4**</td>
<td>83.9 ± 6.7***</td>
<td>3.2 ± 0.5**</td>
</tr>
</tbody>
</table>
3.4.2 Compression isotherm analysis

The monolayer lift off value of pure SC lipids significantly increased with the addition of 30% of each of α-TP, α-T, and α-TA ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively, One-way ANOVA with Dunnett’s multiple comparisons test) (Fig. 3.6, table 3.2). Moreover, the collapse pressure significantly decreased with the addition of 60% α-TP, 30% α-T, and 30% α-TA ($p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively, One-way ANOVA with Dunnett’s multiple comparisons test) (Table 3.2). The collapse area values were not statistically different upon the addition of all the three tocopherols to the SC lipid monolayer compared to the pure SC lipid monolayer ($p > 0.05$) (Table 3.2).

Fig.3.6: $\pi$-A isotherms for stratum corneum (SC) lipids (grey line), three vitamin Es (α-TP, α-T, and α-TA) and their mixtures. a) α-TP and α-TP: SC lipid mixtures, b) α-T and α-T: SC lipid mixtures, c) α-TA and α-TA: SC lipid mixtures, at composition ratio of 10%, 30%, and 60% (v/v) at pH 7. Data represents mean ± standard deviation (n=3).
Table 3.2: Lift off values, collapse area, and collapse pressure for SC lipids, three vitamin Es (α-TP, α-T, and α-TA) and their mixtures. Data represents mean ± standard deviation (n=3).

<table>
<thead>
<tr>
<th></th>
<th>Lift off area (Å²/molecule)</th>
<th>Collapse area (Å²/molecule)</th>
<th>Collapse pressure (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure SC lipid</td>
<td>45.4 ± 5.5</td>
<td>26.6 ± 4.0</td>
<td>48.8 ± 2.2</td>
</tr>
<tr>
<td>10%</td>
<td>47.5 ± 6.7</td>
<td>26.8 ± 2.9</td>
<td>48.1 ± 3.4</td>
</tr>
<tr>
<td>30%</td>
<td>59.2 ± 6.1**</td>
<td>27.2 ± 3.1</td>
<td>48.1 ± 0.4</td>
</tr>
<tr>
<td>60%</td>
<td>65.9 ± 13.0**</td>
<td>27.3 ± 0.0</td>
<td>45.1 ± 0.5*</td>
</tr>
<tr>
<td>100%</td>
<td>79.52 ± 15.2**</td>
<td>31.9 ± 3.5</td>
<td>41.8 ± 1.5**</td>
</tr>
<tr>
<td>α-TP 10%</td>
<td>49.1 ± 8.5</td>
<td>24.6 ± 3.5</td>
<td>45.9 ± 5.0</td>
</tr>
<tr>
<td>30%</td>
<td>60.5 ± 1.6**</td>
<td>20.9 ± 0.2</td>
<td>39.8 ± 5.6**</td>
</tr>
<tr>
<td>60%</td>
<td>75.1 ± 6.3**</td>
<td>21.1 ± 0.2</td>
<td>26.3 ± 0.3**</td>
</tr>
<tr>
<td>100%</td>
<td>85.9 ± 3.4**</td>
<td>25.1 ± 0.1</td>
<td>18.9 ± 0.5**</td>
</tr>
<tr>
<td>α-T 10%</td>
<td>47.8 ± 11.1</td>
<td>25.1 ± 3.8</td>
<td>40.4 ± 7.5*</td>
</tr>
<tr>
<td>30%</td>
<td>61.1 ± 7.8**</td>
<td>22.9 ± 1.0</td>
<td>38.3 ± 5.0**</td>
</tr>
<tr>
<td>60%</td>
<td>80.7 ± 6.7**</td>
<td>22.3 ± 0.5</td>
<td>21.8 ± 0.1**</td>
</tr>
<tr>
<td>100%</td>
<td>91.1 ± 6.1**</td>
<td>29.3 ± 0.2</td>
<td>15.0 ± 0.3**</td>
</tr>
<tr>
<td>α-TA 10%</td>
<td>47.8 ± 11.1</td>
<td>25.1 ± 3.8</td>
<td>40.4 ± 7.5*</td>
</tr>
<tr>
<td>30%</td>
<td>61.1 ± 7.8**</td>
<td>22.9 ± 1.0</td>
<td>38.3 ± 5.0**</td>
</tr>
<tr>
<td>60%</td>
<td>80.7 ± 6.7**</td>
<td>22.3 ± 0.5</td>
<td>21.8 ± 0.1**</td>
</tr>
<tr>
<td>100%</td>
<td>91.1 ± 6.1**</td>
<td>29.3 ± 0.2</td>
<td>15.0 ± 0.3**</td>
</tr>
</tbody>
</table>

3.4.3 Mean molecular area per molecule analysis

Figure 3.7 showed that at all surface pressures (1-30 mN/m) the MMA per molecule of pure SC lipids increased with the addition of 30% α-TP (p < 0.05, unpaired Student t-test). For instance, at surface pressure 30 mN/m, the MMA per molecule of pure SC lipids (30.18 ± 4.75 Å²/molecule) increased with the addition of 30% α-TP up to 38.12 ± 3.61 Å²/molecule (p < 0.05, One-way ANOVA with Dunnett’s multiple comparisons test), this due to increase the repulsive forces and the steric hindrance upon increasing the percentage of α-TP. α-TP weakens the attraction forces between SC lipids hydrophilic head group, thus fluidizing the monolayer. The addition of 30% α-T increased the MMA per molecule of pure lipids at 1-20 mN/m (1-5 mN/m – p < 0.01, 10-15 mN/m – p < 0.05, One-way ANOVA with Dunnett’s multiple comparisons test, 20 mN/m, p = 0.05, One-way ANOVA with Games-Howell test) but there was no significant increase at higher surface pressures.
(25-30 mN/m – p > 0.05, One-way ANOVA with Games-Howell’s test). The 60% \(\alpha\)-TA increased the MMA per molecule of pure lipids at 1-20 mN/m (1-5 mN/m - \(p < 0.0001\), 10-15 mN/m - \(p < 0.01\), One-way ANOVA with Dunnett’s multiple comparisons test) but no significant increase at 20-25 mN/m (\(p > 0.05\), One-way ANOVA with Games-Howell’s test) (see Fig. 3.7 b, and c).

In this study the SC lipids/\(\alpha\)-TP mixtures were not ideally mixed in all compositions studied as shown in figure 3.7a. Their MMAs were higher than those calculated from the equation of additivity rule at each surface pressure except for the addition of 10% \(\alpha\)-TP, which indicates stronger repulsive or weaker attractive interactions between SC lipids and \(\alpha\)-TP compared to the SC lipids/SC lipids and \(\alpha\)-TP/\(\alpha\)-TP interaction. In the equimolar Cer/Chol/PA SC lipid model \(\alpha\)-TP becomes the most abundant single component at molar concentration above 25%. This emphasis that at 30% \(\alpha\)-TP, \(i.e.,\) when \(\alpha\)-TP dominates the composition of the mixture, an inflection point was observed in the MMA curve. This may be a consequence of change the interactions between \(\alpha\)-TP and SC lipids as the concentration of \(\alpha\)-TP increases and becomes the dominant species in the monolayer, which switches from the ordered rigid SC lipids to the more liquid expanded films. The repulsive interactions between the SC lipids and \(\alpha\)-T or \(\alpha\)-TA was to a lesser degree or absent at surface pressure > 15 mN/m (see figure 3.7 b, c).
Fig. 3.7: Mean molecular area for Cer/Chol/PA and α-TP (a), α-T (b), and α-TA (c) mixtures at surface pressure of 1, 5, 10, 15, 20, and 30 mN/m. The solid lines represent the measured MMA from the isotherms, and the dashed lines are the calculated ideal mixing curves. At surface pressure 30 mN/m, the addition of 30% α-TP mixture increased the measure MMA of SC lipid at the inflection point whereas the addition of 30% α-T or α-TA did not.
3.4.4 Monolayer compressibility

The surface compressional moduli were determined from the \( \Pi - A \) isotherms in order to determine the effect of increasing the \( \alpha \)-TP concentration on the elasticity of SC lipid monolayers. This parameter was calculated from the tangent of the isotherms at 10, 20, 30 and 40 mN/m using equation 3.4. Table 3.3 shows that at 30 mN/m, there is an overall trend to decrease of the calculated surface compressional moduli for the SC lipid monolayers as the \( \alpha \)-TP concentration increases above a 10% molar percentage (30% and 60% w/w compared to pure SC control sample, 30% - \( p < 0.001 \) and 60% - \( p < 0.0001 \), One-way ANOVA with Dunnett’s multiple comparisons test), at 30% \( \alpha \)-TP the magnitude of these changes is comparatively small, as the \( K^s \) values for all of the monolayers fall within the liquid condensed (LC) phase (100- 250 mN/m), whereas the magnitude of change was higher at 60% \( \alpha \)-TP, the \( K^s \) value was below 100 mN/m indicates an intermediate phase between LE and LC (50-100 mN/m).

Table 3.4 showed that there was an overall trend to decrease of the calculated surface compressional moduli for the SC lipid monolayers as the \( \alpha \)-T concentration increases (10-30 % w/w compared to pure SC control sample, 10% - \( p < 0.05 \), 30% - \( p < 0.0001 \), One-way ANOVA with Games-Howell’s test). The magnitude of these changes is comparatively moderate, as the \( K^s \) values for of 10% \( \alpha \)-T the \( K^s \) value was below 100 mN/m indicates an intermediate phase between LE and LC (50-100 mN/m), whereas at 30% monolayers the magnitude of change is higher \( i.e. \) shift from the liquid condensed (LC) phase to liquid expanded (LE) (12.5-50 mN/m). The increased concentration of \( \alpha \)-TA (10-30%) % also showed a significant trend to decrease the \( K^s \) similar to \( \alpha \)-T (\( p < 0.0001 \), One-way ANOVA with Dunnett’s multiple comparisons test) (Table 3.5). However, the magnitude of these
changes at 10% is comparatively low, as the Ks values for the monolayer fall within the liquid condensed (LC) phase, whereas at 30% intermediate phase between LE and LC (100-250 mN/m). The collapse of pressure decreased significantly upon increasing the molar percentage of monolayer of α-T or α-TA, making the Ks calculation impossible for higher surface pressure e.g. at 30 mN/m at molar percentage of 60%.

Table 3.3: The compressibility modulus (Ks) for each molar Percentage of α-TP, at surface pressure 10, 20, 30 and 40 mN/m.

<table>
<thead>
<tr>
<th>Compressibility modulus Ks for each molar Percentage of α-TP</th>
<th>Surface pressure (mN/m)</th>
<th>0%</th>
<th>10%</th>
<th>30%</th>
<th>60%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>98.9±10.5</td>
<td>87.8±6.2</td>
<td>66.8±5.3</td>
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</tr>
<tr>
<td></td>
<td>20</td>
<td>140.6±16.9</td>
<td>142.6±15.0</td>
<td>106.7±4.7</td>
<td>76.0±8.9</td>
<td>65.40±11.5</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>169.4±11.7</td>
<td>170.3±17.3</td>
<td>123.7±16.6</td>
<td>87.8±12.4</td>
<td>102.2±16.8</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>134.2±17.9</td>
<td>184.6±49.4</td>
<td>135.6±11.9</td>
<td>70.0±6.1</td>
<td>73.2±17.5</td>
</tr>
</tbody>
</table>

Table 3.4: The compressibility modulus (Ks) for each molar Percentage of α-T at surface pressure 10, 20, 30, and 40 mN/m.

<table>
<thead>
<tr>
<th>Compressibility modulus Ks for each molar Percentage of α-T</th>
<th>Surface pressure (mN/m)</th>
<th>0%</th>
<th>10%</th>
<th>30%</th>
<th>60%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>98.9±10.5</td>
<td>69.6±7.4</td>
<td>62.5±2.9</td>
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<td></td>
<td>20</td>
<td>140.6±16.9</td>
<td>119.0±36.6</td>
<td>38.7±5.2</td>
<td>15.1±1.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>169.4±11.7</td>
<td>62.9±21.9</td>
<td>36.0±4.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>134.2±17.9</td>
<td>141.5±29.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3. 5: The compressibility modulus ($K_s$) for each molar Percentage of $\alpha$-TA at surface pressure 10, 20, 30 and 40 mN/m.

<table>
<thead>
<tr>
<th>Surface pressure (mN/m)</th>
<th>0%</th>
<th>10%</th>
<th>30%</th>
<th>60%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>98.9±10.5</td>
<td>76.3±5.0</td>
<td>70.1±4.8</td>
<td>53.6±4.6</td>
<td>45.9±2.4</td>
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<tr>
<td>20</td>
<td>140.6±16.9</td>
<td>49.3±17.7</td>
<td>10.8±4.7</td>
<td>2.9±0.8</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>169.4±11.7</td>
<td>100.5±13.9</td>
<td>81.2±14.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>134.2±17.9</td>
<td>61.5±2.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.5 Discussion

The injection of amphiphilic $\alpha$-TP into the subphase, in the constant area assay, induced a surface pressure changes in the region of up to 10 mN m$^{-1}$ indicative of $\alpha$-TP insertion into hydrophobic region of SC lipid film. This was similar to a study that determined the penetration of anionic amphiphilic surfactant sodium dodecyl sulfate (SDS) in mixtures of two lipids, 1-palmitoyl-2-oleoyl-$sn$-glycero-3-phosphocholine (POPC) and cholesterol, and 1,2-dipalmytoyl-$sn$-glycero-3-phosphocholine (DPPC) and cholesterol. The injection of SDS at a molar ratio of 0.55 induced up to 5 mN m$^{-1}$ and 10 mN m$^{-1}$ increase in surface pressure in the two lipid mixtures, respectively (Nigam, 2006).

The hydrophobic $\alpha$-T and $\alpha$-TA only induced minimal surface pressure change, less than 3 mN m$^{-1}$, and this indicated that the interactions were less extensive than $\alpha$-TP and occurred with lipid head groups without insertion. In the case of $\alpha$-TA, which immediately precipitated upon injection, its penetration was hindered by monolayer interface and undoubtedly sequestered in (moved into) the subphase. This is similar to the previous study by Marquardt et al, which determined that location of $\alpha$-T in a lipid bilayer was near the lipid–water interface without insertion.
into the core of lipid by means of small-angle neutron diffraction and was unaffected by their lipid composition. In their work, the hydroxyl group of α-T was located high in the membrane, with its branched tail residing far from the core of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayers. In addition, α-T’s hydroxyl group sat above the lipid backbone in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and sphingomyelin bilayers (Marquardt et al., 2015). This minimal change in surface pressure was also similar to that of carboxymethylpullulans (CMP49C8), a polysaccharide derivative, where its penetration was hindered by the phospholipid monolayer dimyristoylphosphatidylcholine (DMPC) (Henni-Silhadi et al., 2007).

In this study, the aqueous monolayers of equimolar mixtures of ceramide, cholesterol, and PA were adopted to mimic a single layer of intercellular SC lipid lamellae. The Π-A isotherms for the SC lipids lifted off ~ 45.38 ± 5.51 Å²/molecule and a collapse at ~ 26.58 ± 4.04 Å²/molecule (collapse pressure, ~ 48.82 ± 2.21 mN/m) following a sharp surface pressure increase. These values are in good agreement with the literature (Flach et al., 2000; Mao et al., 2013) and indicated that the SC lipids form rigid and condensed monolayers at air/aqueous interface.

The isotherm lift off areas of pure SC lipids increased with the addition of 30% α-TP similar to the control (α-T, and α-TA). The pure films of α-T and α-TA are much more expanded, i.e., remain in a liquid-expanded state than those of pure α-TP and pure lipids, which shows higher condensation. Moreover, the transition of the Π-A isotherms of pure films was not due to any change in the phase-state of the α-T and TA at the air/buffer interface, and do not show the presence of the liquid-condensed
phase, as evidenced by the values of the compressibility modulus ($k^3 < 50 \text{ mN/m}$) (13.6 and 45.9 mN/m, respectively, at surface pressure 10 mN/m) (Davies and Rideal, 1981; Elderdfi and Sikorski, 2018; Maget-Dana, 1999). This was possibly due to the $\alpha$-T and TA molecules expelled from the interface directly into the subphase upon increasing the surface pressure, as a result of the electrostatic and steric repulsions that occur due to the branched tilted side chains in the $\alpha$-T or TA molecules (Capuzzi et al., 1996), i.e., at low surface pressures, the $\alpha$-T or TA molecules lie flat on the water surface with the side chains somewhat tilted toward the air. As the pressure increases, the side chains tilt further away from the water. At the knee in the isotherm (long plateau), the side chains are roughly perpendicular to the interface (Hąc-Wydro et al., 2007; Kaku et al., 1992), which is due to their lacking of the amphipathic balance that present in the $\alpha$-TP molecules (fig. 3.8). This amphipathic balance enable the $\alpha$-TP to adopt a more perpendicular (although still tilted) orientation and thus form a stable monolayer at air/liquid interface, which is flexible and easily compressible (Matharu et al., 2012; Petty, 1983). The $\alpha$-T or TA molecules have more interphase parallel (flat) orientation expelled from the interface directly to subphase when the surface pressure increases (Elderdfi and Sikorski, 2018; Kaku et al., 1992).
Fig. 3.8: Scheme of amphiphilic α-tocopherol phosphate (α-TP).

Although the pure α-T and α-TA control isotherms showed similar lift off areas compared to the pure α-TP, the α-T and α-TA (control) pure films started to collapse at lower pressure values, which suggested that these two molecules are too disruptive and cannot pack as tightly in the monolayers as α-TP. This is due to the α-TP structure being similar to the phospholipids and hence can approach the lipids and inset (partition) into the SC lipid. It is also more tolerated than the more hydrophobic controls (α-T/α-TA), as it can change its orientation to fit packing of the lipid monolayers or bilayers. Unlike α-T/α-TA, which is lodged in the lipid bilayer, and hardly passes through the lipids as it might be repelled, α-TP is more mobile and more readily moves through the lipid matrix. Therefore, the more favourable α-TP interaction with SC lipids allows it to pass through the lipid lamellae in the skin and permeate through the intercellular route. This effect was not seen in Chapter 2 studies with the hydrophobic silicone membrane, but can be anticipated since a silicone membrane does not adequately mimic the lipid
domains/lamellae that are found in human skin. However, it could be used to explain the superior ability of α-TP to pass into porcine skin shown in Chapter 2.

The MMA per molecule of pure SC lipids (at surface pressure 30 mN/m) significantly increased with the addition of 30% α-TP, due to an increase in the repulsive forces and the steric hindrance upon addition of α-TP. The consequence of this was weaker attractive forces or stronger repulsive between SC lipid hydrophilic head groups and α-TP, fluidizing the monolayer as a result. It has been reported in the literature that α-TP fluidizes membrane and induces haemolysis in a concentration-dependent manner i.e. acting as a detergent (Rezk et al., 2007). On the other hand, the addition of 30% α-T or α-TA did not change the MMA per molecule of pure lipids. This indicated that these molecules were likely to separate from SC lipid monolayer i.e., there is weaker interaction and poor mixing between the SC lipids and either α-T or α-TA, compared to α-TP. The compression isotherm studies also supported the notion that it is difficult to insert these into the SC lipid monolayer.

The SC lipids/α-TP mixtures were not ideally mixed in all compositions. The experimental MMAs were initially less than the calculated MMAs, which indicated a stronger attraction or weaker repulsion between the SC lipids and the α-TP upon the addition of 10% α-TP. When the α-TP became more dominant (> 25%) an inflection point was observed, where the experimental MMAs were higher than those calculated from the equation of additivity rule at each surface pressure. At these higher molar percentages, the stronger repulsive or weaker attractive interactions between the SC lipids and the α-TP compared to the SC lipids/SC lipids and α-TP/α-TP interaction may have disturbed the packing of lipids. Previous work with oleic acid, a known skin penetration enhancer, found that the addition of 40%
oleic acid (OA), switched the SC monolayer from an ordered (rigid) SC lipid to a more fluid monolayer, indicating a stronger attraction or weaker repulsive interactions between OA and SC lipids than the single component alone (Mao et al., 2013). Another work by Gavin and colleagues, who postulated that Tocopherol phosphate mixture (TPM®) is able to systematically modulate bilayer lipid packing upon the addition of α-TP to a standard membrane forming phospholipid, altering the expected liposomal structures and producing long flexible films of monolayers (Gavin et al., 2017).

A lower compressibility modulus (Ks) indicates a monolayer with lower interfacial stiffness and thus higher elasticity. SC lipids at 30 mN/m displayed a compressibility modulus Ks up to 169.44 ± 11.67 mN/m, while compressibility modulus of amphiphilic α-TP was up to 102.16 ± 16.77 mN/m and could not be determined for both α-T and α-TA.

The addition of 30% α-TP induced a small decrease in Ks which still fell within the LC phase range, whereas at 60% α-TP the monolayer compressibility modulus was further reduced by 0.5 and resulted in an intermediate phase between LC and LE, indicating that α-TP integrated into the SC lipids to change their properties from condensed-liquid film into a more liquid-expanded film, i.e. disrupted the lipid packing and fluidizing it.

However, the hydrophobic α-T or α-TA controls had a greater effect than α-TP in reducing the compressibility modulus with increasing the composition ratio at lower concentrations. For instance, the addition of 10% of α-T significantly reduced the monolayer compressibility modulus but it fell within the intermediate phase and, 30% of α-T caused the monolayers Ks values to shift from the liquid condensed
(LC) phase to the liquid expanded (LE) phase. The $\alpha$-TA addition at 10% had a low change in $K^*$ and still fell within LC phase, whereas the 30% resulted in an intermediate phase between LC and LE. This also supported the kinetic adsorption studies and MMA per molecule data, which indicated that $\alpha$-T and $\alpha$-TA adapted flat (parallel) orientation, and remain phase separated, whereas $\alpha$-TP adopt a perpendicular orientation and mixes well with the SC lipid monolayer.

3.6 Conclusion

Lipid monolayers provide a model system to aid investigation of $\alpha$-TP skin permeation and penetration mechanism. The structural similarity of $\alpha$-TP to the phospholipids allows it to approach and partition into SC lipid monolayer and fluidizes it. The presence of $\alpha$-TP in the SC lipid monolayer was more tolerated than the hydrophobic controls ($\alpha$-T/$\alpha$-TA) as $\alpha$-TP can change its orientation to suit the lipid packing. Unlike $\alpha$-T/$\alpha$-TA which are lodged in the lipid bilayer, and hardly passes through the lipids as it might be repelled, $\alpha$-TP is more mobile and more readily moves through the lipid matrix. Therefore, $\alpha$-TP favourable interaction with SC lipids allows it to pass the lipid lamellae in the skin and permeate through the intercellular route. This data explained why $\alpha$-TP did not pass the silicone membrane in Chapter 2 as it does not mimic or lacks the lipid domains/lamellae and charge, which undoubtedly affect the transport in the skin. These Langmuir constant area and isotherm results suggested $\alpha$-TP nanoaggregates is likely to be transported through intercellular SC lipid matrix and the viable epidermis where it can act as antioxidant and may protect from UVR-induced skin damage. This explains why the charged $\alpha$-TP monomers passed into the skin at pH 9 in Chapter 2 even though they were charged. It also suggests that the $\alpha$-TP aggregates were likely to fuse with
the SC lipids rather than pass through the hair follicles as the α-TP-SC interactions are favourable. The data from both Chapter 2 and Chapter 3, which indicated the nanoaggregates have superior skin deposition and favourable SC lipid interaction compared to α-T, raised further area of interest to investigate the *in vitro* photoprotective effect of α-TP aggregate which was the primary step in the following Chapter.
Chapter 4. Photoprotection by α-tocopherol phosphate in a human *in vitro* skin model

*Chapter summary:*

In this chapter, the cytotoxicity of α-TP and α-T was assessed *in vitro* in human HaCaT keratinocytes. Then, their UVA1 photoprotection potential was determined against (i) cell viability reduction using Alamar blue® and neutral red viability assays and (ii) ROS generation in keratinocytes using the dichlorofluorescin diacetate assay. The results showed that pre-irradiation with α-TP in low serum medium results in superior protection against cell viability reduction compared with α-T. Pre-irradiation treatment with α-TP also reduced the ROS level induced by UVA1 exposure at a similar to α-T. Photodegradation was observed in α-T.
4.1 Introduction

In Chapters 2 and 3, it was shown that α-TP has superior epidermal skin deposition and more favourable SC lipid interaction than α-T. α-TP has been previously shown to protect against UVB-induced damage. However, in all these studies the in vitro photoprotective properties may have been due to an overlap between α-TP absorption spectrum and UVR source spectrum in the UVB region; a possible indication of sunscreen photoprotection by its two aromatic rings. Pure UVA photoprotection studies of α-TP have not been performed. This raised our interest to investigate the protective effect of α-TP especially in the long-wave UVA1 region (375-400 nm) for several reasons; namely this region is (i) poorly protected by many, if not most, sunscreens, (ii) unintendedly ignored when estimating the SPF and UVA-PF using a broad spectrum SSR source that has decreasing output at > 365 nm which may result in overestimation of the protection, i.e., SPF (Diffey and Osterwalder, 2017) and (iii) generates ROS.

The acute clinical effects of terrestrial UVR (~295-400 nm) exposure are mainly erythema (sunburn), pigmentation (tanning), immunosuppression, and enhanced innate immunity (Young, 2006a) and reduction of blood pressure (Johnson et al., 2016; Liu et al., 2014). The long-term (chronic) consequences of exposure to UVR are mainly photoageing and photocarcinogenesis (Matsumura and Ananthaswamy, 2002). The only established advantage of UVR for human is the photosynthesis of vitamin D in the skin by the UVB-induced conversion of epidermal 7-dehydrocholestrol into provitamin D₃, immunomodulation, and photoadaptation (Johnson et al., 2016; Young et al., 2017). The majority of UVR (~95%) is UVA which mostly (~75%) consists of UVA1 (340-400 nm) that penetrates deeper into
various skin layers than UVB (280-320 nm). All the clinical short- and long-term consequences of UVR exposure are caused by molecular and cellular level changes including DNA photolesions, the generation of ROS (singlet oxygen, superoxide, peroxyl radical), melanogenesis, apoptosis, depletion of Langerhans cells, and up or downregulation of many genes and related proteins such as induction the expression of matrix metalloproteinases (e.g. MMP-1) which degrades collagen and thus causes photoageing.

The skin’s epidermis contains several endogenous chromophores, which have absorption spectra within the UVR region. These include the nucleic acids (DNA), urocanic acid, aromatic amino acids, melanin, and their metabolite/precursors (Young, 1997). Photobiological outcomes occur when such a chromophore absorbs UV photon energy and becomes excited and unstable. This consequently may result, for example, in DNA lesions caused by direct absorption of UVR, or an indirectly by another chromophore acting as a photosensitizer generating ROS that damage adjacent macromolecules such as proteins or DNA (Young, 1997, 2006b). The most common DNA photolesion is the cyclobutane pyrimidine dimer (CPD) (Douki et al., 2003; Premi et al., 2015). Other lesions include the (6-4) pyrimidine-pyrimidone photoproduct (6-4 PP), Dewar isomers, and 8-oxo-7,8-dihyoguanine (8-oxoGua). For instance, a study by Premi et al found that UVA exposure of melanocytes led to the formation of peroxinitrite (a product of two free radicals, superoxide and nitric oxide) which are chemically capable of exciting an electron to give triplet state in melanin and thus can induce the formation of dark CPD (by energy transfer) up to 3 h after UVA exposure (Premi et al., 2015). CPD causes many biological effects including cytokine-mediated erythema, immunosuppression, and transition mutations (C to T), which can lead to
keratinocyte cancers (KC) if not repaired (Mouret et al., 2006; Sharma et al., 2018; Vink and Roza, 2001; Young et al., 2017). However, such DNA photolesions may be removed by either nucleotide excision repair (NER) or base excision repair (BER); processes that are essential for protection against DNA damage and the maintenance of DNA integrity. Impairment of DNA damage repair processes, for example due to genetic mutations, greater increases the risk of KC and melanoma which is clearly seen in xeroderma pigmentosum (XP) patients who have defective NER (Bath-Hextall et al., 2007; Bradford et al., 2011; Fajuyigbe and Young, 2016; Sethi et al., 2013; Wei et al., 1995).

Sensitivity to UVR differs with Fitzpatrick skin type (Fitzpatrick, 1988) that ranges from I to VI. For instance, lower number skin types (e.g. fair skins of types I/II) are more susceptible to erythema, with poor tanning ability, and thus a higher risk of skin cancer compared to black skin due to photoprotection by epidermal melanin in darker skin (Fajuyigbe et al., 2018; Fajuyigbe and Young, 2016). The use of sunscreen protection for susceptible individuals is beneficial for acute and long-term effects, but has limitations (Diffey, 2009), such as applying much less sunscreen than the dose (2 mg/cm²) used in the sun protection factor (SPF) testing process (Autier et al., 2001; Azurdia et al., 1999; Bauer et al., 2010; Bech-Thomsen and Wulf, 1993; De Villa et al., 2011; Reich et al., 2009; Yang et al., 2009). Therefore, additional approaches for photoprotection are being sought, such as the use of topical antioxidants. One such topically applied antioxidant is α-T, which can scavenge UVR-induced free radicals, has been recently reported in vitro to inhibit DNA damage (oxidized purines and dark CPD) caused by a UVA1-induced photosensitisation reaction in human keratinocytes, when applied either prior or post the UVA irradiation. Thus, the inclusion of α-T in a topical sunscreen or in an
after sun formulation might mitigate the continuous DNA photoproduct formation even hours after sun exposure ends (Delinasios et al., 2018). In human melanocytes, α-T pre- and post-irradiation treatment abolished the dark CPD induced by UVA exposure by acting as triplet state quencher of melanin fragments and thus may protect from photocarcinogenic lesions that continue to be formed hours after exposure (Premi et al., 2015).

However, α-T has several undesired limitations; (i) poor chemical and photostability (Neunert et al., 2016), (ii) pro-oxidant effect by chromanoxyl radical formation which leads to deleterious free radical reactions (Bowry and Stocker, 1993; Mukai et al., 1993), (iii) hydrophobicity (Abla and Banga, 2014), and (iv) being retained in the stratum corneum (Cichewicz et al., 2013). One approach to this problem is the use of pro-vitamin E, which is more stable chemically, may have greater diffusibility into the skin layers, possibly via hair follicles and/or intercellular route, and may generate α-T and prolong increased α-T intracellular levels even after 24 h of UVR exposure ends. For instance, in comparison to α-T, tocopherol sorbate has been shown to significantly reduce the UVR-induced radical flux in skin by 50% and decrease wrinkling, in short-and long-term UVR exposed mouse models, respectively (Jurkiewicz et al., 1995). α-TP is a naturally occurring compound in human hepatic and adipose tissue. It is commercially produced by phosphorylation to yield a water-soluble chemically stable derivative of vitamin E which is easily incorporated in cosmeceutical formulations without the need of surfactants. Topical application of α-TP on a hairless mouse model for 3 h followed by UVR (63% UVB and 37% UVA) exposure showed that it was adsorbed, converted to α-T and reduced the number of apoptotic SBC by 50%, and reduced DNA photodegradation (Nakayama et al., 2003).
There are many approaches to quantify cell death caused by UVA exposure, *i.e.*, photokilling, as well as photoprotection afforded by antioxidants or sunscreens in cultured UVA-irradiated human skin cells. These include indirect techniques which assess cell viability such as Alamar blue®, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and neutral red assays (Lawrence *et al.*, 2018b, 2018a; Martins *et al.*, 2014; Wu *et al.*, 2014). Alamar blue® and MTT assays are metabolic function (redox) indicators, which measure cell viability indirectly by quantifying reduction within the intercellular environment (Rampersad, 2012). For instance, the Alamar blue® assay measures viable cells’ ability to reduce resazurin to fluorescent resorufin (O’brien *et al.*, 2000). The neutral red assay assesses the ability of viable cells to incorporate the cationic dye into their lysosomes. As the cell surface alters or cell dies, their ability to uptake neutral red dye decreases. Thus the loss of neutral red uptake inside lysosomes correlated to loss of cell viability (Repetto *et al.*, 2008; Zhang *et al.*, 1990).

Necrosis is the most predominant mode of cell death induced by UVA (Pourzand *et al.*, 1999; Zhong *et al.*, 2004). This is due to immediate increases in intracellular labile iron as well as UVA-induced ROS generation. These high levels of labile iron and ROS promote peroxidative membrane damage leading to loss of membrane integrity (Aroun *et al.*, 2012; Pelle *et al.*, 2011; Reed, 1999). Moreover, mitochondrial membrane damage caused by UVA can interrupt the electron transport chain causing further generation of ROS, loss of electrochemical gradient across the inner membrane and adenosine triphosphate (ATP) depletion followed by necrosis (Lemasters *et al.*, 1998; Reelfs *et al.*, 2016, 2010). It has been reported that primary skin cell lines are resistant to UVA-induced apoptotic cell death. The activation of caspases, necessary for apoptosis, requires the presence of ATP to
activate the apoptosis protease activation factor-1 (Apaf-1) (Pourzand and Tyrrell, 1999). UVA induced ATP depletion provides a rational explanation for the predominant mode of cell death being necrosis (Reelfs et al., 2010).

The aim of this Chapter was to investigate the α-TP photoprotection potential against UVA1-induced (i) cell viability reduction in human HaCaT keratinocytes using Alamar blue® and neutral red cell viability assays and (ii) generation of ROS in HaCaT keratinocytes using the 2’,7’-dichlorodihydrofluorescin diacetate (H₂DCFDA) assay. The other aim was to study its in vitro antioxidant potential using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays and compare it with potent antioxidants.

In the cell viability, ROS and UVA photoprotection studies, correction of the α-T concentrations based on the 70% purity was not performed. This was to facilitate easy comparison of this work with the natural compound and the previous work with the synthetic version α-T. The impure natural α-T (1036 IU/g) and the pure synthetic α-T (1100 IU/g) are almost equivalent in terms of biological activity. It is accepted that some impurities are present in the natural α-T and that these may have photoprotection activity, but this was thought to be unlikely.

4.2 Materials

α-TP (all-racemic mixture of 8 isomers or dl-α-tocopherol phosphate, purity: 97%) was supplied by Sigma-Aldrich (Dorset, UK). α-T (type VI, purity: 70%, 695 mg d-α-tocopherol per g, 1036 IU/g), neutral red, and Corning® cell culture flasks were sourced from Sigma-Aldrich (Dorset, UK). DMEM (high glucose, no pyruvate, no glutamine), foetal bovine serum (FBS), L-glutamine, penicillin – streptomycin, phosphate buffered saline (PBS) pH 7.4, TrypLE™ Express (1X, phenol red free)
were sourced from Thermofisher Scientific (Paisley, UK). Alamar blue® was purchased from Fisher Scientific (Leicestershire, UK). Triton-X was supplied from Promega (Southampton, UK). Plastic bottom black wall 96 well plates were purchased from PerkinElmer (Beaconsfield, UK). The H$_2$DCFDA cellular ROS detection assay kit was sourced from Abcam (Cambridge, UK). The ORAC Antioxidant Assay Kit was supplied by Zenbio (North Carolina, USA). The 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Assay was sourced from Alfa Aesar (Lancashire, UK).

4.3 Methods

4.3.1 UVR sources

The 385 nm source (Fig 4.1) was a Loctite LED flood system (Loctite, Henkel Ltd, Hemel Hempstead, UK) using the UVA1 array head (385 nm ± 5 nm – i.e. 10 nm at full width at half maximum (FWHM). The head has an irradiation surface of 97 mm x 96 mm consisting of 144 LEDs. Solar simulating radiation (SSR) (Fig 4.2) was obtained from a Solar® Light 300W-16S xenon arc solar UV simulator (Solar Light, Glenside, USA) with a full solar spectrum UVR setting, complying with that used for sun protection factor (SPF) testing by the International Organization for Standardisation (ISO) Standard 24444 and Cosmetics Europe 2006. UVR was delivered through a liquid light guide with an exit diameter of 7 mm. The spectral irradiiances of the sources are shown in Figure 4.3 and outputs are described in Table 4.1. The SSR spectrum was measured at distance of 0 cm, and the 385 nm spectrum was measured at a distance of 40 cm. Irradiation distances were based on the output of the source being measured and were selected to be within the dynamic range of the spectroradiometer.
Fig. 4.1: The 385 nm UVA1 source (Loctite LED flood systems).

Fig. 4.2: Solar simulating radiation (SSR).
Fig. 4.3: The spectral outputs of the radiation sources. SSR is solar simulating radiation and 385 nm is the UVA1 source. The spectral output of the sources used in all the studies as measured with a Bentham spectroradiometer (details below in Dosimetry section) from 280-460 nm.

Table 4.1: Spectral waveband analyses of the radiation sources. The spectral breakdown of each of the sources used in all the studies as measured.

<table>
<thead>
<tr>
<th>Source</th>
<th>Region</th>
<th>Wavelength (nm)</th>
<th>% of total irradiance</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSR</td>
<td>UVC</td>
<td>250-280</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UVB</td>
<td>280-320</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>UVA</td>
<td>320-400</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Visible</td>
<td>400-500</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>280-500</td>
<td>100</td>
</tr>
<tr>
<td>385 nm</td>
<td>UVC</td>
<td>250-280</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UVB</td>
<td>280-320</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UVA</td>
<td>320-400</td>
<td>94.82</td>
</tr>
<tr>
<td></td>
<td>Visible</td>
<td>400-500</td>
<td>5.18</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>280-500</td>
<td>100</td>
</tr>
</tbody>
</table>
4.3.2 Dosimetry

The spectral irradiances of the sources were measured using a DM120BC double-monochromator spectroradiometer (Bentham Instruments, Reading, UK) with an integration sphere, calibrated by the Centre for Radiation, Chemical and Environmental Hazards (CRCE), Public Health England (PHE) against a UK national standard. Irradiance of the sources was routinely measured with a handheld radiometer. Spectra from the Solar Light solar simulator were measured using a Solar® Light PMA 2100 radiometer (Solar® Light, Glenside, Pennsylvania) after calibration against the spectroradiometric readings, and gave a typical reading of 1120 μW/cm² or 18 s for an MED. For studies with the 385 nm source, a Loctite UVA/Vis radiometer (Loctite, Henkel Ltd, UK) was used, with a typical irradiance of 150 mW/cm² for 385 nm source. An 11 min exposure to 385 nm UVA1 irradiation gave a dose of 56.5 J/cm².

4.3.3 Absorption spectra of photoprotective compounds

The α-TP and α-T were prepared at equivalent molar concentration (400 µM) in methanol. UVR absorbance spectra of α-TP and α-T were determined with a Perkin Elmer Lambda 2 UV / VIS Spectrometer (Perkin Elmer & Co GmbH, Oberlingen, Germany) between wavelengths 250-420 nm, to assess for a possible sunscreening effect.

4.3.4 Photostability

SSR (Fig. 4.2) was used to test the photostability of α-TP and α-T solutions that were exposed to increasing doses of SSR. Protected samples are used as dark control (wrapped with aluminium foil). Absorbance of the dark control A₀, and absorbance of samples (Aₜ) was measured between 250-420 nm (n=3) after each
exposure using a FLUOstar-Omega microplate reader (BMG LABTECH Offenburg, Germany). The percent degradation was calculated from the ratio of the change in absorbances (Ar-A0) after exposure and the absorbance of dark control (A0) at 288 nm for α-TP and 291 nm for α-T, after normalising by subtracting the blank absorbance.

4.3.5 Cell culture

The immortalised human adult low calcium temperature (HaCaT) keratinocyte cell line (purchased from ATCC, Manassas, VA, USA) was used. This cell line has two p53 spontaneously transformed point mutations. HaCaT keratinocytes were cultured in Dulbecco modified Eagle’s medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, Poole, UK), 5% penicillin-streptomycin, and 5% glutamine (Life Technologies, Paisley, UK) and maintained in a humidified incubator at 37°C with 95% air and 5% CO₂. Cells were cultured to around 80% confluence in 75 cm² plastic flasks (Corning, New York, USA). Cells were adherent and so underwent washing with 10 mL PBS then sub-cultured using 3 mL trypsin (TrypLE™). The trypsin was then inactivated by adding complete media (7 mL). The cells were harvested by centrifugation (10000 g for 5 min). The supernatant was aspirated, leaving the pellet intact. Then, the tube was tapped firmly to re-suspend the pellet. After that, 5 mL of media were added and mixed thoroughly with the cell suspension. The cells were counted by adding 90 µL Trypan blue to 10 µL of cell suspension (dilution is x 10). Then, 10 µL of cell suspension was added to haemocytometer with a coverslip. The cells were counted in each of four squares; by calculating average and multiplying by 10,000 (x 10⁴), then the volume of cell suspension that was required to plate the
target number of cells per well was calculated, for instance, if the cell stock suspension count was 2,900,000 cell/mL and 15 mL cell suspension was needed for a target density 10,000 cell per well (equivalent to 100,000 cell per mL), then the required volume from stock cell suspension is calculated according to equation 4.1 and is equal to 0.5 mL and was added to 14.5 mL fresh media. Cells were plated into 96-well plates and left to reach a confluence of 70-80% before being used for experiments.

\[ C_1V_1 = C_2V_2 \]  \hspace{1cm} \text{Equation 4.1}

4.3.6 Cell viability with photoprotective compounds

The cytotoxic potential of α-TP and α-T was evaluated using the Alamar blue® (Fisher Scientific, Loughborough, UK) and neutral red (Sigma-Aldrich, Dorset, UK) and viability assays in HaCaT keratinocytes. The cells were plated in a 96-well plate at a concentration of 10,000 cells per well in full media and left overnight to adhere. The cells were treated 24 h later with doses of α-T, ranging from 0.0001-5 mM in 37°C warmed full media (0.5 % ethanol in full media was used as a control). The α-TP dose ranged from 0.0001-10 mM in warmed full media (full media was used as a control). The range of α-TP treatment was higher than α-T due to the higher solubility of α-TP in the full media (up to 10 mM) compared to α-T (up to 5 mM) in 0.5% ethanol, 95% full media. Plates were returned to the incubator for 24 h. After 22.5 h, the media was aspirated from each well and full media containing 1:10 Alamar blue® or 1:100 neutral red was added to each well for 1.5 or 3 h, respectively as described in Section 4.3.7. The fluorescence intensity or the optical density of each well was measured using Infinite 200 PRO
spectrofluorometer (Tecan Group Ltd, Mannedorf, Switzerland), and cell viability for α-T or α-TP treated cells was calculated as a percentage of the control value.

4.3.7 Cell viability (UVA1 dose-response)

The UVA1 dose-response for HaCaT viability was determined over a range of 385 nm doses at different times. HaCaT were plated on day 1 into black-walled (to prevent UVR scatter) 96-well plates at concentrations of 10,000 cells per well, in full media, and allowed to adhere overnight. On day 2, media was aspirated from all wells and the cells were washed twice with warmed PBS (100 μL/well). Fresh PBS (100 μL/well) was then added to each well before the UVA1 irradiations. The inner six wells (in columns 7, 8, 9, 10, and 11) were irradiated with 56.5, 112.9, 141.2, 169.4, and 225.9 J/cm² of UVA1 (11, 22, 27.5, 33, or 44 min, respectively) (see fig. 4.4) and the remaining wells were covered in foil during this time. On day 3, cell viability was measured using Alamar blue® and neutral red assays.

The media was aspirated 22.5 h after UVA irradiation from each well and replaced with 100 μL of Alamar blue® solution dissolved in warmed, full (10% FBS) media (at a ratio of 1:10). The plate was then incubated in the dark at 37°C with 5% CO₂ for 1.5 h, in order to establish viability 24 h after irradiation. Resazurin is the active ingredient of the Alamar blue® assay; it is a cell-permeable, non-toxic, blue compound that is reduced to resorufin in viable cells. Resorufin is highly fluorescent and hence fluorescence intensity is directly proportional to cell viability. Fluorescence intensity was measured using an excitation waveband of 540–570 nm (λ_{max} = 570 nm) and reading the emission at 580–610 nm (λ_{max} = 585 nm) with a FLUOstar-Omega microplate reader (BMG LABTECH, Offenburg, Germany). The measured fluorescence intensity of the unirradiated (UV-) cells was normalized
to 100%, i.e., for irradiated cells, viability was calculated as a percentage of the unirradiated value.

The neutral red assay was performed by adding 100 μL of neutral red solution dissolved in warmed, full (10% FBS) media (at a ratio of 1:100) into each well with incubation at 37°C, 5% CO₂ for 3 h. Cells were then washed three times in PBS to remove excess neutral red solution and then de-stain solution (50% v/v ethanol, 49% ddH₂O, 1% glacial acetic acid (GAA)) was added. Optical density was measured at 540 nm with a FLUOstar-Omega microplate reader (BMG LABTECH, Offenburg, Germany). Each condition was tested in triplicate from three different passage numbers between a range of 16-20 passages. There is no ideal limit to cell passages reported in the literature and each cell line varies but values of 18 and 25 have been described as ‘low’ passage counts (ATCC., 2010).

Fig.4. 4:Layout for dose-response studies (385 nm UVA1).
4.3.8 Photoprotection effects of α-TP and α-T pre-treatment against cell viability reduction in keratinocytes

4.3.8.1 Preparation of treatment solutions

A 178 µl aliquot of 6 mM α-TP in tris buffer (0.1 M) was added to 5 mL of full-serum (10%) or starved low-serum (2%) (as previously used by Nakayama et al, 2003). Dulbecco’s modified Eagle medium (phenol red free) containing 0.45% glucose (high-glucose) to obtain 200 µM α-TP, and then 50 µL was added to each well that contained 50 µL of the same medium to get 100 µM α-TP in each well. The α-T solution was prepared using a stock solution of 20 mM, then, 200 µM of α-T was prepared by adding 100 µl of the 20 mM α-T stock solution in 10 mL of full-serum (10%) or low serum (2%) media, and then 50 µL was added to each well that contained 50 µL of the same medium to get 100 µM α-T in each well. The low serum (2% FBS) medium which was used Nakamaya et al, was proven to reduce the level of endogenous α-T to half after 24 h incubation (Nakamaya et al, 2003).

4.3.8.2 Pre-irradiation treatment

On day 1, HaCaT cells were plated in black-walled 96-well plates at a concentration of 10,000 cells per well in full media and left overnight to adhere. On day 2, the medium was aspirated from all wells of the plates and washed twice with warmed PBS (100 µL/well). The cells were treated with (100 µM) of α-TP (1.5% tris buffer in medium was used as a control) or α-T (0.5 % ethanol in medium was used as a control) as described in Section 4.3.8.1, and then incubated at 37 °C with 5% CO₂ for 24 h. On Day 3, the treatment solutions were aspirated from all wells of the plates and the cells were washed twice with warmed PBS (100 µL/well). Fresh PBS (100 µL/well) was then added to each well before UVA1 irradiation. Cells were irradiated, without the plate lid, one plate at a time for 33 min, or 44 min to obtain
a UVA1 dose of 170 J/cm² or 226 J/cm², for non-starved full-serum and starved low-serum conditions, respectively. Cells were kept on a cooling platform to keep them at around 37°C. Unirradiated controls (covered with foil) were kept in the same conditions as the longest exposure time to ensure any differences observed were due to the UVA1 exposure rather than any confounding factors. After irradiation, the PBS was removed, and the wells were washed twice with PBS and then medium was replaced, and the plates were returned to the incubator. Then, the cell viability was measured using both Alamar blue® and neutral red at 24 h post irradiation.

4.3.8.3 Post-irradiation treatment

The HaCaT keratinocytes were plated on day 1 in the same way described above (Section 4.3.8.2). On day 2, the cells were washed twice with fresh warmed PBS and fresh PBS (100 μL/well) was immediately added to each well before UVA1 irradiation. Then, cells were immediately irradiated with required UVA1 doses (170 J/cm² for non-starved full-serum condition or 226 J/cm² for starved low-serum condition), which was followed by immediate treatment with 100 μM of α-TP or α-T dissolved in full-serum or low-serum media, respectively. Then, the cell viability was measured 24 h post the irradiation using Alamar blue® or neutral red as the method described in Section 4.3.7.
4.3.9 Reactive oxygen species assays

4.3.9.1 H$_2$DCFDA ROS assay

4.3.9.1.1 Background

ROS production was determined using the 2’,7’-dichlorodihydrofluorescein diacetate (DCFDA, also known as H$_2$DCFDA) cellular ROS detection assay kit (Abcam, Cambridge, UK). The H$_2$DCFDA is a fluorogenic dye used for the quantitative measurement of cellular ROS (hydroxyl, peroxyl and other species) activity within the cell. This dye diffuses into cells and is subsequently deacetylated by cellular esterases to non-fluorescent compound (H$_2$DCF), which is later oxidized by ROS into highly fluorescent 2’, 7’-dichlorofluorescein (DCF). Fluorescence was measured by spectroscopy, with an excitation spectrum at 495 nm and emission spectrum at 529 nm. The ROS can be quantified for up to 6 h (the time period for which intracellular DCFDA is stable).

4.3.9.1.2 Preparation of treatment solutions

A 178 µL aliquot of 6 mM α-TP in tris buffer (0.1 M) was added to 5 mL of low-serum (2%) high glucose phenol red free medium or PBS to obtain 200 µM α-TP, and then 50 µL was added to each well that contained 50 µL of the same medium or PBS to get 100 µM α-TP in each well. The α-T solution was prepared using a stock solution of 20 mM. Then, 200 µM of α-T was prepared by adding 100 µL of the 20 mM α-T stock solution in 10 ml of 2% low-serum high glucose phenol red free medium or PBS, and then 50 µL of was added to each well contained 50 µL of the same medium or PBS to get 100 µM α-T in each well.
4.3.9.1.3 Pre-irradiation treatment

HaCaT keratinocytes were grown to 100% confluence in 75 cm$^2$ flask, washed, trypsinized, centrifuged, counted and then cells were seeded at a concentration of 25,000 cells per well (according to the manufacturer recommended protocol of the ROS detection assay) in full medium in black-walled, clear bottom 96-well plate and left for 24 h to adhere. Then, the medium was aspirated, and the cells were washed in PBS and treated with 100 µL of a 100 µM α-TP or α-T for 24 h and then immediately washed with 1 x PBS followed by incubation with 100 µL of a 20 µM DCFDA solution in the dark for 45 min. Later, the cells were washed by PBS followed by UVA1 irradiation (57 J/cm$^2$ (10 min exposure)). Tertiary Butyl hydroperoxide (TBHP) was added into the assigned positive control wells at concentration 250 µM. Fluorescence intensity from cells of each well was measured using a FLUOstar-Omega microplate reader (BMG LABTECH, Offenburg, Germany) with a 485 nm excitation filter and a 520 nm emission filter.

4.3.9.1.4 Post-irradiation treatment

HaCaT keratinocytes cells were seeded in full medium, followed by incubation with DCFDA in the same way as described in Section 4.3.9.1.3. Then, the DCFDA solutions were aspirated and 100 µM α-TP or α-T solutions dissolved in PBS was added to each well (100 µL/well) and the positive control, negative, unstained wells were covered with aluminium foil, and the remaining designated wells were immediately irradiated with UVA1 (57 J/cm$^2$, (10 min)). Finally, fluorescence intensity from cells of each well was measured as described above.

4.3.9.2 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Assay

DPPH is hydrophobic dye, so its reactions must be run in organic solvents. Literature reports mostly attribute DPPH reactions to hydrogen atom transfer
(HAT), reactions in strong hydrogen-bonding solvents such as methanol interfere with release of hydrogen atoms and thus strongly enhance single electron transfer (SET) over HAT (Barclay et al., 1999; Foti et al., 2008, 2004).

In this Chapter the DPPH assay was used to assess the test compound for their ability to quench free radicals by electron transfer. A 100 µM stock of DPPH (Alfa Aesar, Heysham, UK) was prepared in 100% methanol and 187.5 µL was added to the wells of a 96 well plate. A 100 mM stock of the test compounds was used to prepare 14 serial dilutions (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.20, 0.1, 0.05, 0.025, 0.012, and 0.006 mM) in the solvent in which the test compound was dissolved e.g. ascorbic acid was dissolved in DMSO, α-T was dissolved in methanol, and α-TP was dissolved in water. Thereafter, 12.5 µL of each serial dilution, and the control vehicle, was added to each well and mixed to get final concentrations of (6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05, 0.025, 0.012, 0.006, 0.003, 0.0015, 0.0008, and 0.0004 mM, respectively). The plate was protected from light and placed on a shaker at room temperature for 30 min. Absorbance was measured at 517 nm using an Infinite 200 PRO spectrofluorometer (Tecan Group Ltd, Mannedorf, Switzerland). Each condition was tested in triplicate and the average calculated. The percentage inhibition of DPPH was calculated, and a graph of percent inhibition vs concentration was plotted. Linear regression analysis was carried out to calculate the effective concentration for 50% inhibition (IC_{50}) for each compound.

4.3.9.3 Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay quantifies the anti-oxidant properties of a compound by its ability to inhibit peroxyl radicals (ROO•) from oxidising fluorescein, and consequently
reducing fluorescence. The assay was carried out with the ORAC Antioxidant Assay Kit (Zenbio, Research Triangle Park, North Carolina, USA) according to the manufacturer’s instructions. Trolox standards were prepared in the assay buffer (0-100 µM) along with serial dilutions of the test compounds. A 150 µL aliquot of the fluorescein working solution was added to the inner wells of a 96 well plate, with 25 µL of each of the standards or test compound in duplicate, and the plate was incubated at 37°C for at least 15 min. A 2,2′-azobis-2-methyl propanimidamine dihydrochloride (APPH) working solution was then added to each well (25µL) to start the reaction. Fluorescence was measured in a preheated incubation chamber (37°C) using an Infinite 200 PRO spectrofluorometer (Tecan Group Ltd, Mannedorf, Switzerland) with excitation/emission = 485/530 nm taken immediately and then every minute for 30 min. Standard curves were generated for each compound and the area under the curve (AUC) calculated. Each compound tested was then expressed as a Trolox equivalent concentration.

4.3.9.4 Total antioxidant capacity after supplementation of HaCaT keratinocytes with α-tocopherol phosphate

HaCaT keratinocytes were seeded in 12-well plate with targeted density of 400,000 cell per well (to reach 80-90% confluency in 12-well plate) and incubated for 24 h. Medium was aspirated from each well and then washed with 1 x BPS followed by treatment with 1000 µL of α-TP (31.25, 62.5, 125, 250, 125, 250, 500, and 1000 µM for 10 h. After penetration into the cells, an enzymatic de-phosphorylation of α-TP by phosphatase or esterase can be postulated and with a conversion back to free α-T as this activity has been detected in cell culture and in tissues (Gianello et al., 2005; Kagan et al., 1990; Nakayama et al., 2003; Negis et al., 2005). The cells
were then immediately were washed three times with ice cold PBS and then 1000 µL of 1% triton (prepared in PBS) was added to lyse the cells. The samples were then centrifuged for 10 min at 13,000 rpm, 4°C and supernatant was stored in -70°C until analysis for total antioxidant capacity using ORAC assay.

4.3.10 Statistical analysis

All data are expressed as the mean ± standard deviation (SD) where n=3 unless stated otherwise. The homogeneity of variance (Levene’s test) and the normality (Shapiro-Wilk test) of all sample groups data were assessed prior to statistical analysis. The statistical analysis was performed using statistical package of social sciences, SPSS version 17 (IBM Corp., USA) with a significance level of 0.05. The comparison was done using student’s t-test, ANOVA with multiple comparisons tests (Tukey’s, or Dunnett’s test), and linear regression. The means (n=3) of area under the curve (AUC) of fluorescence intensity over 2 h were calculated for each condition. The mean difference in AUC of fluorescence between the positive control (TBHP), UVA1-irradiated vehicle control, UVA1-irradiated with the treatment, and the unirradiated vehicle control were calculated. Differences of fluorescence intensity AUC between UVA1 irradiated vehicle control and UVA1 irradiated with the pre- or post-irradiation treatment with α-TP or α-T were analyzed using paired t-test analysis.

4.4 Results

4.4.1 UVR emission and absorption spectra of the test antioxidants

The UVR emission spectrum (385 nm Loctite UVA) was compared with the absorption spectra of the test compounds to exclude any possibility of a sunscreening effect. No overlap was seen with either compound (fig 4.5).
Absorption of α-TP (MW: 554.6 g/mole) and α-T (MW: 431 g/mole) is primarily between 270-320 nm (UVC and UVB) with peaks at 287 nm and 290 nm, respectively.

![Absorption spectrum of α-TP and α-T](image)

**Fig.4.5:** The emission spectrum of the “385 nm Loctite UVA” was measured with a Bentham DM 150 double monochromator spectroradiometer. Also shown are the absorbance spectra of 400 µM (0.22 mg/mL) α-TP and 400 µM (0.18 mg/mL) α-T in methanol. There is strong absorbance in the UVB region with a peak absorbance at 288 nm and 291 nm, respectively. α-T refer to natural α-T which is mostly equivalent to synthetic α-T and its molar concentration refer to the whole natural product (see note in page 154).

### 4.4.2 Photostability

The photostability of α-TP and α-T was assessed by UV spectrometry. α-TP was diluted in tris buffer at a concentration of 0.05% w/v (1mM) whereas an equivalent molar concentration of α-T was diluted in ethanol. Both were exposed to increasing doses of SSR, and absorbance was measured between 280- 400 nm after each dose with results displayed in Figure 4.6. Doses used, total absorbance and percentage reduction absorbance are described in Table 4.2. Even at the highest dose of 50 SED there was an increase in the total absorbance, which is probably because of solvent
evaporation and indicates α-TP is a photostable molecule compared to α-T which showed up to 15% reduction in total absorbance at 50 SED.

Fig.4.6: Photostability of α-TP (a) and α-T (b) when exposed to high doses of SSR. A concentration of 0.5 mg/mL (1 mM) of α-TP or 0.43 mg/mL (1 mM) of α-T was exposed to increasing doses of SSR (10-50 SED) and the absorbance subsequently measured by UV spectrometry between 278-320 nm (n=3), there was a minimum level of photodegradation compared to α-T described in table 4.2.
Table 4. 2: The doses of SSR used and percentage reduction in absorbance for α-TP and α-T photostability study. The equivalent doses (J/cm²) for each dose (SED) used to test the photostability of 0.5 mg/mL (1 mM) α-TP or 0.43 mg/mL (1 mM) of α-T. The percentage degradation compared to 0 SED as measured by UV spectrometry between 278-320 nm (n=3).

<table>
<thead>
<tr>
<th>Dose (SED)</th>
<th>Exposure time</th>
<th>Dose (J/cm²)</th>
<th>Abs. at 288 nm</th>
<th>% degradation of α-TP</th>
<th>Abs. at 291 nm</th>
<th>% degradation of α-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 s</td>
<td>0</td>
<td>0.92±0.02</td>
<td>0</td>
<td>0.78±0.08</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>70 s</td>
<td>11.4</td>
<td>0.92±0.02</td>
<td>0.03</td>
<td>0.81±0.04</td>
<td>3.22</td>
</tr>
<tr>
<td>20</td>
<td>140 s</td>
<td>25.5</td>
<td>0.93±0.03</td>
<td>1.41</td>
<td>0.77±0.82</td>
<td>-1.61</td>
</tr>
<tr>
<td>30</td>
<td>210 s</td>
<td>37.8</td>
<td>0.93±0.02</td>
<td>1.92</td>
<td>0.73±0.04</td>
<td>-6.65</td>
</tr>
<tr>
<td>40</td>
<td>280 s</td>
<td>51.9</td>
<td>1.01±0.03</td>
<td>10.18</td>
<td>0.73±0.11</td>
<td>-6.95</td>
</tr>
<tr>
<td>50</td>
<td>350 s</td>
<td>63.3</td>
<td>1.02±0.05</td>
<td>11.34</td>
<td>0.67±0.06</td>
<td>-14.61</td>
</tr>
</tbody>
</table>

4.4.3 Cell viability with photoprotective compounds

The α-TP was well tolerated in HaCaT keratinocytes and the IC₅₀ was comparable using the Alamar blue® and neutral red assays (1676 and 1070 µM, respectively) (figure 4.7). The α-T was also well tolerated by HaCaT keratinocytes dissolved in 0.5% ethanol in full medium (see figure 4.8).
Fig. 4.7: Tolerability of HaCaT keratinocytes to α-TP (0.61-10,000 μM), diluted in Dulbecco's modified eagle's cell culture medium (with FBS) at 37 °C for 24 h. Lethal dose 50 of α-TP on HaCaT keratinocytes was 1676 μM and 1070 μM using Alamar blue® (a) and neutral red (b) cell viability assays, respectively. Data represents mean ± standard deviation (n=3).
Fig.4. 8: Tolerability of HaCaT keratinocytes when treated with (0.005-4,650 μM) of α-T concentrations, diluted in 0.5% ethanol, 95% Dulbecco’s modified eagle’s cell culture medium (with FBS) at 37°C for 24 hours. The α-T was well tolerated on HaCaT keratinocytes (lethal dose 50 > 4650 μM) using Alamar blue® (blue columns) and neutral red (red columns) cell viability assays. All the concentrations show viability ≥ 90%. Data represents mean ± standard deviation, (n=3).

4.4.4 Cell viability (UVA dose-response)

Cell viability was assessed at cell densities 10,000 cells per well using the Alamar blue® and neutral red assays. This was assessed 24 h after incremental doses of 385 nm UVA1 on HaCaT keratinocytes. The Alamar blue® and neutral red assays gave comparable results (Fig 4.9). For example, after 57 J/cm² mean viability was 92.52 vs 89.94%, after 115 J/cm² it was 86.39 vs 88.04%, after 140 J/cm² it was 86.08 vs 81.61%, after 170 J/cm² it was 80.27 vs 77.63% and after 226 J/cm² it was 71.42 vs 72.97%, respectively. The UVA1 dose of 170 J/cm² was selected to perform the photoprotection studies using cell viability reduction as the endpoint.
Fig. 4. 9: Dose-response cell viability for 10,000 cell/well targeted cell density 24 h post irradiation using the Alamar blue® and neutral red assays. Both assays showed a significant dose dependent decrease in cell viability (Alamar blue® – $p < 0.0001$; neutral red $p < 0.01$; n=3, linear regression analysis) with no significant difference between both assays ($p > 0.05$; n=3, linear regression analysis). Data represents mean ± standard deviation of three separate experiments.

4.4.5 Photoprotection effects of α-TP and α-T pre-treatment against cell viability reduction in keratinocytes

4.4.5.1 Pre-irradiation treatment with antioxidants

Studies with full-serum and low-serum medium were done, as low serum has been shown previously to cause intracellular depletion of endogenous α-T after exposure to UVR. Low serum was compared with full-serum studies, as the latter may not induce depletion of endogenous α-T after exposure to UVR and underestimate the photoprotection. Figure 4.10 shows a dose response (57-170 J/cm²) for UVA1 irradiation in the absence of any treatment. It also shows the effects of treatment of keratinocytes with 80 µM α-T or α-TP in 10% full-serum medium prior to 170 J/cm² UVA1 that resulted in equivalent cell viability (93.26 ± 5.14 % and 94.90 ± 2.51 % (n=3), respectively tends to increase Alamar blue® viability compared to the cells irradiated with control vehicle (80.27 ± 8.96 % (mean of n=3 ± SD)) ($p = 0.024$, and $p = 0.012$, respectively, one-way ANOVA with Dunnett’s multiple
comparisons test). However, when the HaCaT keratinocytes were treated with 80 µM α-T or α-TP in low-serum (2%) medium prior to UVA1 irradiation (226 J/cm²) only the treatment with 80 µM α-TP increased the cell viability up to 88-92% (by 14%) compared to cells irradiated with the control vehicle (78%) using both Alamar blue® and neutral red assays (Alamar blue® - p = 0.009, neutral red- p = 0.014, one-way ANOVA with Dunnett’s multiple comparisons test) while α-T did not (70-74%) (Fig. 4.11).

**Fig.4.10: Dose-response viability with pre-UVA1 treatment with α-TP or α-T in HaCaT keratinocytes.** HaCaT keratinocytes were treated with positive control (Triton 1%), unirradiated, pre-UVA1 treated for 24 h with vehicle control exposed to 57, 115, 170 J/cm² with PBS, or pre-UVA1 (170 J/cm²) treated for 24 h with 80 µM α-TP or α-T in full-serum (10%) medium. Cell viability was assessed 24 h later by the Alamar blue® assay (a) or the neutral red assay (b). Both the pre-UVA1 treatment with 80 µM α-TP and α-T significantly protect against cell death (p = 0.024 and p = 0.014 respectively, one-way ANOVA with Dunnett’s multiple comparisons test) compared to irradiated cells treated with the vehicle control by Alamar blue® assay. The effects were not significant with the neutral red assay (p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test). Data represents mean ± standard deviation of three separate experiments. The α-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).
Fig.4.11: Dose-response viability with Pre-UVA1 treatment with \( \alpha \)-TP or \( \alpha \)-T in HaCaT keratinocytes. HaCaT keratinocytes were treated with positive control (Triton 1%), unirradiated, pre-UVA1 treated for 24 h with vehicle control exposed to 57, 115, 226 J/cm\(^2\) with PBS, or pre-UVA1 (226 J/cm\(^2\)) treated for 24 h with 80 \( \mu \)M \( \alpha \)-TP or \( \alpha \)-T in low-serum (2%) medium. Cell viability was assessed 24 h later by the Alamar blue\textsuperscript{®} assay (a) or the neutral red assay (b). Only the pre-UVA1 treatment with 80 \( \mu \)M \( \alpha \)-TP significantly protect against cell death (Alamar blue\textsuperscript{®} - \( p = 0.009 \), neutral red - \( p = 0.014 \), one-way ANOVA with Dunnett’s multiple comparisons test) compared to irradiated cells treated with the vehicle control by Alamar blue\textsuperscript{®} and neutral red assay. Data represents mean ± standard deviation of three separate experiments. The \( \alpha \)-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).

4.4.5.2 Post-irradiation treatment with antioxidants

The treatment of keratinocytes with 80 \( \mu \)M \( \alpha \)-TP or \( \alpha \)-T in full-serum (10%) or low serum (2%) media post UVA1 irradiation (170 J/cm\(^2\) or 226 J/cm\(^2\)) has no effect on 24 h cell viability compared to the irradiated vehicle control (see fig. 4.12 and fig. 4.13).
Fig. 4.12: Dose-response viability with post-UVA1 treatment with α-TP or α-T in HaCaT keratinocytes. HaCaT keratinocytes were treated with Triton 1% (positive control), unirradiated, post-UVA1 treated for 24 h with vehicle control immediately after exposed to 57, 115, 170 J/cm² with PBS, or post-UVA1 (170 J/cm²) immediately treated for 24 h with 80 µM α-TP or α-T in full-serum (10%) medium. Cell viability was assessed 24 h after UVA1 exposure by the Alamar blue® assay (a) or the neutral red assay (b). The post-UVA1 treatment with 80 µM α-TP or α-T did not protect against cell death (Alamar blue®, $p > 0.05$, neutral red- $p > 0.05$, one-way ANOVA with Dunnett’s multiple comparisons test) compared to irradiated cells treated with the vehicle control. Data represents mean ± standard deviation of three separate experiments. The α-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).
Fig. 4.13: Dose-response viability with post-UVA1 treatment with α-TP or α-T in HaCaT keratinocytes. HaCaT keratinocytes were treated with Triton 1% (positive control), unirradiated, post-UVA1 treated for 24 h with vehicle control immedietly after exposed to 57, 115, 226 J/cm² with PBS, or post-UVA1 (226 J/cm²) immedietly treated for 24 h with 80 µM α-TP or α-T in low-serum (2%) medium. Cell viability was assessed 24 h after UVA1 exposure by the Alamar blue® assay (a) or the neutral red assay (b). The post-UVA1 treatment with 80 µM α-TP or α-T did not protect against cell death (Alamar blue®, p > 0.05, neutral red- p > 0.05, one-way ANOVA with Dunnett’s multiple comparisons test) compared to irradiated cells treated with the vehicle control. Data represents mean ± standard deviation of three separate experiments.

4.4.6 Reactive oxygen species assays

4.4.6.1 H₂DCFDA ROS assay (Pre-UVA1 treatment)

The generation of ROS in HaCaTs (figure 4.14) was significantly higher after UVA1 compared to unirradiated cells (p < 0.0001, paired t-test, comparing two conditions). The positive control (250 µM TBHP) generated ROS to a lower extent than by UVA1. The pre-treatment of HaCaT with 100 µM α-TP prior to irradiation with UVA1 caused a significant reduction of ROS generation (ca. 24.1% in ROS generation compared with the vehicle control, p = 0.0026, paired t-test, comparing two conditions) similar to α-T (ca. 23.9% reduction in ROS generation, p = 0.0034,
compared to the vehicle control, paired t-test, comparing two conditions) (see table 4.3 and fig. 4.15).

Fig. 4.14: Pre-treatment studies on HaCat keratinocytes. The generation of ROS in HaCat keratinocytes at 2 min intervals after UVA1 irradiation over 1.8-2 h. HaCat were pretreated with vehicle control or 100 µM of antioxidant (α-TP or α-T) for 24 h and subsequently re-incubated with 20 µM DCFDA for 45 min and then immediately washed with PBS and exposed to a UVA1 dose of 57 J/cm². The TBHP was added into the reserved positive control wells at a concentration of 250 µM. Data represent mean ± standard deviation (n=3). The α-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).
Table 4.3: The generation of ROS in HaCaT keratinocytes pre-treated with antioxidants

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean (n=3) difference (a.u.) over 2 h compared to unirradiated control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive control (TBHP)</td>
</tr>
<tr>
<td>HaCaT + pre-αTP</td>
<td>-8,414,000</td>
</tr>
<tr>
<td>HaCaT + pre-α-T</td>
<td>-7,685,000</td>
</tr>
</tbody>
</table>

Fig.4.15: The ROS induced by UVA1 over 2 h with or without antioxidant pretreatment in HaCaT keratinocytes. HaCaT keratinocytes were pre-UVA1 treated for 24 h with vehicle control or 100 µM antioxidant (α-TP or α-T) for 24 h and subsequently re-incubated with 20 µM DCFDA for 45 min and then immediately washed with PBS and exposed to a UVA1 dose of 57 J/cm² in PBS. The TBHP was added into the reserved positive control wells at a concentration of 250 µM. Fluorescence intensity analysis of area under the curve (AUC) from figure 4.14 (cumulative data) in HaCaT keratinocytes generating ROS over 1.8-2 h. Both the α-TP and the α-T showed significant reduction in ROS production (p = 0.0026, and p = 0.0034 compared to the vehicle control, respectively, paired t-test, comparing two conditions) when added pre-UVA1 exposure. Data represents mean ± standard deviation of three separate experiments. The α-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).
4.4.6.2 H2DCFDA ROS assay (Post-UVA1 treatment)

ROS generation in HaCaT keratinocytes (figure 4.16) was significantly higher after UVA1 compared to unirradiated cells ($p < 0.0001$). The treatment of HaCaTs with 100 µM α-TP immediately post-irradiation has no significant reduction of ROS generation similar to α-T ($p > 0.05$, paired t-test) (see table 4.4 and fig. 4.17).

![Graph](image1)

**Fig.4.16:** Post-treatment studies on HaCat keratinocytes. ROS generation in HaCaT keratinocytes at 2 min intervals after UVA1 irradiation over 2 h. The HaCaT keratinocytes were incubated/stained with 20 µM DCFDA for 45 min and then washed with PBS and then post-treated with vehicle control, or 100 µM of antioxidant (α-TP or α-T) and immediately exposed to a UVA1 dose of 57 J/cm². The TBHP was added into the reserved positive control wells at concentration 250 µM. Data represent mean ± standard deviation (n=3). The α-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).
Table 4.4: ROS generation in HaCaT keratinocytes post-treated with antioxidants

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean (n=3) difference (a.u.) over 2 h compared to unirradiated control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive control (TBHP)</td>
</tr>
<tr>
<td>HaCaT + post-αTP</td>
<td>-6,454,000</td>
</tr>
<tr>
<td>HaCaT + post-α-T</td>
<td>-6,512,000</td>
</tr>
</tbody>
</table>

Fig.4.17: The ROS induced by UVA1 over 2 h with or without antioxidant post-treatment in HaCaT keratinocytes. The HaCaT keratinocytes were incubated/stained with 20 µM DCFDA for 45 min and then washed with PBS and then post-treated with vehicle control, or 100 µM of antioxidant (α-TP or α-T) and immediately exposed to a UVA1 dose of 57 J/cm². The TBHP was added into the reserved positive control wells at concentration 250 µM. Fluorescence intensity analysis of area under the curve (AUC) from figure 4.16 (cumulative data) in HaCaT keratinocytes generating ROS over 2 h. Both the α-TP and the α-T did not show significant reduction in ROS production ($p > 0.05$ compared to the vehicle control, paired t-test, comparing two conditions) when added post-UVA1 exposure. Data represents mean ± standard deviation of three separate experiments. The α-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).
4.4.6.3 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Assay

The α-TP exhibited poor DPPH activity, with an IC₅₀ value of 1038 µM or 575.7 µg/mL. The IC₅₀ values of L-ascorbic acid (L-AA) and α-T were 18.7 and 24 µM, respectively using curve fitting (figure 4.18) but linear regression shows IC₅₀ values of TP, L-AA, and α-T equal to 1380, 21.7, and 21.4 µM, respectively, i.e., the DPPH activity increases as follows: L-ascorbic acid, α-T > α-TP (see figure 4.19).

![Graph showing DPPH radical scavenging ability of potent antioxidants compared with α-TP.](image)

**Fig.4.18:** The DPPH radical scavenging ability of potent antioxidants compared with α-TP. The L-ascorbic acid (L-AA), α-T, and α-TP test solutions were dissolved in DMSO, methanol, and water, respectively. A 12.5 µL aliquot of known antioxidant solutions of increasing concentration (0.006-50 mM) were added to wells of 96-well plate containing 187.5 µL of a 100 µM DPPH stock solution dissolved in methanol. The plate was shaken for 30 min at 25 °C. Then, the absorbance was determined at 517 nm and the graph of % DPPH inhibition vs log final concentration was plotted. The IC₅₀ values of α-TP, α-T, and L-ascorbic acid (L-AA) were determined by gnuplot prism non-linear curve fitting (Gaddum/Schild EC₅₀ fitting). Data represents mean ± standard deviation of three separate experiments. The α-T molar concentration refers to the whole natural product and does not adjust for purity (see note in page 154).
Fig. 4.19: The DPPH radical scavenging/inhibition activity of α-TP (a), L-ascorbic acid (b), and α-T (c). All tested antioxidants show an ability to quench the DPPH radical as a measure of radical scavenging activity in a concentration-dependant manner. All compounds demonstrated significant activity ($p < 0.0001$, linear regression analysis). The standard deviation was too small to be seen (n=3).
4.4.6.4 Oxygen Radical Absorbance Capacity (ORAC) Assay

The concentration of L-ascorbic acid and α-TP equivalent to 100 µM Trolox were 107.02 and 7599 µM, respectively (fig. 4.20). The order of activity was Trolox>L-AA> α-TP. The ORAC assay did not test α-T which is hydrophobic and insoluble in the reaction buffer and any addition of co-solvent may interfere with the assay. Trolox was used instead as it is a water-soluble vitamin E derivative.

![Graphs showing ORAC assay results](Image)

**Fig.4.20:** The oxygen radical scavenging ability of water-soluble Trolox, L-ascorbic acid (vitamin C), and α-TP. Antioxidant solutions, of increasing concentration, were tested for their ability to quench the ROO\(^*\) radical (APPH) as a measure of anti-oxidant capacity. They all demonstrated a significant concentration-dependant ability to quench the ROO\(^*\) radical (APPH) \(p < 0.0001\), linear regression analysis). The standard deviation was too small to be seen \(n=3\).

4.4.6.5 Total antioxidant capacity after supplementation of HaCaT keratinocytes with α-tocopherol phosphate

Supplementation with the exogenous antioxidant α-TP, at different concentrations (0 -1000 µM), which may be converted back to α-T by phosphatase, and thereby increase the intracellular α-T level in HaCaT keratinocytes, has no effect on the total cellular antioxidant capacity using ORAC assay compared to the untreated controls (0 µM α-TP, full-serum DMEM) (fig.4.21). This may be due to serum present in the media that preserves high level of endogenous α-T.
Fig. 4.21: Supplementation with exogenous α-TP (300 -1000 µM) does not enhance total cellular antioxidant capacity using the ORAC assay compared to full-serum medium.

4.5 Discussion

There was no overlap between the absorption spectra of either α-TP or α-T with the 385 nm UVA1 emission spectrum, which excludes any sunscreen effect of the test compounds in the subsequent studies.

The absorbance of α-TP increased with increasing SSR dose probably because of solvent evaporation, whereas the α-T absorbance decreased significantly at 30-50 SED by 7-14.6% which indicates its photodegradation. This is in agreement with reports that α-T is photodegraded by 90% after 1 h irradiation with SSR (dose: 198 kJ/m²), and thus led to reduction in antioxidant activity (Scalia et al., 2013).

The α-TP reduced the cell viability in HaCaT keratinocytes to a greater extent than α-T (LD₅₀ was 1.1-1.8 mM vs > 4.70 mM, respectively) suggesting the phosphate group enhanced the ability of the molecule to enter the cells. The cytotoxicity of α-TP strongly depends on its isomer form (Dolfi et al., 2013) and the cell line tested.
In previous work, it was shown that α-TP has selective toxicity against TPH-1 macrophages and reduces cell viability by 7.7% at 500 µM, while α-TP was well tolerated at 500 µM in human gingival fibroblasts (HGF-1). In the same study, it was shown that α-T was well tolerated and not toxic in both THP-1 and HGF-1 at a concentration of 5000 µM (Harper, 2017). Negis et al found that THP-1 cell proliferation was more sensitive to α-TP than α-T, i.e., α-TP inhibits cell growth, while α-T did not produce any significant effect (Negis et al., 2007). It has been reported that γ-form of TP showing better growth inhibitory activity than the δ-TP (IC50 was 30 vs 55 µM, respectively) in the human colorectal cancer cell (HCT116 and HT29 cells). However, the α-form of α-TP were the least inhibitory compared to other isomers (IC50 > 100 µM) (Dolfi et al., 2013).

Solar UVR radiation causes skin photoageing and skin cancer in humans. Several studies have demonstrated that supplementation with antioxidants decreases UVR-induced skin damage in vitro and in vivo (Swindells and Rhodes, 2004). In this chapter, we demonstrated the ability of α-TP to reduce UVA1-related damage in human HaCaT keratinocytes if applied prior to UVA1 exposure. For instance, treatment of HaCaTs with α-TP for 24 h prior to 170 J/cm² UVA1 exposure tend to increase cell viability comparably to α-T using the Alamar blue® assay. This data suggested that 80 µM of α-TP in non-starved full-media showed to protect HaCaTs against UVA1-induced cell death, as there is a strong evidence in the literature that pre-and post-irradiation treatments with vitamin E are beneficial by increasing the cell viability, scavenging the ROS and protection from DNA photolesions, etc. (Delinasios et al., 2018; Premi et al., 2015; Sethi, 2017; Wu et al., 2014).

Cell survival was 78% when HaCaT keratinocytes were treated with the starved low-serum (2%) vehicle control irradiated with UVA1 dose of 226 J/cm². The 80
μM of α-TP pre-treatment in starved low-serum (2%) medium exhibited superior protection and significantly increased the cell viability fraction by 14% whereas α-T has no significant increase on cell viability (88-92% vs 70-74%, respectively) compared to its irradiated vehicle control (78%) at the highest UVA1 dose (226 J/cm²) tested using both Alamar blue® and neutral red assays. This method have been used by Nakayama and colleagues, and they found that incubation the skin with 2% FBS has significantly depleted the endogenous level of α-T to half after 24 h incubation and they also showed that the treatment of 0.5% α-TP dissolved in 2% DMEM not only inhibited the endogenous α-T depletion, but also increased the level of α-T by two-fold compared to untreated cells even 24 h after incubation. In the same study, the UVB exposure found to significantly reduce the endogenous α-T level in cultured skin by half compared to unirradiated skin. This reduction was inhibited by pre-UVB treatment with α-TP for 3 h which induced a 2-fold rise in α-T level compared to irradiated cells treated with vehicle control (Nakayama et al., 2003). This may be due to the release of α-T from the pro-vitamin E inside the keratinocytes by the phosphatase enzymes and this may prolong the protection effect even after 24 h. Another possible reason is the antioxidant mechanism of α-TP is dependent on its chemical structure that allows its incorporation into biomembranes, and thus it may inhibit the propagation of radical species (Rezk et al., 2004). A recent study demonstrated that treatment of HaCaT with 2.9 IU/mL (2 mg/mL) of α-T in serum-free media for 24 h prior to UVA1 (365 nm) irradiated with 8 J/cm² show a significant increase in the cell survival fraction by 15% compared to irradiated vehicle control (60.2% vs 45%, respectively) (Wu et al., 2014).
It is also reported in the literature that during and following UVA irradiation, the generation of ROS is significantly increased in exposed cells (Morita and Krutmann, 2000; Tyrrell, 1994). As UVA-related biological effects are largely mediated by ROS, their elimination is essential for protection against UVA damage. In this work, pre-irradiation treatment with 100 µM α-TP reduced UVA1-induced ROS generation in HaCaTs similar to α-T (by ~24.1% and 23.9%, respectively) compared to the irradiated vehicle control treated cells. Wu et al., investigated the photoprotection of the HaCaT keratinocytes using SSR with a fixed wavelength of 365 nm as UVA1 source and monitored the ROS generated from UVA exposure using the same dichlorodihydrofluorescein assay. They reported that applying of 2.9 IU/ml of α-T in serum free medium caused significant reduction (80%) in ROS generation compared to irradiated vehicle treated cells (742.5 vs. 3,952.2 fluorescence intensity/protein, 1/mg protein, p < 0.05) (Wu et al., 2014). The difference in the degree of protection with our findings may be due to the differences in UVA1 spectrum and dose, the correction of fluorescence intensity per amount of protein as well as the treatment protocol (the level of supplements in growth medium). As Wu, et al used low (8 J/cm²) UVA1 dose (less oxidative stress) compared to our relatively high (57 J/cm²) UVA1 dose. This low dose of 8 J/cm² may generate lower rate and extent of ROS generation i.e. lower fluorescence intensity and thus the protective effect of exogenous tocopherol would be more efficient and easily scavenge ROS that generated at low rate. In addition, the supplement levels were also different as Wu et al, used serum free media to dissolve the α-T which will deplete the level of endogenous α-T in the irradiated vehicle control, and thus, more ROS generation reduction is produced by pre-treatment with exogenous α-T prior to irradiation.
In this work, there are limitations to the indirect approaches which we used to quantify cell death: (i) the HaCaT keratinocyte model, which was shown previously to be highly resistant to UVA-induced necrotic cell death, due to low level of LIP (Zhong et al., 2004) was the only model used in the photoprotection studies. Primary fibroblasts and keratinocytes cell lines were not used, (ii) Fibroblasts are more susceptible to UVA-induced necrotic cell death than HaCaT keratinocytes, and hence the main target for UVA and they should be used along HaCaT keratinocytes in all future photoprotection studies, (iii) the cytotoxicity assays that were used in this work are indirect approaches to quantify cell death caused by UVA1 irradiation, i.e., neutral red assay monitors lysosomal membrane integrity rather than cytotoxicity; the Alamar blue® assay is an indirect enzymatic measurement for oxidative damage. In future work, it would be beneficial to use more direct approaches such as flow cytometry using dual-staining with Annexin V-FLUOS/propidium iodide 24 h after UVA treatment to score the percentage of live, apoptotic, and necrotic cells. Moreover, other human skin models, such as 3D organotypic skin cultures and skin explants could be used to validate UVA photoprotection by α-TP before moving to human studies. It should be noted that animal studies are banned in UK (and EU) for validation of cosmeceutical produces, thus the previously mentioned approaches are used by cosmetics companies prior to obtaining ethical approvals for pilot topical studies in human volunteers (in vivo).

Antioxidants protect cells from the damage caused by free radicals (Blot et al., 1993) which are involved in many disorders such as skin inflammatory disease, cancer, and aging. The antioxidant activity of α-TP was tested with the DPPH assay. The α-TP exhibited a poor DPPH activity, with an IC₅₀ value of 1038 µM or 575.7 µg/mL compared to ascorbic acid and α-T (IC₅₀: 24 and 19 µM, respectively).
new dienamide, (−)-kunstleramide (1) was found to exhibit a poor DPPH activity, with an IC$_{50}$ value of 179.5 ± 4.4 μg/mL compared to ascorbic acid (Mollataghi et al., 2012). In the literature the IC$_{50}$ of Ascorbic acid was higher compared to our study and variable, i.e., 34 μM (Manojlovic et al., 2014), 54.6 μM (Maldonado et al., 2005), 49.5 μM (Kweon et al., 2001). Also the α-T IC$_{50}$ in the literature was higher compared to our finding 40.6 μM (Kweon et al., 2001). Factors such as concentration of DPPH and test compound, purity and stability of the test compound can influence the variations in the antioxidant activity using DPPH assay.

The ORAC assay was used to test the antioxidant activity of α-TP and water-soluble ascorbic acid and compared to Trolox. 100 μM Trolox was equivalent to 107 μM ascorbic acid whereas it was equivalent to 7599 μM α-TP, i.e., Trolox is 1.1 and 75-fold more effective than ascorbic acid and α-TP, respectively. Promden, et al. found that 5 μM Trolox was equivalent to 10 μM ascorbic acid, i.e., Trolox was 2-fold stronger than ascorbic acid (Promden et al., 2014). Our findings agreed fairly well with those of Rezk et al. using the Trolox equivalent antioxidant capacity (TEAC) assay, in which they determined the TEAC for Trolox and α-TP (1.0, and 0.01, respectively) i.e., Trolox was 100-fold stronger antioxidant than α-TP (Rezk et al., 2007).

As discussed, supplementation with the exogenous antioxidant α-TP may increase the intracellular level of α-T in HaCaT keratinocytes. However, such supplementation failed to increase in the total cellular antioxidant capacity using ORAC assay. This result agrees with the hypothesis that the total cellular antioxidant capacity is maintained under tight regulation and is not easily manipulated. For example, one study examined adding several types of antioxidants
including ascorbic acid phosphate to HaCaT, Hep3B, PC3, and Caco-2 cells. A significant increase of intracellular antioxidant concentration had no effect on overall antioxidant capacity which was assessed using several methods, and in some cases it decreased (Koren et al., 2008).

4.6 Conclusions

In conclusion, α-TP had a weak in vitro antioxidant activity when assessed by the DPPH and ORAC assays. α-TP was well tolerated in HaCaT keratinocytes and, in starved conditions, had superior protection against UVA1-induced cell viability reduction compared to the α-T control. Pre-irradiation treatment of HaCaT keratinocytes with α-TP resulted in a similar reduction in ROS generation as α-T. The biological activity of α-TP is likely to be a consequence of its bio conversion to α-T by endogenous cellular phosphatases, which increase the intercellular level of α-T even after UVA1 exposure ends. Photostability studies showed α-T was photodegraded by SSR compared to α-TP which was very photostable. The data this chapter have shown that α-TP had (i) superior photostability, (ii) greater protection against cell viability reduction and (iii) comparable reduction in level of ROS generated by UVA1 exposure compared to α-T. This raised a further area of interest which was to investigate the influence of a physical sunscreen such as nanoparticle addition of α-TP in lotion and semisolid cosmetic product, which was the key step in the following chapter. This screening process is a primary step in sunscreen development as the nanomaterial sunscreen additive may negatively influence the delivery or release of topical antioxidant such as α-TP, and thus, reduce its efficacy, if the latter is being adsorbed on the surface of nanomaterial sunscreen resulting in physical instability.
Chapter 5. Characterising the influence of nanoparticles on the transport of alpha tocopherol phosphate through the membrane

Chapter summary

In this Chapter, the influence of combining a topical antioxidant, α-TP, with sun screening nanoparticles was assessed in a lotion and semisolid gel formulations to exclude any active-excipient interaction. This was done by assessing the change in hydrodynamic size, change in rheological properties, and studying the release profile. The results showed that α-TP adsorbed on the surface of solid lipid nanoparticles as a monomer via hydrophobic interaction whilst it adsorbed onto silica nanoparticles as aggregate via hydrogen bonding. SLN showed an improvement in the release of α-TP that was greater compared to silica nanoparticles indicating that the SLN disrupted the aggregates. The skin deposition showed that α-TP delivery from the gel into the skin was not hindered by presence of SLN indicating that α-TP could be translated into an effective cosmeceutical sunscreen product.
5.1 Introduction

In chapter 4 it was shown that α-TP protects against UVA1 induced cell damage and ROS generation in human skin cells. However, if the α-TP was added to a topical product, it may be combined with sun screening agents such as nanoparticles. The high surface area of nanomaterials can adsorb the amphiphilic α-TP, and thus, may influence its photoprotective effects. Hence, it is important to understand the α-TP-nanomaterial interaction to translate α-TP into an effective cosmeceutical product. The Amphiphilic molecules such as α-TP can self-assemble into extended nanoscale structures when dispersed in topical formulations that are applied to the skin (Harper et al., 2018). This provides another complication when attempting to produce a cosmeceutical product. For example, α-TP micelles can entangle with each other via weak hydrophobic interactions to form long chains, known as ‘worm-like micelles’ (often at concentrations ≥ 1 mM). These nanoscale structures have been shown to influence the viscoelastic properties of the formulation in which they are dispersed, and thus the drug release by the formulation (Raghavan and Douglas, 2012). Unlike the polymer chains, which are hard to break, the α-TP ‘worm like’ micelle chains can form loops that can reversibly associate into a complex 3D structure (Molchanov et al., 2018). The α-TP formulation phase behaviour and interactions with other colloidal systems present in the formulation, e.g., nanoparticles, are influenced by the particular structure taken up by α-TP nanoscale objects (Nettesheim et al., 2008a; Petekidis et al., 2002). Reversible formulation interactions, especially those that may be responsive to an extrinsic or intrinsic stimulus such as light, UV, pH, temperature, etc. are of great interest as they can form dynamic delivery systems (Lin et al., 2009).
The physical (Chapters 2 and 3) and the UVR protection properties (Chapter 4) of α-TP have been described in simple co-solvent solutions, but there is little data to indicate if these properties will change if administered to the skin as a traditional semisolid formulation. It is anticipated that the α-TP will be used in a cosmetic semi-solid product as a topical antioxidant in addition to other nanomaterials that act as physical sunscreens. Like CTAB (cetyltrimethylammonium bromide) with its counter ion (NaBr or KBr) and lecithin-bile salt mixture (Lequeux, 1996) α-TP ‘worm-like’ micelles and their interactions with sunscreens nanomaterials may be consequential on the ability of the formulation to deliver the α-TP into the epidermis of the skin.

α-TP delivered in a commercial cosmetic formulation will be at a relatively high concentration (e.g. 2% w/w or 36 mM), i.e., above the CMC (Kato et al., 2011; Nakayama et al., 2003). Although this aggregation may hinder the transport of α-TP through an artificial model membrane, combining it with nanomaterials could enhance the percutaneous penetration through barrier disruption (Lin et al., 2011), skin occlusion (Dingler et al., 1999; Jenning et al., 2000), and drug de-aggregation (Cai et al., 2016; X. J. Cai et al., 2016) which may counteract this problem to facilitate effective drug release. Silica nanoparticles (diameter of 200 nm, zeta potential -23 mV) have shown previously to adsorb and de-aggregate of a model drug ‘tetracaine’, and produced 3.6-fold enhancement in percutaneous penetration across porcine epidermis compared drug solution without nanoparticles (X. J. Cai et al., 2016). In addition, silica coatings of zinc nanomaterials have been shown to be effective sunscreens (Sotiriou et al., 2014) hence the silica-α-TP interactions could be interesting to assess in the context of cosmetic products for UVR protection.
The aim of this Chapter was to assess how α-TP’s interactions with silica nanomaterials in a cosmetic lotion and semi-solid gel of α-TP would influence the topical formulation and drug release characteristics. The 200 nm diameter silica nanoparticles (with different functional group/surface charges) were selected as a model of the possible silica coated zinc particles, which were not commercially available. The silica particles were compared to 50 nm diameter solid lipid nanoparticles as they provided a highly inert surface. In addition, SLNs act as a physical sunscreen as they have the ability to scatter the UVR (Müller et al., 2000) and thus boosting the sun protecting (Wissing and Müller, 2002). The effect of nanoparticles addition to α-TP lotion and gel formulations was characterised using DLS and with rheology, respectively. Furthermore, the release of the drug from both formulations with and without nanoparticles was understood using a model membrane in a Franz diffusion cell. The potential for the best performing α-TP formulation co-administered with candidate nanoparticles to modify the porcine skin deposition was compared to the α-TP formulation without the nanoparticles.

5.2 Materials

α-TP (all-racemic mixture of 8 isomers or dl-α-tocopherol phosphate, purity: 92.7%, cosmetic grade) was a gift from Showa Denko K.K., Japan. Propylene glycol, ethanol, tris base, tris hydrochloric acid, iso-propanol, HPLC grade water and trifluoroacetic acid (TFA) were purchased from SigmaAldrich, U.K. Medium chain triglycerides of caprylic (C8) and capric (C10) acids (Labrafac® WL 1349, pharmaceutical grade, average MW 512 Da), purified phosphatidylcholine (>90%) from soybean lecithin (Lipoid S75-3N) and PEG 15 hydroxystearate (Solutol® HS 15) were supplied by Gattefosse S.A (Saint-Preist, france). NanoSiO2, NanoSiO2COOH, NanoSiO2NH2 (50 mg/mL) with a nanodiameter of 200 nm were purchased from
Kisker Biotech GmbH and Co., Germany. Ultrapure water (18.2 MΩ) was used. Biotech Cellulose ester (CE) membrane with MWCO 1000 KDa was purchased from Spectrum Lab, USA.

5.3 Methods

5.3.1 Alpha tocopheryl phosphate nanostructures

The nanostructures were formed in a 2% α-TP lotion, prepared from α-tocopherol phosphate (α-TP) disodium salt dissolved in 5 mL of 20% propylene glycol, 20% ethanol, 20% water, 0.1 M tris.HCl at pH 7.4. A 2% α-TP gel was prepared at pH 9, by dissolving a 20 mg of α-tocopherol phosphate disodium salt in 1 mL the ultrapure water and stirred at 32°C in A water bath for 24 h until α-TP completely dissolved and the solubility reached equilibrium. Note that the two pHs were used because the α-TP aggregation differed with composition and pH of formulation, i.e., it forms liposomes in lotion at pH 7.4 and complex 3D structure most likely worm(rod)-like micelles in ultrapure water at pH 9. Moreover, α-TP negatively charge phosphate group was pH-dependant which may have an impact on its interaction with the sunscreen nanomaterials.

The concept of critical packing parameter (C_{pp}) by Israelachivili was initially employed to predict the structures formed by α-TP in water. The C_{pp} is commonly used to predict the geometry of self-assembled structures in aqueous vehicle. It is calculated from equation 5.1, where V_o and l_e are the effective volume occupied in the aggregate core by, and the maximum effective length (critical chain length) of the hydrophobic tail, respectively, and A_0 is the effective hydrophilic head group surface area at the aggregate-solution interface. The amphiphilic compounds are expected to self-assemble into spherical micelles when C_{pp} ≤ 1/3,
worm-like micelles when $1/3 \leq C_{PP} \leq 1/2$, and lamellar/bilayer structures for $C_{PP} \sim 1$ (see Fig. 5.1). Equations 5.2 and 5.3 were used to calculate $V_o$ and $l_c$ (Tanford., 1972). Where $\eta_c$ is the number of carbon atoms in the hydrophobic tail (Israelachvili, 1985; Lombardo et al., 2016; Tao et al., 2015).

$$C_{PP} = \frac{V_0}{A_0 l_c} \quad \text{Equation 5.1}$$

$$V_o = 27.4 + 26.9 \eta_c \quad \text{Equation 5.2}$$

$$l_c = 1.5 + 1.265 \eta_c \quad \text{Equation 5.3}$$
Fig. 5.1: The morphology of self-assembled amphiphilic molecule can be predicted from the critical packing parameter ($C_{pp}$) (Israelachvili, 1985; Lombardo et al., 2016).

### 5.3.2 Solid lipid nanoparticles

The solid lipid nanoparticles (Fig. 5.2) were manufactured via precipitation from a stable emulsion following repeated phase inversion, as previously described by Heurtault et al (2002) (Heurtault et al., 2002). Medium chain triglycerides (MCT) (Labrafac® WL 1349) (17% w/w), phosphatidylcholine soybean lecithin (Lipoid S75-3N) (1.75% w/w), PEG hydroxystearate (Solutol® HS 15) (17% w/w) and a 3% w/v sodium chloride (NaCl) aqueous solution (64.25% w/w) were mixed at room temperature and then heated to 85°C at a rate of 4°C per minute, with continuous magnetic stirring using Stuart™ UC 152 hotplate and stirrer (Cole-Parmer, Stone, UK). The mixture was then allowed to cool to 60°C. The
temperature of the mixture was cycled between 60°C and 85°C a total of three times. Within this temperature range phase inversion occurred, with an oil-in-water emulsion being converted to and from a water-in-oil emulsion. Following the final heating cycle, the emulsion was allowed to cool to 72°C, at which point 25 mL of ice-cold water was added, causing nanoparticle generation. The suspension was stirred for 5 min then made up to a final volume of 50 mL using deionised water. Solid Lipid shell nanoparticle suspensions were purified of excess excipients and larger particulate matter via centrifugation using 1.5 mL amicon tube and washed 4 times using ultra-pure water. The purified suspension of SLN was used for further transport experiments.

![Fig.5.2: Schematic representation of solid lipid nanoparticles prepared by phase-inversion temperature method (Heurtault et al., 2002).](image)

**5.3.3 Nanoparticle size characterization**

The nanoparticle size was determined by the Nanosizer (Malvern Nanoseries Zetasizer ZS 632.8 nm, Malvern Instruments Ltd., U.K). Detection of the light scattering signal was achieved at a 173° backscattering angle with samples equilibrated at 32 °C for 30 seconds. For all the silica nanoparticles samples water
at pH 7.4 (tris.HCl buffer) was used as a dispersant (refractive index 1.33, viscosity 0.8872 cP). For the nanoparticle-α-TP combinations, PG/EtOH/water at pH 7.4 (0.1 M Tris) 10:10:80 was used as the dispersant (additives were propylene glycol, weight%: 10.36 and ethanol, weight%: 8.03, refractive index 1.346, viscosity 1.6064 mPa.s). For the α-TP lotion, PG/EtOH/water 20:20:60 was used as the dispersant (additives were propylene glycol, w/w%: 22.89 % and ethanol, w/w%: 16.36%, refractive index 1.365, viscosity 1.6122 mPa.s). Due to the high viscosity of the 2% gel, sizing measurements could not be determined in the undiluted vehicle. All the samples of NanoSiO2, NanoSiO2COOH, NanoSiO2NH2, solid lipid nanoparticle were mixed with 2% α-TP in a ratio 1:1 (for silica mixture- 400 μL of 2% α-TP lotion, 200 μL of 5% silica nanoparticles and 200 μL of tris buffer at pH 7.4) (for SLN mixture-400 μL of 2% α-TP lotion and 400 μL of SLN), 2% α-TP with water (0.1 M tris buffer at pH 7.4 (400 μL 2% α-TP lotion and 400 μL tris buffer at pH 7.4), NanoSiO2, NanoSiO2COOH, NanoSiO2NH2 and solid lipid nanoparticle were characterized in terms of their size. The AFM was used to image the unmodified silica nanoparticles as method described in Section 2.3.4.

5.3.4 Rheology of formulations

The rheological measurements of each formulation were performed using a cone and plate rheometer (Physica MCR 51, Anton Paar, U.K.) with plate diameter of 50 mm and cone angle of 1.005° and truncation (gap) of 49 μm. Test performed over 1-10 Hz frequency range at constant stress of 0.798 Pa. Ten data point were recorded for each rheogram and triplicates were performed for each formulation. The 2% α-TP gel control (2 mL of 2% α-TP gel with 0.8 mL water), 2% α-TP gel with NanoSiO2, NanoSiO2COOH, NanoSiO2NH2 and SLN (2 mL gel and 0.8 mL nanoparticles) were tested at ambient temperature 20°C to exclude the effect of
changing the temperature which may interfere with nanoparticles interaction with α-TP gel. A slight change in temperature may impact the rheological behaviour of α-TP gel. As temperature increases, molecular thermal energy increases which reduces the intermolecular forces and hence viscosity decreases (Santos et al., 2016).

5.3.5 Drug release

Franz cells were employed to measure the drug release from the α-TP lotion and gel formulations. The average area of Franz cell was 2.08 ± 0.13 cm² and the average volume of Franz cell was 9.12 ± 0.40 mL. A water bath was set to 37 °C to provide a membrane surface temperature of 32 °C. The donor and receptor solution were filtered and sonicated before use to reduce air bubbles (to make the vehicle touch the membrane and make sure the results correct). A cellulose ester membrane was cut, mounted and sealed with parafilm between two chambers of the glass diffusion cell with a 13 mm magnetic flea in the receiver chamber (to make the solution evenly). The cells were inverted and filled with the receptor solution up to the calibration line. The system equilibrated for 0.5 h before the start of the test. After equilibration, an integrity test was performed by inverting each cell and visually checking if there was any fluid leakage or receiver fluid backflow. Leaking cells were excluded from the study. Samples at 0 h were collected prior to the addition of the donor solution. The samples were mixed using syringe 10 times prior to sampling. A finite dose of 2.11 mg/cm² from α-TP lotion (200 µl), 5.26 mg/cm² (500 µl) from α-TP gel formulations was applied uniformly to the surface of each membrane and the donor compartment was covered with parafilm to minimise donor phase evaporation. A 200 µL dose of unmodified silica, carboxy modified, and amine-modified silica (25 mg/mL) nanoparticles, SLN or placebo were added
to the α-TP lotion or gel formulation in drug: nanoparticle ratio of 1:1 immediately upon application to prevent any physical issues. Due to a fast release profile (low viscosity) of the 2% α-TP lotion, the time intervals were 0, 30 min, 45 min, 60 min, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h. These time intervals were extended for the 2% α-TP gel up to 6 h (0, 30 min, 45 min, 60 min, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 5 h and 6 h). After sampling the Franz cells 1 mL of the receiver compartment fluid was transferred to a HPLC glass vial and the sample was replaced with fresh receiver fluid to keep the liquid volume in the Franz cell receiver compartment constant. After collection of the sample, the cells were inverted and gently agitated to prevent the formation of bubbles. The collected samples were analysed by HPLC (HPLC was consisted of pump with autosampler (Hewett-Packard series 1050, Agilent Technologies U.K. Ltd., U.K.) and UV detector (detector-UV HP 1050 system4, PK Dept)). The mobile phase was isopropanol: water: TFA 85:15:1. The stationary phase was a 00G-4435-E0 Gemini 5u C18 110A (5 μm, 250 mm × 4.60 mm). Detection wavelength (λ) was 287 nm and the retention time of α-TP was 5.5 min (flow rate was 1 mL/min). A total of 5 replicates of each experiment were performed.

Cumulative amount of α-TP penetrating the unit surface of the membrane area (cm²) were corrected for sample removal and plotted against time (h). The steady-state flux (J) was calculated from the slope of the linear portion of the curve (R² ≥ 0.98) using at least 3 points with values above the assay limit of detection (LOQ) based on equation 5.4, where Q is the quantity of α-TP traversing the membrane in time t, and A is the area of exposed membrane in cm² and flux unit is (μg/cm².h). The permeability coefficient of α-TP was calculated using equation 5.5 and unit was (cm/h):
\[ J = \frac{Q}{At} \]  
Equation 5.4

\[ K_p = \frac{J}{C_f} \]  
Equation 5.5

Where \( J \) represented the flux, \( K_p \) was the permeability coefficient of the permeant across the membrane and \( C_f \) was the concentration of the drug in the formulation. The flux enhancement ratio (ER) of the different formulations was determined by the equation 5.6 (Patel et al., 2010):

\[ ER = \frac{K_{p(2)}}{K_{p(1)}} \]  
Equation 5.6

Where \( K_{p(1)} \) and \( K_{p(2)} \) were the permeability coefficient of \( \alpha \)-TP from \( \alpha \)-TP gel or lotion formulation alone and with the addition of nanoparticle, respectively in a ratio of 1:1, respectively.

The accumulative mass of \( \alpha \)-TP per area transported through the membrane was calculated at 210 min (3.5 h) and the permeation lag time was estimated from the X-axis intercept from the linear regression of the model applied to permeation data in order to determine the flux (method as per Cai et al., 2016). All data were checked to ensure sink conditions, i.e., sample donor solution never fall < 90% of its initial concentration and the receiver solution never showed > 10% of its permeant drug concentration. All the reported data obeyed these sink conditions.

5.3.6 Skin deposition studies

The skin deposition was carried out using upright calibrated Franz diffusion cell was set up as described in Section 5.3.6. The porcine skin was cut, mounted and sealed with parafilm between two chambers. Tris.HCl of pH 7.4 was employed as receiver fluid to mimic the skin environment. After cell equilibrium for 1 h at 37 C
in pre-heated water bath, the cells were checked for leaks by inversion and visually inspected for back diffusion and leaking cells were excluded. An infinite dose (1 mL) of 2% gel-like α-TP formulation at pH 9 was uniformly applied on the apical surface of full-thickness skin and donor compartment was covered with parafilm to minimise evaporation of topical application. A 500 µL aliquot of SLN or water was added to α-TP gel formulation immediately (0 h) upon its application to the skin to avoid any potential issues induced by physical instability. The permeation study was allowed to proceed for 3 h and at the end the amount deposited of α-TP in SC, epidermis, dermis was determined as described in Section 2.3.8.

5.3.7 Statistic analysis

All values were shown as their mean ± one standard deviation (SD). The statistical analysis of data was performed using the Statistical Package for Social Sciences, SPSS version 24 (IBM Corp., USA), with a significance level of 0.05. All the data was initially analysed by Levene’s test so that the normally distributed data and non-normally distributed data could be treated appropriately. Two-group comparisons of means were performed using Student’s independent t-test for data with equal variance and with Welch’s t-test correction or Mann-Witney’s test for non-equal variance. Normally distributed was analysed using one-way or two-way analysis of variance (ANOVA) or with Turkey’s test or Bonferroni’s test and Mann-Whitney test for non-normally distributed data.

5.4 Results

5.4.1 α-TP nanomaterial structure

The value of $A_o$ used was 91.46 Å², which was calculated from Marvin Sketch. The number of carbon atoms in the hydrophobic tail ($\eta_c$) values for α-TP was
17 for $V_0$ (volume), and 13 for $l_0$ (length) and consequently the $V_0$ and $l_0$ values are 484.7 Å$^3$ and 17.945 Å, respectively; and thus, the critical packing parameter ($C_{pp}$) was calculated to be 0.295 (~ 1/3). This $C_{pp}$ parameter suggested that $\alpha$-TP would form spherical or worm-like (cylindrical) micelles in the aqueous vehicle used in this experimental work.

5.4.2 Nanomaterial size

The mean diameter of the $\text{NanoSiO}_2$, $\text{NanoSiO}_2\text{COOH}$, and $\text{NanoSiO}_2\text{NH}_2$ nanoparticles significantly increased ($p < 0.05$) upon mixing with the 2% $\alpha$-TP lotion (165.0 ± 9.14 nm vs 196.8 ± 6.26 nm, 154.1 ± 6.37 nm vs 234.6 ± 13.14 nm and 138.6 ± 2.34 nm 188.2 ± 1.35 nm, respectively, Table 5.1). In the 2% $\alpha$-TP lotion, the solid lipid nanoparticles showed a very small but significant decrease ($p < 0.05$) in size from 49.63 ± 1.58 nm to 45.28 ± 0.12 nm (See Table 5.1). The AFM tapping mode images showed that silica nanoparticles stock solution (5 mg/mL) had a diameter size of 206.6 ± 12.06 nm and height of 100 nm (see Fig 5.3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (d.nm)</th>
<th>PDI</th>
<th>Sample</th>
<th>Size (d.nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{NanoSiO}_2$</td>
<td>165.0 ± 9.14</td>
<td>0.05±0.02</td>
<td>$\alpha$-TP + $\text{NanoSiO}_2$</td>
<td>196.8 ± 6.26</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>$\text{NanoSiO}_2\text{COOH}$</td>
<td>154.1 ± 6.37</td>
<td>0.04±0.02</td>
<td>$\alpha$-TP + $\text{NanoSiO}_2\text{COOH}$</td>
<td>234.6 ± 13.14</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>$\text{NanoSiO}_2\text{NH}_2$</td>
<td>138.6 ± 2.34</td>
<td>0.04±0.01</td>
<td>$\alpha$-TP + $\text{NanoSiO}_2\text{NH}_2$</td>
<td>188.2 ± 1.35</td>
<td>0.44±0.02</td>
</tr>
<tr>
<td>SLN</td>
<td>49.63 ± 1.58</td>
<td>0.22±0.01</td>
<td>$\alpha$-TP + SLN</td>
<td>45.28 ± 0.12</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>$\alpha$-TP alone</td>
<td>9.1 ± 0.37</td>
<td>0.21±0.02</td>
<td>$\alpha$-TP + water</td>
<td>14.6 ± 0.12</td>
<td>0.26±0.01</td>
</tr>
</tbody>
</table>
5.4.3 Rheology of formulations

The rheology of the gel α-TP formulation was examined to understand the interaction between α-TP aggregates and nanoparticles. The lotion formulation was not examined as it was too free flowing to be measured using the cone and plate rheometer. The addition of NanoSiO$_2$NH$_2$, NanoSiO$_2$, SLN, and NanoSiO$_2$COOH to the 2% α-TP gel formulation (see figure 5.4) showed a comparable average viscosity to the α-TP gel ($p > 0.05$). The addition of SLN to the 4% gel decreased the average viscosity at frequency of 1-4 Hz ($p < 0.001$ at 1-2 Hz, $p < 0.01$ at 3-4 Hz, two-way ANOVA with Bonferroni’s multiple comparisons test) (see figure 5.5). Moreover, the addition of SLN to the 4% gel decreased the storage modulus at all frequencies range 1-10 Hz (at 1-3 Hz - $p < 0.05$, at 4-5 Hz - $p < 0.01$, and at 6-10 Hz $p < 0.0001$, two-way ANOVA with Bonferroni’s multiple comparisons test). Both the 2% and 4% gel exhibited gel-like rheology at 20°C as the elastic and viscous moduli ($G'/G''$) ratio was less than 10 as shown in fig. 5.6 and 5.7, respectively.
Fig. 5.4: Complex viscosity of 2% α-TP gel control (●) with the addition of solid lipid nanoparticles (♦), NanoSiO2COOH (▲), NanoSiO2NH2 (▼), and NanoSiO2 (■). Data points represent mean ± standard deviation (n=3).
Fig. 5.5: Complex viscosity of 4% α-TP gel control (●) with the addition of solid lipid nanoparticles (♦). Data points represent mean ± standard deviation (n=3).
Fig. 5.6: Storage (elastic) modulus (G’) and loss (viscous) modulus (G’’) measured as a function of frequency (Hz) for α-TP 2% gel control (●) with the addition of solid lipid nanoparticles (♦), NanoSiO2COOH (▲), NanoSiO2NH2 (▼), and NanoSiO2 (■). Data points represent mean ± standard deviation (n=3).
Fig. 5.7: Storage (elastic) modulus (G’) and loss (viscous) modulus (G’”) measured as a function of frequency (Hz) for α-TP 4% gel control (●) with the addition of solid lipid nanoparticles (♦). Data points represent mean ± standard deviation (n=3).

5.4.4 Drug release studies

For the 2% α-TP lotion control, the steady-state flux lag time (t_{lag}) of (84.74 ± 27.32 min) significantly decreased by 2.9-fold (by 65%) and 206-fold (by 99.5%) respectively after the addition of the Nano_{SO_{2}CO_{2}OH} (29.33 ± 20.60 min) and SLN (0.41 ± 15.06 min) (p < 0.05 and p < 0.001, respectively, one-way ANOVA with Dunnett’s multiple comparisons test). The flux of α-TP, the K_p, and the accumulative amount of α-TP at 210 min, all significantly increased in the presence of SLN by 2-fold compared to the lotion control (p < 0.05, student independent t-test). The flux, the K_p, and accumulative mass per area at 210 min of α-TP from the
lotion with the addition of all the silica nanomaterials did not show any significant difference compared to the lotion control ($p > 0.05$, one-way ANOVA with Dunnett’s multiple comparisons test) (see Table 5.2, Figure 5.8).

For the 2% αTP gel formulation, the flux lag time ($t_{lag}$) of α-TP in the presence of the SLNs was significantly reduced by 2.7-fold (by 63%) compared to the gel control ($23.81 \pm 5.86$ vs $63.77 \pm 26.19$ min) ($p < 0.05$, Student unpaired t-test with Welch’s correction). The flux of α-TP from the gel with the addition of the SLN was 11-fold higher than the gel control (without the nanoparticles) ($628.89 \pm 122.37$ vs $56.32 \pm 41.36$ µg/cm$^2$/h, respectively) ($p < 0.001$, One-way ANOVA, Dunnett’s multiple comparisons test). The $K_p$ of α-TP from the gel significantly increased by 11-fold with the addition of SLN formulation compared to the gel control ($43.98 \pm 856$ vs $3.94 \pm 2.89 \times 10^{-3}$ h/cm, $p < 0.001$, One-way ANOVA with Dunnett’s multiple comparisons test). The accumulative amount at 210 min (3.5 h) of α-TP released from the SLN formulation was 8-fold higher compared to the gel control ($1190.09 \pm 190.66$ vs $146.77 \pm 125.24$ µg/cm$^2$, respectively) ($p < 0.001$, One-way ANOVA with Dunnett’s multiple comparisons test). Three formulations containing the unmodified, carboxy- and amine-modified silica nanoparticles slightly reduced the flux, permeability coefficient, and accumulative amount at 210 min (3.5 h) of α-TP but were not significantly different over the gel control ($p > 0.05$, One-way ANOVA, Dunnett’s multiple comparisons test). The amine-modified silica nanoparticles significantly increased the lag time by 1.9-fold over the gel control ($118.73 \pm 87.19$ vs $63.77 \pm 26.19$ min) ($p < 0.01$, Student unpaired t-test with Welch’s correction) (see Table 5.3 and Figure 5.9).
Table 5.2: Steady-state drug release (flux) and flux lag time ($t_{lag}$), cumulative amount at 210 min (3.5 h) across synthetic cellulose ester membrane after topical application finite dose of α-TP lotion (2.11 mg/cm²) at pH 7.4. The enhancement ratio (ER) was calculated as a ratio of permeability coefficient ($K_p$) of α-TP from lotion formulation alone and with the addition of nanoparticles at a ratio of 1:1. Data represent mean ± standard deviation (n=5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flux (µg/cm².h)</th>
<th>$K_p \cdot 10^{-3}$ (cm/h)</th>
<th>$t_{lag}$ (min)</th>
<th>3.5 h (210 min) Cumulative amount per area</th>
<th>Enhancement ratio (ER)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% lotion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% lotion</td>
<td>99.17±5.52</td>
<td>9.92±5.52</td>
<td>84.74±27.32</td>
<td>211.24±122.37</td>
<td>-</td>
</tr>
<tr>
<td>NanoSiO₂</td>
<td>144.03±82.94</td>
<td>14.40±8.29</td>
<td>37.60±43.18</td>
<td>375.66±155.44</td>
<td>1.5</td>
</tr>
<tr>
<td>NanoSiO₂COOH</td>
<td>145.71±85.09</td>
<td>14.57±8.51</td>
<td>29.33±20.60*</td>
<td>344.09±203.34</td>
<td>1.5</td>
</tr>
<tr>
<td>NanoSiO₂NH₂</td>
<td>123.23±51.04</td>
<td>12.32±5.10</td>
<td>29.31±28.36</td>
<td>298.92±99.09</td>
<td>1.2</td>
</tr>
<tr>
<td>SLN</td>
<td>183.17±94.72*</td>
<td>18.32±9.47*</td>
<td>0.41±15.06***</td>
<td>445.33±155.13*</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 5.3: Steady-state drug release (flux) and flux lag time ($t_{lag}$), cumulative amount at 210 min (3.5 h) across synthetic cellulose ester membrane after topical application finite dose of α-TP gel (5.26 mg/cm²) at pH 9. The enhancement ratio (ER) was calculated as a ratio of permeability coefficient ($K_p$) of α-TP from gel formulation alone and with the addition of nanoparticles at a ratio of 1:1. Data represent mean ± standard deviation (n=5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flux (µg/cm².h)</th>
<th>$K_p \cdot 10^{-3}$ (cm/h)</th>
<th>$t_{lag}$ (min)</th>
<th>3.5 h (210 min) Cumulative amount per area</th>
<th>Enhancement ratio (ER)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Gel-like</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% gel</td>
<td>56.32±41.36</td>
<td>3.94±2.89</td>
<td>63.77±26.19</td>
<td>146.77±125.24</td>
<td>-</td>
</tr>
<tr>
<td>NanoSiO₂</td>
<td>41.10±11.13</td>
<td>2.87±0.78</td>
<td>58.15±21.03</td>
<td>106.44±40.37</td>
<td>0.7</td>
</tr>
<tr>
<td>NanoSiO₂COOH</td>
<td>25.62±29.17</td>
<td>1.79±2.04</td>
<td>68.35±22.70</td>
<td>61.83±69.11</td>
<td>0.5</td>
</tr>
<tr>
<td>NanoSiO₂NH₂</td>
<td>24.51±15.91</td>
<td>1.71±1.11</td>
<td>118.73±87.2**</td>
<td>51.94±69.73</td>
<td>0.4</td>
</tr>
<tr>
<td>SLN</td>
<td>628.89±122.4***</td>
<td>43.98±8.56***</td>
<td>23.81±5.86*</td>
<td>1190.09±190.66***</td>
<td>11***</td>
</tr>
</tbody>
</table>
Fig. 5.8: The transport of α-TP across a cellulose membrane when applied as a 2% lotion formulation (●) with the addition of solid lipid nanoparticles (♦), NanoSiO2COOH (▲), NanoSiO2 (■), and NanoSiO2NH2 (▼) at pH 7.4. Each represents the mean ± standard deviation (n=5). The linear lines represent steady-state portion of the permeation profiles from where the flux was calculated.
5.4.5 Skin deposition studies

The % total recovery of α-TP from the skin layers for the 2% α-TP gel with the addition of SLN did not show any significant difference compared to gel control was (94.48 ± 4.26 % and 89.40 ± 4.9 %, respectively). The amount of α-TP penetrated the SC of porcine skin from 2% α-TP gel formulation with the addition of SLN was not statistically different compared to gel control (269.30 ± 12.40 vs 245.40 ± 22.66 µg) (p > 0.05, unpaired t-test with Welch’s correction). There was also no difference in the amount of α-TP recovered from epidermis and dermis for 2% α-TP gel formulation with the addition of SLN compared to the gel control.
(27.35 ± 5.81 vs 28.91 ± 9.51 µg and 20.79 ± 4.26 vs 21.04 ± 2.30 µg, respectively) 

\( p > 0.05 \). (see fig. 5.10).

![Graph showing amount of α-TP in SC, Epidermis, and Dermis](image)

**Fig.5.10: Amount of α-TP in the stratum corneum (SC), epidermis and dermis after application of 2% α-TP gel alone was similar to the 2% α-TP gel with SLN (SLN). SLN refers to solid lipid nanoparticles. Data represent mean ± standard deviation (n=3).**

### 5.5 Discussion

α-TP is an anionic amphiphilic compound that can be considered as a single tailed surfactant. The calculated critical packing parameter for α-TP was 0.3, which indicated that the α-TP was expected to form spherical or worm-like micelles in aqueous vehicles. These theoretical calculations do not account for pH of the vehicle which was important for α-TP as it is ionisable. The DLS data revealed that in 2% lotion the α-TP aggregates were 9 nm with PDI of 0.21. In Chapter 2, the AFM images showed that α-TP at a concentration of 0.9 mM at pH 7.4 form spherical micelles or vesicles (liposomes) with diameters of 52 nm, height of 17 nm, whereas at higher concentration (6.3 mM) it formed rod-like structures with
diameters of 300-570 nm and height of 10 nm. At pH 9, α-TP at 36 mM (2 % w/v) concentration dissolved in water forming a gel structure, which suggested that the nanostructures may have aggregated. It has been reported that vitamin E derivatives such as α-tocopherol polyethylene glycol succinate (TPGS) 400, and TPGS 1000, and Triton X-100 (MW 647 Da) surfactant form spherical micelles in term of critical micelle concentration (CMC), with CMC ca. 1.5 mM, 0.02 mM, and 0.19 mM, respectively, and number of aggregates for TPGS 1000 and Triton X-100 was 10, and 63, respectively. Moreover, the microviscosity was high for all three surfactants and similar in spite differences among CMC (Sadoqi et al., 2009).

Another study has studied the size and morphology of TPGS 1000 micelles using dynamic light scattering (DLS), AFM, and TEM. The DLS revealed that TPGS micelles are between 12 and 14 nm with PDI of 0.2-0.3. The AFM revealed that TPGS form smooth-surface spherical micelles in shape, without any noticeable pinholes or cracks, with size ca. 20 nm close to DLS and TEM also showed that TPGS were spherical micelles with size of ca. 60 nm but appear greater than DLS due to combined assembly of couple of micelles (Muthu et al., 2012).

The shape and size of nanomaterials may vary depending on vehicle environment, depending on concentration, vehicle ethanol ratio, formulation composition, temperature, pH, salinity, etc (Oda, 2006). The rheology supported the visual evidence that 3D network structure of α-TP was formed in the pH 9 vehicle as the elastic (storage) modulus remained higher than viscous modulus (G’> G’’)) independent of frequency. In addition, at high frequency, the G’ increased up to ~100 Pa while G’’ remained the same, which indicated elastic gel formation. However, unlike the cross-linked polymeric gel, which have G’: G’’ equal or higher than 10, the G’/G” of 2% and 4% α-TP gel only reached to 6.3 and 6.6, respectively,
i.e., less than 10 but higher than 1, hence the α-TP at pH 9 did display a weak gel-like structure. It is most probably that worms under these conditions may cause the formation of an entangled network that were temporally persistent, i.e. worms may frequently break and recombine very slowly (Cates and Candau, 1990; Raghavan and Douglas, 2012). At 25°C, all the solid-like (elastic) modulus G’ values of α-TP gel were higher than the liquid-like (viscous) modulus (G’’) over entire range of tested frequency, this suggested that the α-TP gel behaved more solid-like. These results were similar to a study by Kumar et al. who showed that 50 mM of the surfactant erucyl dimethyl amidopropyl betaine (EDAB), which has a long C22 tail form worms in water. However, EDAB at 60°C the dynamic moduli intersect at low frequency which implies a viscoelastic response with finite relaxation time, due to the worm become shorter but still entangled but break and reform quickly (milliseconds) transition from gel-like to viscoelastic solution (Kumar et al., 2007). This solid-like behaviour was similar to poly (ethylene oxide) hydrogel films synthesized by UV cross-linking with pentaerythritol tetra-acrylate (PETRA) as the cross-linking agent (Wong and Dodou, 2017).

The rheological data indicated that α-TP gel displayed a solid-like (gel-like) behaviour with visco-elastic properties, but it is insufficient as the frequency was done on one decade instead of three decades and the shear thinning may be due to probably an artefact. The SLN appeared to reduce the average viscosity and storage (elastic) modulus when added to 4% gel formulation of α-TP. This may be due changing the rheological properties which possibly due to adsorption of α-TP monomer to SLN breaking of the α-TP aggregates in the gel. Further studies are needed to confirm that.
At pH 7.4, α-TP was negatively charged, the functional groups on the surface of the unmodified, carboxyl, and amine-modified silica nanoparticles (see fig. 5.11) were O\(^{-}\) (Nano\(_{\text{SiO}}\_2\)), COO\(^{-}\) (Nano\(_{\text{SiO}}\_2\text{COOH}\)), and NH\(_3^+\) (Nano\(_{\text{SiO}}\_2\text{NH}_2\)), respectively. Mixing of 20 mg/mL of α-TP with the silica nanoparticles increased the hydrodynamic size (188-234.6 nm) by 30-80 nm. Such an increase in size is unlikely to be a result of α-TP monolayer adsorption onto the surface of all the silica nanomaterials in a physically stable system as this would generate a much smaller size increase (the theoretical length of one α-TP molecule was expected to 2.2 nm according to the Harper \textit{et al} who assumed that the theoretical length of two α-TP molecules has a size of 4.4 nm) ((Harper \textit{et al.}, 2018). It is possible that the size changes were a result of α-TP aggregates adsorption onto the surface of the silica particles (α-TP aggregate diameter was 9.1 nm). It has been reported that cationic CTAB has good adsorption to counter charged silica nanoparticles, but it decreased the effective charge (zeta potential) bring about aggregation and destabilize the system, whereas unexpectedly, equal or like-charged anionic SDS adsorb to silica surface increasing the effective charge and electrostatic repulsion and stability (Ahualli \textit{et al.}, 2011).

This adsorption phenomenon may justify the increase in size of nano-silica due to adsorption of hydrophilic negatively charged head group of α-TP to hydrophilic silica nanoparticles. This adsorption could be due to hydrogen bonding between α-TP and hydroxyl group at silica nanoparticles surface. This increase in silica nanoparticles hydrodynamic size was not due to aggregation of silica nanoparticle itself (PDI was 0.17-0.44) as the change in size is \textit{ca.} 30-80 nm which less than the diameter of silica nanoparticles. However, amine-modified silica nanoparticles were the least negatively charged due protonation of amine functional group at their
surface (zeta potential is -20 mV) compared to others carboxy-modified and unmodified silica nanoparticles (zeta potential was -30 and -40, respectively). The increase in the PDI up to 0.44 may as a result of more adsorption of negatively charged α-TP aggregates to the positively charged amine group at the surface amine-modified silica nanoparticles (5.12). Recent study investigated the interaction between like-charged and counter-charged silica nanoparticles and cationic wormlike micelles composed of cetyltrimethylammonium bromide (CTAB) and sodium salicylate (NaSal) and proposed that endcap of wormlike micelles can absorb on the surface of silica nanoparticles, \( i.e., \) complex cross-linking networks constructed by wormlike micelles and silica nanoparticles (Dai et al., 2017; Helgeson et al., 2010; Nettesheim et al., 2008b).

![Fig.5. 11: Schematic diagram of the surface structure for (a) unmodified, (b) carboxyl, and (c) amino modified silica nanoparticles.](image)

![Fig.5. 12: Schematic diagram of interaction between amine-modified silica nanoparticles and α-TP aggregates.](image)
When mixing the $\alpha$-TP with the SLNs there was a slight decrease in the hydrodynamic size of SLN. This was presumably due to the SLN-$\alpha$-TP surface interactions increasing the stability of SLNs. As the SLN consists of lipid core with emulsifier at the shell it could be that $\alpha$-TP hydrophobic tail is pulled and adsorbed to the SLN and may break the aggregates of $\alpha$-TP and the same time their size is reduced as a result of stabilization effect (fig.5.13). Previous studies investigated the effect of co-surfactant (e.g. Pluronic F68, bile salts, amino acid tyrosine, phynylalanine, and Tween 60 and 80), on the physical stability of SLN and found that bile salts and aromatic amino acids have distinct amphiphilic structure which act as reservoir of molecules available for adsorbing fast on the SLN interface, i.e., interfacial stabilisation, preventing aggregation of SLN at pH 9 as all these amphiphilic co-surfactant has net negatively charge at pH > pK$_a$ and the molecular interaction would be repulsive once absorbed to SLN minimizing its calescence and prevent polymorphic transition (Salminen et al., 2014). This agreed with another study found that SLN hydrodynamic average size reduced and offer better stability to small lipids preventing its coalescence upon increase the concentration of tween 80 (Shafique et al., 2017).
Fig. 5. 13: Postulated adsorption of α-TP molecule on the surface of SLN and through lipophilic (hydrophobic) interaction.

The $K_p$ of α-TP from the lotion was 2.5-fold higher than the gel ($9.9 \pm 5.5 \times 10^{-3}$ vs $3.9 \pm 2.9 \times 10^{-3}$ cm/h, respectively). The effect of nanomaterials on transport rate of α-TP from a 2% α-TP lotion and gel formulations was assessed using a 1000 KDa cellulose ester (CE) semipermeable membrane. For cellulose ester membranes, drugs with a diameter less than the membrane molecular weight cut-off (MWCO) can pass the membrane, and thus the major diffusion rate limiting step was assumed to be drug release from the formulation.

The addition of the SLN to 2% α-TP lotion formulation statistically increased the flux, $K_p$, and accumulative amount by 2-fold and decreased the flux lag-time by 206-fold over the lotion control. This suggested that SLN interaction with α-TP increased is its availability to pass through the membrane, i.e., α-TP de-aggregated by SLN to become monomers which have less restriction to passively diffuse through the membrane. Furthermore, this interaction possible could also lead to
increase the thermodynamic activity, thus would effectively increase the driving force of diffusion. However, other silica nanoparticles had little effect.

The addition of SLNs to the 2% α-TP gel also significantly increased the α-TP drug release, but to a larger extent than the lotion (ER was 11 vs 2, respectively). It was assumed to be as the same mechanism as the lotion but here the effect of nanoparticles was probably more pronounced as they interfered with the rheological properties of α-TP aggregate without the presence of other viscosity modulator excipients such as ethanol or propylene glycol that found in lotion formulation. This enhancement effect was similar to a previous study in which the lipid nanocarrier enhanced the release of α-T at 2 h across dialysis cassettes (2000 KDa) by 7.5-fold (Abla and Banga, 2014).

SLN addition to the α-TP gel reduced the lag time by 2.7-fold compared to the gel control. However, the silica nanoparticles did not affect the flux, k_p, accumulative amount or the lag time over the gel control. These results may be due stronger interaction of silica nanoparticles with α-TP aggregates resulted in the hindrance of the α-TP monomers transport through the cellulose membrane. This stronger surface interaction between drug aggregates and silica was noted previously was due to endcap adsorption of aggregated nanostructures on the surface of silica nanoparticles which constructed a complex cross-linking network (Helgeson et al., 2010; Nettesheim et al., 2008b). As previously noted the SLN most likely interacted with α-TP monomer as the hydrophobic tail could be adsorbed to SLN surface. The effect of SLN on enhancing the transport of α-TP by deaggregation was similar to a previous study which found that introduction of NanoSiO_2 (-23 mV) at pH 8 to tetracaine solution resulted in flux enhancement ratio of 3.6 compared to the control; by breaking the drug aggregates without the particle penetrate into the skin.
Moreover, addition of NanoSiO2 (-23 mV) to 151 mM HPMC gel of tetracaine result in enhancement ratio of 3.6 compared to the gel control (X. Cai et al., 2016; X. J. Cai et al., 2016). The differences in penetration enhancement induced by nanoparticle addition may be due to differences in drug-nanoparticle interactions. It was hypothesized by Cai, et al, that the adsorption of positively charged tetracaine to negatively silica nanoparticles were due electrostatic (attractive) interactions, while in case of α-TP-SLN interactions in our results may be due to hydrophobic interactions.

The amount deposited of α-TP in skin layers with the addition of SLN was similar to gel control. This indicated sunscreen additives such as SLN did not deteriorate the α-TP delivery into the skin layers which may reduce its photoprotective efficacy. Furthermore, the addition of SLN to α-TP gel in cosmetic product was beneficial for both SLN stability and α-TP release from the gel formulation. Hence, this indicated that α-TP-nanomaterial interaction could be translated into cosmetic product.

5.6 Conclusion

The addition of α-TP to SLN reduced their hydrodynamic by the adsorption of α-TP monomer on the SLN surface probably through hydrophobic interaction. The SLN improved the transport of α-TP from both the lotion and gel formulation which most probably indicated that SLN broke the α-TP aggregates. On the other hand, the addition of α-TP to silica nanoparticle increased their hydrodynamic size indicated the adsorption of α-TP aggregate on the silica nanoparticle surface. Silica nanoparticles did not improve the transport of α-TP from lotion and even hindered it from the gel formulation indicated stronger interaction than the SLN-α-TP
interaction, probably through hydrogen bonding. Solid lipid nanoparticle showed superior de-aggregation of \( \alpha \)-TP in the gel compared to the lotion due to absence of viscosity modulators. The skin deposition of \( \alpha \)-TP from SLN formulation was not hindered by the presence of SLN indicated that the \( \alpha \)-TP still effective if added to a sunscreen additive. Hence, the addition of SLN to \( \alpha \)-TP gel in cosmetic product was beneficial for both in term of SLN stability and \( \alpha \)-TP release from the gel formulation.
Chapter 6. General discussion
Effective delivery of photoprotective antioxidants into viable skin layers after topical application is essential to maximise their activity against UVR-induced oxidative skin damage, photoageing and photocarcinogenesis, when used alone or in combination with sunscreens. α-T has antioxidant activity and is considered to be a good candidate photoprotective agent. However, it is chemically unstable and susceptible to photodegradation. In addition, α-T is too hydrophobic to pass into the viable skin epidermis, which is its site of antioxidant activity upon UVR exposure, and it is prone to retention in the upper layer of dead skin (SC). At present there is no drug delivery solution that effectively overcomes the α-T problems and thus it is not currently used effectively, even when included in topical preparations.

An alternative, but α-T related antioxidant, is α-TP, which is water-soluble and chemically stable. α-TP can self-assemble in nanosized aggregates in a hydro-alcoholic vehicle, allowing it to have the potential to pass the SC, despite its charge, and reach pass the viable epidermis. Previous studies have shown that α-TP can pass into the skin, but this previous work has not used a preparation that is suitable for administration into the human tissue. Therefore, at present it is not easy to translate the preliminary work on α-TP into a cosmeceutical formulation suitable for human use (Nakayama et al., 2003). For example, in one study α-TP was applied to full-thickness ex vivo skin in culture media, hence the amount α-TP absorbed into the skin (18-188 nmol/g skin) is unreliable (Nakayama et al., 2003). The previous studies also did not report whether α-TP nanoaggregates were formed or not, which would likely affect deposition in various human skin layers. Consequently, knowledge on the mechanism of how α-TP permeates the skin appeared to be still lacking in the current literature.
The aim of this project was to develop an α-TP formulation that was suitable to administer to humans. In order to achieve this, the first objective was to investigate α-TP aggregation and its influence on epidermal skin deposition (Chapter 2). The data generated from the initial experiments indicated that α-TP solubility increased in an aqueous vehicle as the pH increased and this was most notable when the pH was higher than the α-TP pKa. This indicated that the percentage of α-TP’s dianionic form was critical to its aqueous solubility. The CAC of α-TP in a 20% ethanol, 20% propylene glycol, 60% water at pH 7.4 (100 mM Tris.HCl) vehicle was in the millimolar concentration range, which was similar to the prodrug dexamethasone phosphate (Shah et al., 2009), but no aggregation was observed at pH 9. It was thought that α-TP aggregation at pH 7.4 was a result of mono-ionic microspecies, which was in the majority, whilst the lack of aggregation at pH 9 was a result of the di-ionic microspecies being the most dominant form. The pH dependant aggregation of α-TP provided an opportunity to test the influence of aggregation on chemical penetration into the skin.

It was notable from the data in Chapter 2 that both α-T and α-TP were found to aggregate with the latter forming small nanostructures. The aggregation of α-T is rarely noted in the literature when topical products are generated using this agent, and the fact that previous work has not characterised this physical phenomenon prior to topical application may mean that previous understanding of this molecule’s diffusion into the skin may not be completely correct. The fact that the addition of the phosphate head group to α-T to form α-TP reduced the aggregated size suggested that the charged phosphate probably oriented to the surface of nanostructures to enhance its interaction with an aqueous vehicle (Fig. 6.1). The AFM images which were obtained by tapping mode, confirmed that the α-TP...
aggregates were smaller than the α-T aggregates, and that both were spherical in shape. The shape of α-TP aggregates was close to TPGS aggregates previously reported in the literature (Muthu et al., 2012). Through varying the concentration of α-TP in the vehicle, it was shown that the size and shape of the aggregates changed. For example, at 6.3 mM the α-TP formed larger rod-like aggregates (diameters 306-571 nm), which suggested that aggregates were liposomes that grow in the presence of more α-TP, but future work is required to confirm this. When relating the aggregation concentration effects with the typical concentrations of α-TP used in topical products, e.g., 35 mM (2% w/v) in a typical α-TP in lotion formulation (this concentration was too high to measure in the light scattering instrument), it appeared likely that the rod-shaped nanostructures would be present in these products and hence it was perceived important to understand their effects on skin penetration.

Fig.6. 1: Theoretical nanostructure of α-TP.
The short-term physical stability study of α-TP showed that the aggregate size and PDI did not change over 2 days, but there was a slight increase in both size and PDI (by 3 nm and 0.07, respectively) over 7 days. This suggested that these aggregates were stable during the studies reported in this thesis, but it is accepted that long-term stability studies would be required in order to develop a product that could be applied to humans. This short-term physical stability was similar to that seen in a previous study that used 1.1 % α-T to stabilize 1% phytosphingosine in a ME which showed only a 2 nm and 0.02 increase in size and PDI, respectively over a period of 7 days (Cichewicz et al., 2013).

In order to perform the skin permeation studies, a suitable α-T and α-TP analytical technique was required. HPLC was selected as the method as there was precedent for this approach in the literature and the equipment was readily available. The HPLC quantitative method that was employed for both α-T and α-TP was shown to be ‘fit for purpose’ based on the LOD, LOQ, precision and linearity. A full validation was not performed as elements such as user reproducibility, etc. were not required in this study. The HPLC assay also provided a quick and reliable method to determine the short-term chemical stability α-TP. The degradation of a 100 µg/mL in 20% ethanol, 20% propylene glycol, 60% water at pH 7.4 (Tris buffer, 100 mM) of α-TP was 2.26 µg/mL/week (i.e. a degradation rate of 2.3%), with no degradation over the period of the transport and skin deposition studies. This indicated that α-TP was chemically stable at concentrations of ≥ 100 µg/mL, 97.7-100% of its initial concentration remained over the period of the study, and as was within the acceptable chemical degradation rate limit for a topical product (10% degradation for 500 µg/mL active ingredient over 12 weeks at 40° C) (Stephane, 2015). However, to ensure consistency in the PhD project that samples used were
always freshly prepared for each experimental study and stored for not longer than 48 h. Harper et al showed that 20 μg/mL (+) α-TP in a 20% ethanol, 80% water at pH 7.4 (Tris buffer, 25 mM) vehicle degraded at a rate of 1.2 μg/mL/week, which suggested the (+) α-TP pure stereoisomer was even more stable than the α-TP racemic mixture that was employed in this work (Harper, 2017; Harper et al., 2018). It may be beneficial in future work to assess the ability of the (+) α-TP pure stereoisomer to penetrate the skin and assess its UVR protection effects. These studies were not possible in the remit of the current PhD as there was not enough time to synthesise, purify and characterise the (+) α-TP, which is not commercially available.

The model membrane transport data suggested that the diffusion of α-TP nanostructures across 20 KDa and 1000 KDa cellulose membranes at pH 7.4 was significantly less than at pH 9. This indicated that α-TP nanoaggregate slows down the diffusion of α-TP molecules through the membrane, compared to non-aggregated form. However, the ex vivo skin permeation studies showed that the amount of α-TP deposited from topical application of an infinite dose at pH 7.4 is comparable to pH 9 in the epidermis and dermis. The synthetic membrane studies were indicative of the effects of aggregation on the self-diffusion coefficient, i.e., the diffusion through the vehicle in the aggregated and non-aggregated forms. The skin permeation data would be subject to the change in self-diffusion caused by aggregation, but the fact that the penetration of the two physical forms of α-TP showed no difference indicated that the penetration into the barrier, which was probably the rate limiting step ‘normalised’ these diffusion effects.

α-TP showed superior SC and epidermal deposition compared to α-T and this suggested that α-TP could show better affinity for the skin, although in Chapter 2
this was not actually measured. However, the fact that α-TP at pH 9 did not show superior skin deposition over pH 7.4, despite the former being in the unaggregated form (this would be expected to pass through the skin more efficiently due to the small molecular size) raised additional questions about the route by which α-TP passed into the skin. It was not clear if the α-TP aggregates used the hair follicular route and α-TP molecule used the intercellular route or if both molecules used the same route of transport (Fig. 6.2). It is very difficult to understand the route of transport of molecules through the skin, but without this information topical formulation development may not be optimal hence it was thought important to investigate the interactions of the α-TP with the skin in Chapter 3 in order to gather further evidence of how α-TP passed into the cutaneous tissue.

In Chapter 3, a SC lipid monolayer, which was composed of an equimolar ratio of ceramide, cholesterol, and palmitic acid, was employed to model the SC intercellular lipids in human skin in order to study the α-TP skin interactions. The simplification of the skin to study its interactions with molecules is a common method used in the field of skin research to understand chemical permeation. The model is far from ideal however because it only looks at SC drug interactions and not epidermal tissue. This limitation was accepted in this work as the SC was thought to be the major barrier for the hydrophobic tocopherols. In further work it would be important to expand the interaction studies to models of the epidermis and combine the studies with some imaging work using dyes that could localise within the α-TP liposomes.
The monolayer subphase injection studies in Chapter 3 showed that α-TP showed a better interaction with the lipid monolayer than α-T. The data indicated that α-TP inserted into hydrophobic regions of SC lipid film, whereas α-T did not. α-TP also showed a faster interaction with the lipid monolayer in a concentration dependant manner. These two results together suggested that α-TP compared showed amphiphilic surfactant type interactions the lipid monolayer (Nigam, 2006).

The Langmuir compression isotherm studies showed that the measured MMA of SC-α-TP mixtures were higher than the calculated values and this indicated that α-TP disrupted the lipid packing of the monolayer used in this work. Previous work with oleic acid, a known skin penetration enhancer, found that the measured MMA
of SC lipid-OA mixtures were significantly smaller than the calculated MMA at each surface pressure. Comparison of this previous work with the current study data demonstrated that the interactions of α-TP were probably not strong enough to actually disrupt the SC (Mao et al., 2013). The α-T and α-TA mixtures again showed weaker interactions compared to α-TP in the compression isotherm studies which supported the notion that they found it difficult to insert into the SC monolayer.

The addition of 60% α-TP significantly reduced the compressibility modulus $K^*$, which provided further evidence that α-TP integrated into the SC lipids and changed their properties from liquid condensed into more liquid expanded film, i.e., disturbed the lipid packing and fluidized the membrane (see fig. 6.3). α-T or TA could not adopt a liquid condensed state, i.e., they could not be compressed at high surface pressure. This meant that both controls could not be packed easily as α-TP into the membrane. The amphiphilic balance present in α-TP seemed to allow it to adopt a perpendicular orientation to form a stable monolayer at the interphase and to move or diffuse through lipids (See fig. 6.3), whereas α-T and α-TA lack the amphiphilic balance and thus probably display a more parallel (flat) orientation at the SC interface. The plateau observed in the compression isotherm of the pure α-T and α-TA monolayers was similar to that of a pure fengycin monolayer, reported in a study by Eeman et al. In this previous work, the fengycin was squeezed out of the mixed monolayers when the monolayer pressure was increased and thus it was unable to diffuse through extracellular lipid matrix of SC model (Eeman et al., 2009).
Fig. 6: Diagram representing the disrupting of SC lipid packing and increase in its fluidity caused by α-TP

The data obtained from Chapter 3 indicated that α-TP had a favourable interaction with the SC lipids, which was thought to explain why, although displaying a charged head group, α-TP could pass through the SC lipid lamellae in the skin to reach the epidermis possibly by fusion with SC lipids. The data also indicated that α-TP showed superior interactions with the skin compared to the more commonly employed vitamin E derivatives (such as α-TA) in skin cosmetic products. As a consequence of the positive results in Chapters 2 and 3, it was thought prudent to examine the photoprotective effects of α-TP in Chapter 4. However, unlike previous studies, which have added pro-vitamin E, such as α-TA, and tocopherol glucoside to UVR filters in broad spectrum sunscreen formulations to maximize the benefit (Wu et al., 2011; Young et al., 2018), Chapter 4 details a more focused study on the UVA1 region of the solar spectrum as the properties of α-TP appeared well suited to protect against potential damage (e.g. ROS) caused by this type of UVR.
A limited amount of studies has shown that α-TP is a promising protective agent against UVB-induced damage. However, there has been an overlap of the α-TP absorption spectrum and UVR source spectrum in the UVB region, hence it is not clear from these preliminary studies if the effects of α-TP arise as a consequence of its sunscreen photoprotection (its two aromatic rings absorb UVB) or its antioxidant properties. No α-TP photoprotection studies using UVA1, which does not overlap with the absorption spectrum of α-TP, have been performed to date. The long-waveband UVA1 region (375-400 nm) is a major component of terrestrial UVR and it has recently been the focus of a number of research studies because: (i) most sunscreens, especially in the USA, currently do not protect well against UVA1 (ii) UVA1 is often unintentionally ignored when estimating SPF and this may result in an overestimation of protection, i.e., SPF and (iii) UVA1 is mainly responsible for generating ROS. In contrast, a UVB type source, i.e., a source that is rich in short wavelength UVA (UVA2) mainly causes skin damage by direct absorption by skin chromophores not by oxidative stress. UVB was classically the focus of photoprotection studies as although it only represents 5% of the sunlight spectrum it is mainly responsible for causing sunburn, an effect that is traditionally associated with skin damage. When people apply sunscreen, that block UVB, this allows them to stay longer under the sun without getting sunburn. However, it is important to consider the other longer wavebands since they may still reach the skin and cause long-term consequences due to the higher doses of oxidative stress. Therefore, researchers and the industry are now driven towards developing sunscreen products to protect skin from the damage and effects of longer waveband.

For a cosmetic formulation to be approved in the market, it requires data on cytotoxicity at the early stages of development. However, the toxicity of α-TP in
human keratinocytes has not been reported in the literature to date. Therefore, Chapter 4 initially determined the LD$_{50}$ of $\alpha$-TP, which provided an indication of the maximum concentration that can be used before detrimental effects occur to the skin cells. This would provide a further indication of the possibility of using the $\alpha$-TP and developing the agent as a novel cosmetic formulation, on an industrial level.

The results obtained from Chapter 4 indicated that $\alpha$-TP was well tolerated in HaCaT keratinocytes (LD$_{50}$ was 1.1-1.7 mM) and hence the subsequent photoprotection studies were not hindered by the test compounds causing cell toxicity. In the photoprotection studies both the Alamar blue® and neutral red assays showed a significant dose-dependent decrease in keratinocyte viability at 24 h after incrementally increasing doses of UVA1 were given to the cells. The pre-irradiation treatment with $\alpha$-TP in starved low-serum medium showed a superior protection and significantly increased the cell viability fraction by 14% compared to the no antioxidant control, whereas $\alpha$-T had no significant increase on cell viability compared to the same control. The incubation of keratinocytes with the low-serum medium was thought to induce the depletion of the endogenous level of $\alpha$-T after 24 h incubation. The pre-irradiation treatment of $\alpha$-TP may have prevented this reduction of endogenous $\alpha$-T (Nakayama et al., 2003). A recent study demonstrated that treatment of HaCaTs with 2.9 IU/mL of $\alpha$-T in serum-free media for 24 h prior to UVA (8 J/cm$^2$ 365 nm) showed a significant increase in the cell survival fraction by 15% compared to irradiated vehicle control (60.2% vs 45%, respectively) (Wu et al., 2014). However, in this Chapter the post-irradiation treatment with either $\alpha$-TP or $\alpha$-T did not show any photoprotection against cell viability reduction.

The generation of oxidative stress by long waveband UVA1 has many serious consequences in human skin. ROS can lead to oxidation of lipid membrane, DNA,
and proteins and DNA oxidation has been associated with signature mutations and subsequent onset of skin cancer. Thus, the photoprotection offered by α-TP on reducing the level of ROS generated by UVA1 was also investigated in Chapter 4. The DCFDA ROS detection assay indicated irradiation of the vehicle-treated cells with long waveband UVA1 led to an increase in the level of ROS. Furthermore, the pre-irradiation (dose of 57 J/cm²) treatment with 100 μM α-TP in low-serum medium significantly reduced the ROS generation in HaCaT keratinocytes cells by 24.1%. This α-TP antioxidative effect was comparable to α-T (23%), when compared to the irradiated vehicle control. It is possible that the antioxidant activity of α-TP is due to its conversion to α-T, possibly via phosphatase enzyme, intracellularly. However, the conversion rate in cell line has previously been reported to be < 5% hence this was unlikely (Kato and Takahashi, 2012; Nakayama et al., 2003). However, this conversion may be very sensitive to the experimental conditions. Another possible reason is the antioxidant mechanism of α-TP is dependent on its chemical structure that allows its incorporation into biomembranes, and thus it may inhibit the propagation of radical species (Rezk et al., 2004). The photoprotective effect of α-T was to a lower degree, compared to the findings of Wu et al., as they found a significant reduction in ROS generation by 80% in HaCaT keratinocytes when treated with α-T in serum-free medium prior UVA1 irradiation with 8 J/cm² dose compared to irradiated vehicle control (Wu et al., 2014).

Photostability studies were performed to verify the amount of active that was present after the UVR. The data showed that α-TP was photostable whereas α-T degraded by 15% after exposure to 50 SED dose of SSR. The 15% degradation could have been consequential if the concentration of the actives were not at their
optimal levels. This agreed with reports that α-T is photodegraded by up to 90% after 1 h irradiation with SSR (dose of 198 kJ/m²), which leads to a reduction in its antioxidant activity (Scalia, et al. Marchetti and Bianchi, 2013).

The results from Chapter 4 in general, indicated that α-TP showed superior protection against cell viability reduction, similar reduction in ROS level (Fig. 6.4), and possessed superior photostability compared to α-T. Hence, it appeared that α-TP could be translated into an effective cosmeceutical product. However, it is unlikely that a pure antioxidant product would be produced by the cosmetics industry, because consumers often demand protection from sunburn and oxidative damage arising from UVB and UVA respectively, in a single product. When combined in mixtures with sunscreening agents antioxidants have the potential to interact and possibly lose some of the beneficial effects that have been observed when the products are used alone. Hence in Chapter 5 the effects of combining sunscreening agents like nanoparticles with to α-TP in a lotion and semisolid cosmetic formulations were assessed.
Fig. 6.4: The photoprotection of α-TP nanomaterial in Tris formulation against UVA1 induced cell viability reduction and ROS generation compared to irradiated vehicle-treated cells in HaCaT keratinocytes.

Previous studies have conventionally used nanoparticles as a drug carrier to deliver α-T into the skin (Moddaresi et al., 2010). However, Chapter 5 reports on the mixing of nanoparticles, which can act as a physical sunscreen, with α-TP aggregates to understand these interactions and the consequences on product performance. A 2% α-TP lotion at pH 7.4, with effective skin deposition and SC lipid interaction, was compared with 2% TP which naturally formed a gel at pH 9, in order to observe the effects in the two different vehicles. Negatively charged unmodified, carboxyl-modified, and amine-modified silica nanoparticles (200 nm) were used to model the silica coated zinc oxide nanomaterial, which has been used previously as a physical sunscreen (Sotiriou et al., 2014). These were compared to 50 nm SLN, which has also previously shown to act as a physical sunscreen (Müller et al., 2000; Wissing and Müller, 2002). The data from Chapter 5 indicated that when α-TP was mixed with silica nanoparticles, the α-TP flux, the permeability
coefficient, and the 3.5 h accumulative mass from a lotion formulation did not change. In an α-TP gel, the silica nanomaterial slowed the α-TP penetration through a 1000 KDa cellulose ester membrane. The SLN generated a 2.0-fold and 11-fold enhancement in α-TP penetration across 1000 KDa cellulose ester membrane from the lotion and gel-like formulation, respectively (see figure 6.5.a).

To understand the nanoparticle-drug interactions, the dynamic size of nanoparticles or the viscosity was monitored immediately after mixing with the α-TP in lotion or in the gel, respectively. The hydrodynamic size of negatively-charged hydrophilic silica nanoparticles alone was 138-165 nm. This increased by 30-80 nm after mixing with negatively-charged α-TP (ratio 1:1) indicating that negatively charged α-TP aggregates interacted with the negatively charged head group found on the nanoparticle (α-TP aggregate size in 9.1 nm). This agreed with recent studies which proposed that worm-like micelles (aggregates) of cationic CTAB, via their endcap, can adsorb to the surface of like-charged (negatively charged) or counter charged (positively charged) silica nanoparticles (Dai et al., 2017; Helgeson et al., 2010; Nettesheim et al., 2008a). However, mixing α-TP with SLN slightly decreased the hydrodynamic size of SLN. This was thought to be as a result of adsorbing the hydrophobic tail of α-TP to the SLN surface; stabilizing the SLN and breaking the α-TP aggregates (see figure 6.5 b). This was similar to amphiphilic amino acid co-surfactant, which at a pH higher than pKa2, formed negatively charged molecules that once adsorbed on the SLN interface, increase their stability by increasing the repulsive interaction and minimizing its coalescence (Shafique et al., 2017). The effects observed in the regenerated cellulose membrane did not manifest in the pig skin permeation studies. This is likely due to the favourable interaction of α-TP with the skin, thus causing self-enhancement for its own permeation, possible by
slight structural alteration in the lipid lamellae found in SC which was not seen in the cellulose membrane passive diffusion studies.

Fig.6. 5: The dynamic α-TP gel mixed with SLN provided higher transport across cellulose ester porous membrane compared to gel control (a) possibly by breaking the aggregation (b).

In combination, the data from this thesis provided evidence that α-TP aggregates formulated in polar vehicles can penetrate into the skin and protect against UVA1. The α-TP can be formulated with a physical sunscreen, such as SLN, without deteriorating the product efficacy.

There were a few limitations to the studies. The first was that a synthetic α-TP was used. Ideally the natural compound should have been used as developed by Harper et al. However, the time was not available in this project to undertake the synthesis, purification and characterisation required to use this non-commercially available material (Harper et al., 2018). In addition, in the α-TP-skin interaction studies only a very simplistic SC membrane model was used. Work with whole tissue including keratin binding and epidermal sheet partitioning work could be undertaken in future...
work. Porcine skin was used in the permeation studies. Ideally, the gold standard model for such studies is *ex vivo* human skin, which provides an even greater barrier compared to porcine skin, should be used. At the time of the study the ethics permission to use human skin was not in place in the research group. In the UVR protection work, a limitation to the data interpretation is lack of knowledge of $\alpha$-T to $\alpha$-TP conversion during the studies. Although this has previously been measured by other workers, a repeat of these measurements in the exact cell culture media used in the current PhD would have strengthened the data. In addition, using more than one cell type in the UVR would have strengthened the data. Another limitation to this current work was the insufficient data for the rheological study. The frequency sweep during the measurement was done only over a decade instead of three decades, which would have provided more detail on the shear thinning effects. The rheology of the gel-type structure of $\alpha$-TP can be compared with that of carbomer (poly-acrylic acid) gel to assess the mechanical properties, *i.e.*, the solid-like (elastic) and liquid-like (viscous) properties when compared to a well characterised material. However, these limitations open opportunities for future work and investigations.

**Future work**

The aim of this thesis was met successfully, but the data generated provided many new opportunities for future work. For example, further *in vitro* permeation studies, using excised human skin could be performed to smooth the translation of the product into human use. In addition, although this study developed a cosmetic hydro-alcoholic lotion formulation of $\alpha$-TP nanomaterial for sun protection against
UVA1, future work could look into developing different types of formulations. In addition, *in vivo* work should be conducted to investigate the photoprotection of the developed α-TP formulation *in vivo*. To facilitate studies in human ethical approval is required, participants enrolled to the study and all the active ingredients and the excipient in the formulation should be in cosmetic grade. The *in vivo* work should test if the α-TP photoprotective formulation is effective alone to protect the skin from long wavebands or combined with other UVR filters to maximize the protection.
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