Branka Rozman

Topical microemulsions containing vitamins C and E: from formulation optimisation to evaluation using reconstructed human epidermis

Dermalne mikroemulzije z vitaminoma C in E: od optimiranja sestave do vrednotenja na modelu človeških keratinocitov

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The articles included in the dissertation were not used as a part of a doctoral dissertation of any of the coauthors.
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The author herewith declares that the content of the thesis is a result of his own work supervised by professor Mirjana Gašperlin as mentor. Results achieved in collaboration with other coworkers are published in the presented papers.

Branka Rozman

Izjavljam, da je pričujoča doktorska disertacija samostojno delo, ki sem ga izvedla pod mentorstvom prof. dr. Mirjane Gašperlin. Rezultati, ki so nastali v sodelovanju s sodelavci, so objavljeni v pričujočih člankih.

Branka Rozman
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TABLE OF CONTENTS

I. ABSTRACT 1

II. RAZŠIRJENI POVZETEK 5

1. INTRODUCTION 19

1.1. Microemulsions (ME) 21
   1.1.1. Definition, composition and structure 21
   1.1.2. Characterization of ME 22
   1.1.3. ME as colloidal drug delivery vehicles for topical application 23

1.2. Skin, free radicals and antioxidants 26
   1.2.1. Role of free radicals in skin ageing and disorders 26
   1.2.2. Skin antioxidant defence 27
   1.2.3. Oxidative stress 32

1.3. Vitamins C and E in human skin 33
   1.3.1. Vitamin E 33
   1.3.2. Vitamin C 35
   1.3.3. Interactions of vitamins C and E 37

1.4. Antioxidant delivery approaches 38
   1.4.1. Chemical modifications 39
   1.4.2. Novel drug delivery systems 41

1.5. Alternative methods for skin toxicity testing 45
   1.5.1. Skin irritation testing 45
   1.5.2. Skin corrosion testing 46
   1.5.3. Skin phototoxicity testing 47

2. OBJECTIVES OF THE THESIS 55

3. RESULTS AND DISCUSSION 59

3.1. Chapter 1

Stability of vitamins C and E in topical microemulsions for combined antioxidant therapy 61

3.2. Chapter 2

Simultaneous absorption of vitamins C and E from topical microemulsions using reconstructed human epidermis as a skin model 85

3.3. Chapter 3

Dual influence of colloidal silica on skin deposition of vitamins C and E simultaneously incorporated in topical microemulsions 109

3.4. Chapter 4

Temperature sensitive microemulsion gel: an effective topical delivery system for simultaneous delivery of vitamins C and E 134
3.5. Chapter 5

Key role of structure in microemulsion skin irritation and phototoxicity potential 162

4. CONCLUSION 185

III. APPENDIX 189

Bibliography 191
Curriculum vitae 193
I. Abstract
The novel role of oxidants and antioxidants in skin ageing as well as in various skin disorders and diseases has opened new research areas in several disease states and their therapeutic strategies. Successful prophylaxis and therapy of reactive-oxygen mediated skin ageing and photogenesis would necessitate control of oxidant/antioxidant balance in the affected site, which can be achieved by external supply of skin endogenous antioxidants. However, antioxidants’ skin bioavailability is usually low, mainly due to their poor aqueous solubility, inefficient permeability or instability during the storage. Colloidal drug delivery systems, like microemulsions (ME), nanoparticles or liposomes, are applicable to overcome the stability and pharmacokinetic problems associated with antioxidant delivery. These systems offer better targeting to upper skin layers, with faster onset and lower concentrations of the antioxidants needed. Moreover, liposomes and ME have a unique ability to simultaneously deliver both lipid- and water-soluble antioxidants.

The thesis focuses on topical ME simultaneously loaded with lipophilic vitamin E, skin’s major membrane antioxidant and hydrophilic vitamin C, the main component of skin water-soluble antioxidant defence. Initially, viscosity of liquid o/w and w/o ME both composed of the same ingredients has been optimized for topical application. Among thickening agents tested carbomer, colloidal silica and mixture of xanthan and alginate revealed as suitable thickeners for o/w ME and white wax and colloidal silica for w/o ME. Transparent microemulsion gel (gel-like ME) that has been formed upon the addition of specific amounts of water to liquid o/w ME has been investigated as an alternative solution to viscosity problem.

Gel-like ME was found to offer the best protection for both vitamins, although other ME also significantly increased their stability compared to solution. In the presence of vitamin C no decrease in vitamin E content occurred. The addition of thickeners to ME changed the stability of at least one vitamin, but the systems still protected vitamins better than solutions, the exception being o/w ME thickened with mixture of xanthan and alginate.

By varying the composition of ME, absorption of vitamins C and E in reconstructed human epidermis was significantly modulated. The absorption of vitamins C and E in epidermal and collagen layer was in general enhanced by ME when compared to solutions. The location of the antioxidants in the ME and affinity for the vehicle appeared to be crucial in the case of non-thickened ME. Addition of colloidal silica to ME enhanced the deposition of vitamins E and C in the reconstructed human epidermis.

The addition of colloidal silica to either o/w or w/o ME also increased skin bioavailability of both vitamins. Dual influence of colloidal silica on skin delivery of vitamins C and E from
topical ME was confirmed: it affected formulation characteristics and had a direct impact on the skin.

The temperature-driven changes in the microstructure of gel-like ME together with their influence on skin deposition of vitamins C and E were confirmed by rotational rheometry, viscosity measurements, droplet size determination and pig ear permeation studies. The release studies have shown that the vitamins’ release at skin temperature from gel-like ME were comparable to those from o/w ME and were much faster and more complete than from o/w ME conventionally thickened with polymer (o/w ME carbomer). Concerning effectiveness in skin delivery of both vitamins o/w ME was found the most appropriate, followed by gel-like ME and by o/w ME carbomer.

Finally, the irritation potential of ME was assessed in comparison to commercially available ME. Two different models were used: reconstructed human epidermis as ECVAM validated model for skin irritation testing and NCTC 2544 human keratinocyte cell cultures. Both models confirmed our ME less irritant than commercial product.

In summary, ME have been proved effective and non-irritant vehicles with functionally suitable consistency for simultaneous topical delivery of a hydrophilic vitamin C and a lipophilic vitamin E.
II. Razširjeni povzetek
Uvod

Mikroemulzije

Mikroemulzije so bistre, termodinamsko stabilne disperzije vodne in oljne faze, stabilizirane z medfaznim filmom emulgatorja. Kot nosilni sistemi za dermalno dostavo učinkovin izkazujejo številne prednosti, saj lahko vanje zaradi njihove specifične zgradbe vgradimo tako vodotopne kot tudi lipofilne in/ali amfifikne učinkovine z namenom povečanja topnosti, hitrosti in obsega absorpcije učinkovine, doseganja prirejenega sproščanja, zaščite nestabilne učinkovine, zmanjšanja variabilnosti biološke uporabnosti ter zakrivanja neprijetnega vonja. Kljub naštetim prednostim je število registriranih mikroemulzij majhno, in sicer predvsem zaradi velikega deleža deleža emulgatorjev, ki so potencialno toksične snovi. Mikroemulzije so pogosto Newtonske tekočine z nizko viskoznostjo, ki so neprimerne za direkten nanos na kožo. Viskoznost mikroemulzij lahko vpliva z dodajanjem zgoščeval, ki ustrezno spremenijo reološko obnašanje sistema, ne da bi pri tem vplivala na druge lastnosti mikroemulzij, kot so bilistentnost, bistrost in spontan nastanek. Druga možnost prirejanja viskoznosti je izdelava mikroemulzijskih gelov z izbiro ustreznih sestavin mikroemulzij in določitvijo koncentracij, kjer spontano tvorijo lamelarne strukture, za katere je značilna viskoznost.

Koža, radikali in antioksidanti

predelih celice, vse več dejstev dokazuje, da je njuno delovanje sinergistično. Vitamin C potencira antioksidativno učinkovitost vitamina E preko regeneracije tokoferoksilnega radikala v aktivno obliko vitamina E. Antioksidativni obrambni mehanizmi so torej medsebojno prepletenci, zato je ravno preseje med posameznimi kožnimi antioksidanti zelo pomembno. Ker oksidiran vitamin E za svoje obnavlanje troši druge antioksidante, je priporočljivo kombinirano dajanje vitamina E z antioksidantom, ki sodeluje pri njegovi obnovi, npr. vitaminom C, glutationom ali karotenoidi.

**Vloga nosilnega sistema pri dostavi antioksidantov v kožo**

Na trgu obstaja veliko formulacij z antioksidanti, najpogosteje z vitamini, ki pa so vgrajeni v klasične farmacevtske oblike, saj so le-te praviloma enostavne za izdelavo in relativno poceni. Učinkovitost teh pripravkov je pogosto vprašljiva zaradi slabih fizikalno-kemijskih in biofarmacevtskih lastnosti antioksidantov, kot so nizka topnost, slaba permeabilnost in/ali visoka nestabilnost. Eden od načinov zaščite antioksidantov in izboljšane dostave na mesto delovanja je njihova vgraditev v različne koloidne nosilce: micle, mikroemulzije, nanodelce, liposome. Za doseganje ustreznih koncentracij antioksidantov v koži peroralno jemanje običajno ne zadostuje, nasprotno pa sta se dermalna dostava in kombinacija dermalne in per os dostave antioksidantov, vgrajenih v ustrezne nosilne sisteme, pokazali kot učinkoviti. V zadnjih letih so antioksidanti postali marketinški hit v kozmetični industriji. Uporabljajo se predvsem z namenom upočasnjevanja staranja kože. V tabeli 1 so navedeni nekateri primeri komercialnih izdelkov, ki vsebujejo kot aktivne učinkovine antioksidante, vgrajene v koloidne nosilne sisteme.

**Tabela 1. Komercialno dostopni izdelki proti staranju, ki vsebujejo antioksidante vgrajene v koloidne nosilne sisteme.**

<table>
<thead>
<tr>
<th>Zaščiteno ime</th>
<th>Proizvajalec</th>
<th>Antioksidant</th>
<th>Dostavni sistem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revitalift</td>
<td>L’Oreal</td>
<td>Pro-retinol</td>
<td>nanosomi</td>
</tr>
<tr>
<td>Bioperformance Crème Super</td>
<td>Lancome</td>
<td>Gama linolenska kislina</td>
<td>nanokapsule</td>
</tr>
<tr>
<td>Agera microemulsion antioxidant serum</td>
<td>Agera</td>
<td>Vitamin E, vitamin A, beta karoten</td>
<td>mikroemulzija</td>
</tr>
<tr>
<td>Advanced night repair protective</td>
<td>Estee Lauder</td>
<td>retinilpalmitat, tokoferilacetat, ekstrakt Acuminata</td>
<td>liposome</td>
</tr>
<tr>
<td>Radical sponge</td>
<td>BioResearch</td>
<td>C₆₀ nanodelci</td>
<td>/</td>
</tr>
</tbody>
</table>

Dokaz, da je to področje zelo aktivno tudi z raziskovalnega vidika so številne publikacije.
Alternativne metode za vrednotenje varnosti dermalnih pripravkov

Najboljši sistem za vrednotenje varnosti in učinkovitosti dermalnih pripravkov je človeška koža in vivo. Za ovrednotenje draženja kože se je tradicionalno uporabljal Draizeov test na kuncih, ki pa je tarča številnih kritik, tako zaradi uporabe živali kot tudi z vidika prenosljivosti podatkov na človeka. Ker postaja uporaba živali v laboratorijske namene čedalje strožje regulirana, je bilo veliko truda vloženega v razvoj novih in vitro modelov, med katerimi so najbolj obetajoče celične kulture. Čeprav je koža heterologena membrana, sestavljena iz več vrst celic, je bilo ugotovljeno, da monokulture človeških keratinocitov (rekunstruirani človeški epidermis) dobro posnemajo tridimenzionalno zgradbo človeške kože. Tudi na področju morfologije, sestave lipidov in biokemičnih označevalcev celične kulture izkazujejo precejšnje podobnosti s človeško kožo, tako da so v zadnjem desetletju postale vodilni model za preučevanje draženja kože, povzročenega z dermalnim nanosom učinkovine ali formulacije. Leta 2007 je Evropski center za validacijo alternativnih metod (ECVAM) EpiSkinov model celičnih kultur keratinocitov priznal kot prvi in vitro model, ki se lahko uporablja kot enakovredna zamenjava za Draizeov test draženja kože, leto kasneje pa še dva modela (SkinEthic in EpiDerm). Vsi trije modeli so tudi validirani za določevanje kožne korozivnosti. Poleg tridimenzionalnih celičnih kultur so razvijali tudi druge modele, ki so osnovani predvsem na izolirani živalski koži, vendar pa sta bila uspešno validirana samo merjenje električne prevodnosti na izolirani podganji koži kot model za kožno korozivnost in določevanje prevzema barvila v celice mišjih fibroblastov (3T3 NRU test) kot model za določevanje fototoksičnosti.

Cilji naloge

V doktorskem delu smo ovrednotili primernost mikroemulzij kot nosilnih sistemov za sočasno dostavo vitaminov C in E v kožo.

Izhodiščna sestava nezgoščenih mikroemulzij je rezultat predhodnega raziskovalnega dela katedre za Farmacevtsko tehnologijo Fakultete za farmacijo v Ljubljani. Mikroemulzije so sestavljene iz izopropilmiristata kot lipofine faze, Tweena 40 kot emulgatorja, Imwitorja 308 kot koemulgatorja in prečiščene vode kot hidrofilne faze. Vse komponente so primerne za uporabo na koži, delež površinsko aktivnih snovi pa je najnižji, ki še omogoča nastanek mikroemulzij.

Delo je bilo razdeljeno na več sklopov.

V poglavju 1 smo optimirali osnovno sestavo tekočih mikroemulzij z namenom prilagoditi viskoznost dermalni aplikaciji. V ta namen smo preskusili velik nabor farmacevtsko
sprejemljivih zgoščeval. Zgoščevala, ki so se izkazala za kompatibilna z osnovnimi sestavinami mikroemulzij tipa o/v in v/o, smo ovrednotili z reometrično analizo. Vpliv izbranih zgoščeval na mikrostrukturo mikroemulzij smo nadalje preučili z uporabo termične analize. Ker je znano, da je vitamin C v formulacijah, ki vsebujejo vodo, zelo nestabilen, in da je vitamin E ob izpostavitvi svetlobi podvržen fotooksidaciji, smo preučevali (foto)stabilnost obeh vitaminov v nezgoščenih in zgoščenih sistemih. Iskali smo povezave med stabilnostjo antioksidantov, njuno lokacijo v mikroemulziji, strukturo mikroemulzije in fizikalno-kemijskimi lastnostmi zgoščevala.

V **poglavju 2** smo ovrednotili sproščanje obeh vitaminov iz mikroemulzij in njuno porazdelitev v kožo. Absorpcijo vitaminov v in preko kože smo spremljali s pomočjo celičnih kultur človeških keratinocitov. Poleg vpliva mikroemulzij na celične kulture smo ovrednotili tudi vpliv lokacije vitamina v mikroemulziji ter njegovo mobilnost in afiniteto do nosilnega sistema na njegovo porazdeljevanje v različnem plasti celičnih kultur.

Študije stabilnosti in absorbicije vitaminov na celičnih kulturah človeških keratinocitov so pokazale izstopajoče lastnosti iz dveh sistemov: mikroemulzij zgoščenih s koloidnim silicijevim dioksidom in mikroemulzijskega gela, spontano nastalega ob povečanju deleža vode v o/v mikroemulziji. Lastnosti teh dveh sistemov smo zato podrobneje proučevali v poglavjih 3 in 4. V **poglavju 3** smo ovrednotili dva vidika delovanja koloidnega silicijevega dioksida v mikroemulzijah: prvič, njego sposobnost, da vpliva na lastnosti dostavnega sistema (porazdelitveni koeficient vitaminov, njuna topnost in sproščanje) in drugič, njegov neposredni vpliv na kožo preko merjenja prehoda vode preko kože (TEWL), slikanja kože z vrstnim elektronskim mikroskopom in viabilnosti celičnih kultur keratinocitov. Vpliv omenjenega zgoščevala na nalaganje vitaminov C in E v kožo smo dodatno proučili na izolirani koži prašičega ušesa kot najbolj ustreznem in vitro živalskem modelu za človeško kožo.

V **poglavju 4** smo raziskovali temperaturo pogojene spremembe strukture mikroemulzijskega gela v primerjavi s tekočo hidrofilno mikroemulzijo in mikroemulzijo, klasično zgoščeno s polimerom. Primerjali smo njihove reološke lastnosti in velikost kapljic notranje faze. Učinkovitost vseh treh formulacij kot nosilnega sistema za sočasno dostavo vitaminov C in E smo raziskali z in vitro testom permeacije na izolirani živalski koži.

V **poglavju 5** smo ocenili, v kakšni meri naši nosilni sistemi povzročajo draženje kože ter njihovo fototoksičnost. Poleg testiranja mikroemulzij na dveh različnih celičnih kulturah keratinocitov, kjer smo se osredotočili na celično viabilnost, smo uporabili še testiranje na izoliranem prašičjem ušesu, kjer smo spremljali spremembe barijerne funkcije prašičega
epidermisa. Za testiranje fototoksičnosti formulacij smo celične kulture po nanosu formulacij obsevali z necitotoksičnim odmerkom UVA svetlobe.

Metodologija

- **Primernost zgoščeval** iz nabora farmacevtsko uporabnih snovi smo ovrednotili z rotacijsko in oscilacijsko reometrijo s senzorskim sistemom stožec-ploščica (MK22).

- **Mikrostrukturo zgoščenih sistemov** smo določevali s termično analizo vzorcev (DSC, zamrzovanje vzorcev s hitrostjo 10K/min), fotonsko korelacijsko spektroskopijo in meritvami lomnega količnika.

- **Stabilnost** obeh vitaminov v različnih tipih mikroemulzij smo določili s HPLC metodo, ki jo smo predhodno razvili in validirali za vsak vitamin posebej. Vzorce smo shranjevali v tesno zaprtih vsebnikih na sobni temperaturi, razen tistih, ki so bili namenjeni študiju vpliva svetlobe. Za študije fotostabilnosti vitamina E smo vzorce 48 ur obsevali z UVA svetlobo.

  Kromatografski pogoji za vitamin C:
  Stacionarna faza: Nucleosil NH2 (5 μm, 250 x 4 mm)
  Mobilna faza: metanol: acetonitril: 0.02 M fosfatni pufer pH 3.5 = 20: 30: 50
  Pretok: 1 ml/min
  Valovna dolžina detekcije: 243 nm

  Kromatografski pogoji za vitamin E:
  Stacionarna faza: Nucleosil (5 μm, 120 x 4 mm)
  Mobilna faza: metanol: acetonitril= 70: 30
  Valovna dolžina detekcije: 292 nm

- **Sproščanje vitaminov** iz mikroemulzij smo izvedli preko umetne membrane (celulozniacetat) s pomočjo Franzovih celic. Receptorski medij za vitamin C je predstavljala 0,9% raztopina NaCl. Receptorski medij za vitamin E je bil sestavljen iz izopropilmiristata z dodatkom 0,5% Tweena 40 in 0,5% Imwitorja 308. Temperatura receptorskega medija je bila 32+/−1°C. V naprej določenih intervalih smo vzorčili 300 μl receptorskega medija. Vsebnost vitaminov v vzorcih smo določili s HPLC analizo.
Kinetiko sproščanja vitaminov iz mikroemulzij smo ugotavljali s pomočjo primerjave eksperimentalnih vrednosti z regresijskimi premicami za posamezne kinetike.

- **Topnost vitaminov** v posamezni formulaciji in njun porazdelitveni koeficient vodna/oljna faza smo določali s HPLC analizami vitaminov v formulacijah in posameznih fazah.

- **Perkutano absorpcijo** vitaminov smo proučevali na celičnih kulturah keratinocitov (EpiSkin® large model) in na izolirani koži prašičega ušesa. Kot receptorsko tekočino smo uporabili 0.9% vodno raztopino NaCl z dodanim 3% kokošjim albuminom. Poskus je potekal 6 ur; v vnaprej določenih intervalih smo vzorčili receptorsko tekočino. Po 6 urah smo koncentracije vitaminov v različnih plasteh kože oz. celičnih kultur in v receptorski tekočini Franzove celice po ekstrakciji določili s HPLC. Parametre permeabilnosti smo določili iz linearnega dela odvisnosti kumulativne količine sproščenega vitamina od časa sproščanja.

- **Spremembe morfologije rožene plasti kože** po nanosu mikroemulzij smo proučevali z vrstičnim elektronskim mikroskopom.

- Za določevanje celične viabilnosti, ki smo jo uporabili kot kriterij za določevanje draženja kože in fototoksičnosti nezgoščenih in zgoščenih mikroemulzij, smo uporabili MTT test, ki je osnovan na redukciji rumenega tetrazola v modri formazan s pomočjo mitohondrijskih encimov, ki so aktivni samo v živih celicah. Nastanek formazana smo kvantificirali s pomočjo UV spektroskopije. Celično viabilnost po izpostavitvi celičnih kultur testiranim mikroemulzijam smo primerjali z viabilnostjo celic, ki so bile pod enakimi pogoji izpostavljene raztopini standardnega irritanta in komercialno dostopni mikroemulziji. Poskuse smo izvedli na dveh celičnih kulturah keratinocitov: EpiSkin® large model in celični liniji NCTC 2544. Za testiranje fototoksičnosti smo celične kulture keratinocitov (EpiSkin® large model) istočasno izpostavili mikroemulzijam in necitotoksičnem odmerku UVA svetlobe in nato celično viabilnost primerjali z viabilnostjo celičnih kultur, ki so bile izpostavljene samo mikroemulzijam.
Pri testu draženja, ki je bil izveden na prašičji koži na Franzovih difuzijskih celicah, smo merili razliko v TEWL (transepidermal water loss) pred in po izpostavitvi kože mikroemulzijam.

- **Morfoligo celic** smo opazovali z uporabo invertnofaznega kontrastnega mikroskopa. Po inkubaciji celičnih kultur z mikroemulzijami smo obarvali celična jedra in aktinska vlakna.

- Za **statistično vrednotenje** rezultatov smo uporabili analizo variance enega faktorja in dvostranski Studentov t-test pri stopnji tveganja 0.05.

**Rezultati**

*Stabilnost vitaminov C in E v dermalnih mikroemulzijah za kombinirano antioksidantno terapijo*


Ugotovili smo, da je gelirana mikroemulzija najbolje ščitila oba vitamina, čeprav sta tudi drugi dve mikroemulzij bistveno povečali njuno stabilnost v primerjavi z raztopino. V prisotnosti vitamina C se vsebnost vitamina E ni zmanjšala. Dodatek zgoščeval v mikroemulzijo je spremenil stabilnost vsaj enega vitamina, vendar pa so tudi zgoščeni sistemi še vedno bolje ščitili vitamina kot raztopine, iz jemo mikroemulzije, zgoščene z zmesjo ksantana in alginata. Zelo verjetno so spremembe v notranji strukturi mikroemulzij, ki so posledica dodajanja zgoščeval, skupaj s spremembami v topnosti kisika v zunanjji faze najpomembnejši dejavniki, ki vplivajo na stabilnost vitaminov v zgoščenih mikroemulzijah.
Študija istočasne absorpcije vitaminov C in E iz dermalnih mikroemulzij na celičnih kulturah keratinocitov kot modelu kože

Z uporabo tridimenzionalnih celičnih kultur keratinocitov kot alternativnega modela za študije perkutane absorpcije zdravilnih učinkovin smo proučevali absorpcijo vitaminov C in E iz nezgoščenih mikroemulzij, mikroemulzij zgoščenih s koloidnim silicijevim dioksidom ter iz oljne raztopine vitamina E in vodne raztopine vitamina C. Mikroemulzije so se, v primerjavi z raztopinami, izkazale kot učinkovitejši nosilni sistem. Tudi med proučevaniami mikroemulzijami smo opazili znatne razlike. Vgradnja vitaminov v zunanjbo fazo mikroemulzij je povzročila večjo absorpcijo kot pri vitaminih, ki so se nahajali pretežno v notranji fazi. Pri vitaminu C je lokacija v mikroemulziji manj vplivala na njegovo porazdeljevanje v kožo kot pri vitaminu E. Posledično je za učinkovite vodne nosilne sistema pri vitaminu C in E boljše dostavo obeh vitaminov v celične kulture keratinocitov bolj primerna v o/mikroemulzija kot obratni tip.

Dodatek koloidnega silicijevega dioksida v mikroemulzijo je znatno povečal absorpcijo obeh vitaminov, kar je podstavilo lahko vodno vodeno raztopino vitaminov E in C v celične kulture, kar je posledica rušenja njihove barierne funkcije in sprememb pH vehikla.

Vpliv koloidnega silicijevega dioksida na sočasno prehajanje vitaminov C in E iz dermalnih mikroemulzij v kožo

Študije perkutane absorpcije na celičnih kulturah so pokazale, da je dodatek koloidnega silicijevega dioksida v mikroemulzijo drastično povečal absorpcijo obeh vitaminov, zato smo poskus ponovili na izolirani koži prašnega uhlja kot najboljšemu in vitro modelu za človeško kožo. Rezultati so potrdili vpliv koloidnega silicijevega dioksida na nalaganje vitaminov v prasičko kožo. Ugotovili smo, da je znatno vplival na lastnosti nosilnega sistema, kar smo dokazali z določevanjem topnosti in porazdelitvenega koeficienta vitaminov. Učinki so bili izraziteji pri vitaminu E, kar se odražalo tudi v študijah sproščanja. Medtem ko smo pri nezgoščenih sistemih pri vitaminu E opazili počasnejšo sproščanje iz o/v kot iz v/o mikroemulzije, pri sproščanju iz obeh tipov mikroemulzije, zgoščenih s koloidnim silicijevim dioksidom ni bilo razlike. Sproščanje vitamina C je bilo tako pri nezgoščenih mikroemulzijah kot pri mikroemulzijah zgoščenih s koloidnim silicijevim dioksidom hitrejše iz o/v kot iz v/o tipa. Potrdili smo tudi njegov neposredni vpliv na kožo. Prehod vode preko kože, na katero smo aplicirali mikroemulzijo zgoščeno s koloidnim silicijevim dioksidom, se je zmanjšal; predpostavili smo, da je koloidni silicijev dioksid zadržal vodo v roženi plasti kože. S pomočjo SEM slik smo namreč ugotovili, da se je masovno kopčil v zgornjih plastah kože. Primerjava celične viabilnosti kultur človeških keratinocitov ni pokazala občutne razlike med
toksičnostjo vodne disperzije koloidnega silicijevega dioksida in mikroemulzijo, zgoščeno s koloidnim silicijevim dioksidom.

**Temperaturno občutljiv mikroemulzijski gel: učinkovit nosilni sistem za sočasno dostavo vitaminov C in E**

S spreminjanjem deleža vode v tekoči hidrofilni mikroemulziji smo razvili mikroemulzijski gel s temperaturno odvisnimi reološkimi lastnostmi, katerega lastnosti in učinkovitost smo primerjali s tekočo o/v mikroemulzijo in o/v mikroemulzijo, klasično zgoščeno s polimerom (karbomerom). Pri sobni temperaturi je mikroemulzijski gel izražal reopeksno reološko obnašanje, ki ga v farmaciji redko zasledimo. S fotonsko korelacijsko spektroskopijo smo potrdili, da ga pri sobni temperaturi sestavljajo lamelarne faze, z zvišanjem temperature na fiziološko pa se struktura podre in preide v klasično mikroemulzijo z nanometrskimi kapljicami olja, razpršenimi v vodni fazi. Temperaturno pogojene spremembe mikrostruktura so bile dodatno potrjene z rotacijsko reometrijo in meritvami absolutne viskoznosti. Študije sproščanja so pokazale, da je sproščanje vitaminov iz gelirane mikroemulzije podobno sproščanju iz o/v mikroemulzije in je veliko hitrejše in popolnejše kot iz mikroemulzije zgoščene s karbomerom. Glede učinkovitosti dostave obeh vitaminov v kožo je bila najboljša o/v mikroemulzija, sledil ji je mikroemulzijski gel, najslabša pa je bila mikroemulzija zgoščena s karbomerom. Študija citotoksičnost je pokazala dobro preživetje celic po izpostavljenosti mikroemulzijam in potrdila vse preskušane mikroemulzije kot primerne za dermalno aplikacijo.

**Vpliv strukture mikroemulzij na njihovo draženje kože in fototoksičnost**

V zadnjem poglavju smo ocenili potencial mikroemulzij sestavljenih iz izopropilmiristata, Tweena 40, Imwitorja 308 in prečiščene vode, za draženje kože v primerjavi s tržno dostopno mikroemulzijo. Z uporabo dveh različnih celičnih kultur keratinocitov (tridimenzionalni model EpiSkin in NCTC 2544 celična linija) smo potrdili, da so naše mikroemulzije manj dražeče kot komercialna. Nadalje je vrednotenje na EpiSkinovem modelu pokazalo, da je mikroemulzijski gel bolj dražeč kot o/v in v/o mikroemulziju, čeprav so vsi sistemi vsebovali enako količino istih emulgatorjev (Tween 40, Imwitor 308), snovi, ki jih običajno povezujemo z draženjem kože. Nasprotno pa NCTC 2544 celice niso razlikovale med o/v, v/o mikroemulzijo in mikroemulzijskim gelom. Razlog smo pripisali različnemu protokolu testiranja – medtem ko smo pri EpiSkinovem modelu vzorce nanesli direktno na površino celičnih kultur, smo morali vzorce pred testiranjem na NCTC celicah razredčiti, s čimer smo
podrli mikrostrukturo vzorca. Iz tega smo sklepali, da je poleg količine in kemizma površinsko aktivne snovi, ki ga formulacija vsebuje, za dermalno sprejemljivost bistvena struktura pripravka. Rezultati dobljeni na EpiSkinovem modelu so dobro korelirali z oceno draženja kože z vrednotenjem barierne funkcije prašičjega ušesa.

Zaključek

Za optimiranje dermalne aplikacije smo izhodni tekoče o/v in v/o mikroemulziji, sestavljeni iz izopropilmiristata kot lipofilne faze, Tweena 40 kot emulgatorja, Imwitorja 308 kot koemulgatorja in prečiščene vode kot hidrofilne faze, zgostili z dodatkom ustreznih zgostil. Kot primerna zgostitev so se izkazali karbomer, zmes ksantana in alginate in koloidni silicijev dioksid za o/v mikroemulzijo ter beli vosek in koloidni silicijev dioksid za v/o mikroemulzijo. Kot drugo možnost za povečanje konsistence smo izbrali mikroemulzijski gel, sistem pri katerem se emulgatorji uredijo v plasti in tvorijo lamelarne strukture, zaradi česar se poveča viskoznost.

Vgradnja vitaminov C in E v nezgostočene mikroemulzije je signifikatno povečala njuno (foto)stabilnost. Prisotnost vitamina C v mikroemulzijah je povečala UVA stabilnost vitamina E. Stabilnost vitaminov v zgoščenih sistemih je bila odvisna od fizikalno-kemijskih lastnosti zgoščevala in njegovega vpliva na strukturo mikroemulzije. Z vidika stabilnosti vitaminov so bile preskusane zgoščene mikroemulzije, z izjemo o/v mikroemulzije zgoščene z zmesjo ksantana in alginate, primeri nosilnih sistemov za sočasno vgradnjo obeh vitaminov.

Mikroemulzije so, v primerjavi z raztopinami, povečale nalaganje obeh vitaminov tako v tridimenzionalne celične kulture človeških keratinocitov, ki smo ga preskusili kot potencialen model za študije perkutane absorpcije kot tudi v izolirano kožo prašičjega uhlja, ki je najboljša in vitro alternativa za človeško kožo. Na obeh modelih sta se zlasti mikroemulziji, zgoščeni s koloidnim silicijevim dioksidom, izkazali kot zelo učinkovit sistem na za dermalno dostavo obeh vitaminov. Ko smo podrobnije proučili vlogo silicijevega dioksidna na perkutano absorpcijo vitaminov, smo potrdili njegov vpliv na lastnosti mikroemulzij (kar smo dokazali preko določevanja porazdelitvenega koeficienta vitaminov in njune topnosti) in tudi neposredno na kožo, saj je masovno kopčil v zgornjih plasteh roženega sloja.

Rezultati absorpcije vitaminov iz mikroemulzijskega gela v celične kulture keratinocitov so nakazali, da se struktura gela pri povišani temperaturi podre in postane podobna strukturi tekoče o/v mikroemulzije, saj so bile razlike v absorpciji vitaminov iz obeh sistemov zelo majhne. Čeprav smo dokazali prehod lamelarne strukture mikroemulzijskega gela v kapljično
ob izpostavitvi fiziološki temperaturi z reometrijo, merjenjem viskoznosti in fotonsko korelacijsko spektroskopijo, je bil sistem manj učinkovit kot o/v mikroemulzija pri dostavi vitaminov v kožo prašičjega uhlja; razlog je verjetno manjši delež oljne faze, ki je znan pospeševalc kožne penetracije. Mikroemulzijski gel pa je bil bolj učinkovit v primerjavi s klasično zgoščeno mikroemulzijo s karbomerom.

Izdelane mikroemulzije so manj dražile kožo kot komercialno dostopna, kar smo dokazali s proučevanjem celične viabilnosti na dveh modelih človeških keratinocitov - na tridimenzionalnih celičnih kulturah in na celični liniji. Noben model ni pokazal razlike med mikroemulzijami s kapljično strukturo (o/v mikroemulzija, o/v mikroemulzija zgoščena s karbomerom, v/o mikroemulzija, v/o mikroemulzija zgoščena z belim voskom). Mikroemulzijski gel z lamelarno strukturo je bil na tridimenzionalnih kulturah bolj citotoksičen kot ostale mikroemulzije, čeprav je vseboval enake količine površinsko aktivnih snovi. Na celičnih linijah nismo opazili razlike, kar smo pripisali uničenju lamelarne strukture pri redenju s celičnim medijem, saj testiranje na pripravkih na celičnih linijah, v nasprotju s tridimenzionalnimi celičnimi kulturami, ne omogoča direktnega nanosa formulacije. Ko smo mikroemulzije ovrednotili še s testom barierne funkcije prašičjega ušesa, na katerega smo nanašali nerazredčene pripravke, smo ponovno opazili povečano draženje kože po nanosu mikroemulzijskega gela. Zaključili smo torej, da struktura mikroemulzije eden odločilnih faktorjev pri dermalni toksičnosti.
1. Introduction
1.1. Microemulsions (ME)

1.1.1. Definition, composition and structure

Microemulsions (ME) are clear, thermodynamically stable dispersions composed of water, oil and surfactants. These spontaneously formed systems possess specific physicochemical properties, such as transparency, optical isotropy and low viscosity. The transparency of ME arises from their small droplet diameter, which is usually within the range of 10-140 nm. An essential requirement for their formation and stability is the attainment of a very low interfacial tension between oil and water, which is achieved by surfactants [1, 2]. Recently attempts have been made to use natural surfactants, mainly alkyl polyglycosides, phospholipids and sucrose esters that are known for their good biocompatibility. Pegylated fatty alcohols are also surfactants of interest, since they offer the possibility of forming co-surfactant-free ME. However, in most ME the combination of surfactant and cosurfactant is used. The purpose of using the cosurfactant – usually a short chain alcohol – is to increase the interfacial film fluidity. The choice of oil depends mostly on its solubilisation capacity for the drug used [3, 4]. Frequently used components of ME are given in Table 1 (adapted from [3]).

Table 1. Frequently used components of ME.

<table>
<thead>
<tr>
<th>surfactant</th>
<th>sorbitan fatty acid esters, polysorbates, (pegylated) glycerol fatty acid esters, pegylated fatty alcohols and acids, poloxamers, alkyl polyglycosides, sucrose esters, phospholipids, fluorosurfactants</th>
</tr>
</thead>
<tbody>
<tr>
<td>cosurfactant</td>
<td>ethanol, isopropanol, butanol polypropylene glycol, tetruglycol, octanediol, polyglyceryl fatty acid esters, alkyl ethers</td>
</tr>
<tr>
<td>lipophilic phase</td>
<td>vegetable oils, esters (isopropyl myristate, isopropyl palmitate), medium chain triglycerides, benzyl alcohol</td>
</tr>
<tr>
<td>hydrophilic phase</td>
<td>water with or without electrolytes</td>
</tr>
</tbody>
</table>

The simplest representation of the structure of ME is the droplet model in which ME droplets are surrounded by an interfacial film consisting of surfactant molecules. As shown on Fig. 1 there are three basic structural types of ME: water-in-oil (w/o), oil-in-water (o/w) and bicontinuous [1, 5].
ME formation is dependent on oil-water-surfactant ratio, and the region of existence is determined from ternary and pseudoternary (in the presence of cosurfactants) phase diagrams. A highly schematic pseudoternary phase diagram is presented on Fig. 2.

**1.1.2. Characterization of ME**

The common feature of ME is that their structures are highly dynamic, undergoing continuous and spontaneous fluctuations. As structures can also be altered by changes in composition and
temperature, their direct examination is very difficult. However, as drug delivery properties of ME are related to their inner structure, there is a need to correctly characterize their structure [3, 6, 7]. Many techniques have been employed with varying success in the analysis of ME [1], but generally the combination of techniques is required to fully characterize these systems [8, 9]. At the macroscopic level viscosity, conductivity, density and dynamic scanning calorimetry (DSC) determination provide useful information [10]. Nuclear magnetic resonance (NMR) [11] and small angle x-ray scattering (SAXS) are powerful tools for elucidating the microstructure of ME [12]. The most used technique for droplet size calculation is dynamic light scattering (DLS) [13-16].

1.1.3. ME as colloidal drug delivery vehicles for topical application

Topical ME are experiencing a very active development as reflected by numerous publications and patents being granted on these systems [17]. These systems have because of their specific structure considerable potential to act as drug delivery vehicles by incorporating lipophilic, water-soluble and/or amphiphilic drugs. They have been shown to be able to increase drug solubility, increase rate and extent of absorption, modify drug release, protect labile drug, reduce patient variability and mask unpleasant odour [1, 2, 14, 18, 19]. ME demonstrated a significant increase of bioavailability compared to emulsions, gels and solutions, which is mainly attributed to extended and sustained absorption due to the reservoir effect of inner phase. A number of mechanisms are hypothesized as the basis for increased topical drug delivery, but up to now the mechanism is not yet fully understood. Most likely it is attributable to a variety of factors depending on the composition and the resulting microstructure of ME. The high solubilisation capacity allows a large amount of drug to be included. Continuously and spontaneously fluctuating interfaces of ME enable high drug mobility and might enhance drug diffusion process [20, 21]. Finally, it is possible that the surfactants may reduce the diffusion barrier of stratum corneum [14]. However, the influence of surfactant content on drug flux has been studied several times and it turned out that the more surfactant is not always the better.

Distinctive property of ME for topical application is their increased viscosity, which is of practical relevance to the administration. ME are by definition low viscosity Newtonian fluids, making proper skin application difficult. However, from phase diagrams constructed for the selected components, areas with lamellar structures and consequently increased viscosity can be formed [8, 22, 23]. These systems possess a structural organisation very
similar to that of ME and retain the properties of ME such as thermodynamic stability and transparency. Since they do not fulfil the criteria of Danielsson-Lindman definition for ME (optical isotropy, liquidity) they are often named ME gels. Most frequently used ME gels used in topical delivery are lecithin or sorbitan ester based systems in biocompatible solvents [6, 14]. When different lecithin ME gels containing indomethacin and diclofenac were studied in comparison to oily solutions of both drugs, in permeation studies higher drug fluxes were obtained by ME gels [24]. A study investigating the relationship between the colloidal structure of ternary phospholipid-containing formulations and kinetics of diclofenac diethylamine release as well as its stratum corneum permeability confirmed that drug release and permeation depend on the vehicle’s microstructure. Depending on the ratio of components liposomes, lamellar liquid crystals and ME were formed. Whereas in liposomes and lamellar phases drug was strongly bound and the phospholipids hampered the interactions with stratum corneum, incorporation into ME enhanced its permeability [25].

The more usual way to solve viscosity problem is to add a suitable thickening agent to liquid ME. Ideally it should modify the rheological behaviour without significant influence on other features of ME, such as stability, transparency and spontaneous formation [26]. Thickened ME are usually composed of two distinct structural elements, a network formed by thickener in the outer phase that coexists with the microemulsion droplets [23] as shown on Fig. 3.

![Proposed structure of ME thickened with gelatine based on small angle neutron scattering](image_url)

Fig. 3. Proposed structure of ME thickened with gelatine based on small angle neutron scattering [27].

Thickening agents like carrageenan, carbopols, xanthan, gelatine and colloidal silica have been used to thicken topical ME [26, 28, 29]. The permeability of estradiol from
microemulsion thickened with carbopol was less than from non-thickened ME, which was attributed to increased viscosity of the vehicle and consequently highly ordered microstructure [28]. On the contrary the addition of colloidal silica to w/o ME led to increased amount of released sodium ascorbyl phosphate. The authors assumed that colloidal silica modified the physicochemical characteristics of the external phase and hence the diffusion of highly hydrophilic sodium ascorbyl phosphate from the internal through the external phase of ME [26]. Addition of carrageenan transformed liquid ME into stable semisolid gels exhibiting increased thixotropy. An enhancing effect of carrageenan for sodium fluorescein was found for all ME compared to fluid ones. The good adhesiveness on the skin due to the polysaccharide was given as possible explanation [29]. Chen et al. added xanthan to ME and obtained stable system, but the systems were not further evaluated [30]. Thickening of ME containing basil oil, antiseptic used in the treatment of acne, with hydroxyethyl cellulose led to slightly lower antibacterial activities when compared to non-thickened formulation [31].

An interesting approach is the development of patches from pure ME by adding polymers. Compared to a commercial product, this adapted formulation showed increased drug fluxes similar to the fluid ME and improved the drug deposition into dermis layer [32].

In summary, ME as topical drug delivery systems offer a number of advantages, but their use in practice is still limited, mainly due to the high amount of surfactants, which are potential irritants. The development of “biocompatible” ME is a relatively new research area, stressing the importance of minimal irritation potential of the vehicle, which would hopefully increase their clinical use [33].
1.2. Skin, free radicals and antioxidants

1.2.1. Role of free radicals in skin ageing and disorders

The free radical theory is one of the most widely accepted theories to explain the cause of skin ageing, diseases and disorders. This theory suggests that lipid peroxidation, DNA damage and inflammation, all caused by free radicals as shown on Fig. 4, play a key role in skin pathologies [34]. This discovery has led to medical revolution that is stressing the role of antioxidants as prophylactic and therapeutic agents [35, 36].

Fig. 4. Negative impact of free radicals on cell constituents [37].

ROS – reactive oxygen species; RNOS - reactive nitrogen species; MDA – malondialdehyde; 4-HNE – 4-hydroxynonenal.
A free radical is any species that contains one or more unpaired electrons. Reactive oxygen species (ROS) is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, H₂O₂, ¹O₂). The most important ROS are summarized in Table 1 [38]. RNS – reactive nitrogen species - is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as non-radicals such as HNO₂ and N₂O₄. The third reactive species that contribute to development of several diseases are RCS – reactive chlorine species.

Table 2. The most important ROS (3).

<table>
<thead>
<tr>
<th>REACTIVE OXYGEN SPECIES (ROS)</th>
<th>FREE RADICALS</th>
<th>NON-RADICAL REACTIVE SPECIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide O₂⁻</td>
<td>Hydrogen peroxide H₂O₂</td>
<td></td>
</tr>
<tr>
<td>Hydroxyl OH</td>
<td>Hypochloric acid HOCl</td>
<td></td>
</tr>
<tr>
<td>Hydroperoxyl HO₂⁻</td>
<td>Ozone O₃</td>
<td></td>
</tr>
<tr>
<td>Peroxyl RO₂⁻</td>
<td>Singlet oxygen O₂¹</td>
<td></td>
</tr>
<tr>
<td>Alcoxyl RO</td>
<td>Organic peroxydes ROOH</td>
<td></td>
</tr>
<tr>
<td>Carbonate CO₃⁻</td>
<td>Peroxynitrite ONOO⁻</td>
<td></td>
</tr>
<tr>
<td>Carbon dioxide CO₂⁻</td>
<td>Peroxynitril acid ONOOH</td>
<td></td>
</tr>
</tbody>
</table>

Free radicals are ubiquitous in our body and are generated by normal physiological processes, including aerobic metabolism and inflammatory responses [38]. Recent evidence suggested that ROS may be the final common signal for a number of stimuli and that the cell membrane is an important initiation site of cell signaling induced by ROS [39]. Skin is an exception, where the most important source of ROS is UV radiation [40]. UV rays are known to produce harmful effects in the skin. The induction of oxidative damage by UV radiation and ozone was demonstrated to occur in lipids, proteins and DNA [41, 42]. Adverse reactions include sunburn in short-term exposure, Langerhans cells depletion and local immunosuppression caused by longer UV exposure and long-term effects – cutaneous photoageing and skin cancer [41, 43, 44].

1.2.2. Skin antioxidant defence

Because free radicals can inflict cellular damage, organisms have evolved several defense lines both to protect our cells from radicals – such as antioxidant scavengers and enzymes –
and to repair DNA damage [41]. These defense lines include various mechanisms as shown on Fig. 5.

![Fig. 5. General defense mechanisms against reactive species [41].](image)

The most important lines of defence against damaging reactive metabolites in skin are enzymatic (e.g. superoxide dismutase, catalase) and non-enzymatic (e.g. glutathione, α-tocopherol, ascorbate, β-carotene, ubiquinone) antioxidant systems – Fig. 6 [41].

![Fig. 6. The antioxidant defense system of the living cell [41].](image)

*Antioxidant* is a much misused word. Almost any chemical can exert antioxidant effect *in vitro* by choosing appropriate assay conditions. Halliwell & Gutteridge in 1999 defined an antioxidant as any substance that, when present at low concentrations compared to those of an
oxidizable substrate, significantly delays or prevents oxidation of that substrate. The term oxidizable substrate includes every type of molecule found in vivo [38].

Enzymatic antioxidant system

Several enzymatic antioxidant systems have been identified in the skin: superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase, lipoamide system, NADPH ubiquinone reductase, haem oxygenase. The most important constituents of enzymatic antioxidant system are briefly discussed below [35, 45-47]:

- **Glutathione peroxidase (GSH-Px).** The Se-dependent enzyme GSH-Px is active against $\text{H}_2\text{O}_2$ and lipid peroxides by catalyzing the reactions of GSH with these compounds. It is considered to be the most important antioxidant enzyme.

- **Catalase (CAT)** is another enzyme that scavenges $\text{H}_2\text{O}_2$. Since inside the cell CAT is primarily localized in peroxisomes, a possible strategy to increase cellular CAT might be the use of drugs that are known to induce peroxisome proliferation.

- **Superoxide dismutase (SOD)** catalyzes the reduction of superoxide anion to the less reactive $\text{H}_2\text{O}_2$. There are three types of SODs in humans; cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD and extracellular SOD. Since the product of SOD, $\text{H}_2\text{O}_2$, is toxic, any increase in SOD activity should ideally be accompanied by an increase in CAT and/or GSH-Px.

- **Haem-oxygenase** is involved in the breakdown of haem to biliverdin which is then converted to bilirubin, which are both powerful antioxidants.

- **Glucose-6-phosphate dehydrogenase (G6PD)** is the rate-limiting enzyme in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the level of glutathione.

- **The lipoamide system** is a constituent of the mitochondrial dehydrogenase complex. The system contains lipoamide, a form of lipoic acid which is attached to a lysine side chain of enzymes dihydrolipoyl transacetylase and lipoamide dehydrogenase. There is a redox coupling between lipoate and dihydrolipoate which gives the system its antioxidant properties. Lipoate can scavenger singlet oxygen whereas dihydrolipoate is capable of reducing glutathione disulfide, tocopheroxyl radical and dehydroascorbate.
Non-enzymatic antioxidant system

The group of non-enzymatic antioxidants can be further subdivided into physiological and dietary antioxidants. For the physiologic antioxidants we have an absolute requirement. Some of them like uric acid, glutathione and Coenzyme Q\textsubscript{10} are synthesized in the human body and can also be derived from dietary sources. However, as humans lack the ability to synthesize ascorbic acid, tocopherols and β-carotene endogenously their needs can be met only by the dietary sources. Other antioxidants like carotenoids and polyphenols that are obtained from external sources also play an important role in maintaining human health [35].

The water-soluble group of non-enzymatic antioxidant system consists of three types of compounds: thiols, ascorbic acid and urates. There are three groups of lipophilic antioxidants that are essential for life: tocopherols, carotenones and ubiquinones [46].

- **Glutathione (GSH)** is an endogenous tripeptide, which is by far the most abundant thiol in most tissues. It plays a pivotal role in the cellular defence against oxidative damage. GSH is active at various levels. It can act as a direct free radical scavenger or quench radicals by hydrogen atom donation, in both cases forming GSH disulphide (GSSG). It also functions as a hydrogen donor for several other endogenous antioxidants such as ascorbate and coenzyme Q\textsubscript{10}. In this way, GSH can potentiate the protective efficacy of other endogenous antioxidants. Oxidized GSH (GSSG) is recycled by GSH-reductase (GR), with NADPH acting as hydrogen donor. The central position of GSH in the defense against oxidants makes it the focus of many attempts aimed at stimulation of endogenous antioxidative protection [45, 47, 48].

- **Ascorbic acid (ascorbate, vitamin C):** Primates, including humans, do not synthesize ascorbate from glucose due to a deficiency of α-gulono-γ-lactone oxidase, the final enzyme in ascorbate synthesis. The mechanism of its antioxidant activity seems to be two-fold. Ascorbate directly quenches or reacts with singlet oxygen, hydroxyl radicals and superoxide anions. A second important function is the support of the action of α-tocopherol by regenerating tocopheroxyl radicals [46, 47].

- **α-tocopherol (α-TOC, vitamin E),** a lipophilic endogenous antioxidant, provides protection against oxidative membrane damage, presumably by scavenging ROS and free radicals. It functions as a chain breaking antioxidant and is a scavenger of lipid peroxide radicals [41, 46, 47].

- **β-Carotene,** a precursor for vitamin A and the most important member of the carotenoid family of antioxidants, is known for its anticarcinogenic properties. It is
capable of quenching excited triplet state and singlet oxygen and scavenging lipid peroxide radicals. The chronic use of carotene, even at high doses, is considered to be relatively non-toxic [46, 47].

- **Ubiquinon (coenzyme Q10)**, and its reduced form (ubiquinol) are lipophilic chain breaking antioxidants that are present in most cellular organelles. Studies performed on the mechanism of its action suggest that ubiquinol acts by affecting the initiation process and preventing the formation of lipid peroxyl radicals. It functions in conjunction with α–TOC [46, 47, 49].

- **Miscellaneous other non-enzymatic endogenous antioxidants.** Finally, mannitol, xanthine, sorbate, urate, melanin precursors 5-S-cysteinyldopa and 5,6-dihydroxyindole were shown to be scavengers and quenchers of various ROS, but the importance of these compounds seems to be very limited compared to the mechanisms described above [47].

Apart from above described physiological antioxidants a range of dietary antioxidants is known, with plant-derived polyphenols leading the group. The most important dietary antioxidants are shown on Fig. 7. Given the great structural diversity of dietary antioxidants it is not feasible to define structure – activity relationship to deduce underlying molecular mechanisms. In addition, it is very likely that many of these compounds are, besides their antioxidant activity, also involved in cell proliferation, differentiation and apoptosis [50].
1.2.3. Oxidative stress

The capacity of endogenous antioxidative systems is not unlimited. An imbalance between free radicals and antioxidants, with the former prevailing, leads to oxidative stress. The antioxidant defense in cutaneous tissues can be overwhelmed by either an increased exposure to exogenous (e.g. UV-exposure) or endogenous (e.g. inflammatory disorders) sources of ROS, or by a primarily depleted antioxidant defense (e.g. by genetic defects or malnutrition). In these conditions, external supply of antioxidants is essential to countervail the deleterious consequences of oxidative stress [35]. A promising strategy for enhancing skin protection from oxidative stress would be to support the endogenous skin antioxidant system [42, 51, 52].
1.3. Vitamins C and E in human skin

The most intensively studied antioxidants for preventing skin oxidative damage have been vitamin C, vitamin E, glutathione and coenzyme Q<sub>10</sub> and lipoic acid, so called “network antioxidants” which work synergistically to regenerate each other (Fig. 8). Vitamin C, coenzyme Q<sub>10</sub>, lipoic acid or glutathione can recycle vitamin E by donating electrons to its radical to return it to its natural state. Vitamin C can be recycled by lipoic acid or glutathione and coenzyme Q<sub>10</sub> by glutathione [53]. While glutathione, coenzyme Q<sub>10</sub> and lipoic acid can be synthesized by humans, levels of vitamins C and E depend on their oral intake or topical delivery.

![Regeneration pathways of “network antioxidants”](image)

Fig. 8. Regeneration pathways of “network antioxidants”.

1.3.1. Vitamin E

Vitamin E is the most important chain-breaking radical scavenger in the liposoluble compartment, so constituting the major specific defence line against lipid peroxidation. The
initial oxidation product of tocopherol is metastable tocopheroxyl radical, which can be reduced to tocopherol or reacts with another lipid peroxyl radical, yielding tocopherol quinone. Because the physiologic molar ratio of tocopherols to polyunsaturated lipids is less than 1:1000 in most biologic membranes, regeneration of tocopherol by other antioxidants (vitamin C, glutathione, coenzyme Q10...) is essential for its high antioxidant efficacy in vivo [54, 55].

The main natural sources of vitamin E are fresh vegetables, vegetable oils, cereals and nuts. As shown on Fig. 9 the term vitamin E collectively refers to eight naturally occurring molecules (four tocopherols and four tocotrienols) that exhibit vitamin E activity [54].

![Chemical structures of tocopherols and tocotrienols.](image)

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<thead>
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<td>beta</td>
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Fig. 9. Chemical structures of tocopherols and tocotrienols.

α-tocopherol is the predominant vitamin E homologue in human skin. Higher vitamin E levels were found in human epidermis than in dermis. The epidermis of humans is reported to contain 189 - 675 ng α-tocopherol/ mg protein, while whole skin contains 200 pg α-tocopherol /mg protein [46].

Irradiation of human skin with UV doses below minimal erythema dose deplete vitamin E human stratum corneum by almost 50%, indicating that its depletion from uppermost skin layer is a very early and sensitive event of photooxidative damage. The high susceptibility of stratum corneum vitamin E to UV depletion might be, at least in part, due to a lack of co-antioxidants, e.g. ascorbate, which is present only at very low levels in stratum corneum compared to dermal and epidermal levels [56].

It remains to be clarified whether the epidermal uptake and transport of vitamin E after oral application is an unspecific and passive process, or, as described for human hepatocytes is regulated by a mechanism involving a specific binding enzyme (α-tocopherol transfer protein). Sebaceous gland secretion is a relevant physiological delivery pathway of vitamin E
to sebaceous gland-rich skin regions, such as facial skin [57, 58]. Although oral vitamin E has been used to treat many dermatological diseases, including yellow nail syndrome, claudication, ulcers and wound healing, the efficacy has never been convincingly proven [59]. Nevertheless, it was shown that combined topical and oral use of vitamin E increases its levels in stratum corneum compared to topical treatment alone [60].

Topical application of vitamin E has been shown to protect against UV-induced cutaneous damage, carcinogenic and mutagenic activity of ionising radiation and chemical agents [54]. Animal and human studies have demonstrated significant photoprotective effects of natural and synthetic vitamin E when applied topically before UVA or UVB exposure [55]. Vitamin E provides protection against UV-induced skin photodamage through the combination of antioxidant and UV-absorptive properties [61]. It is also widely used in cosmetic preparations since it has been shown to decrease fine lines and wrinkles induced by photoageing. Vitamin E has been touted as an excellent moisturizer, causing increased softness and smoothness of the skin [62, 63].

While topical vitamin E is mostly used at concentrations of 5% or less, products with concentrations of 0.0001% and more than 20% of vitamin E and its esters have been developed and marketed in Europe and USA. For commercial use vitamin E esters, mainly succinate and acetate, are often used because of their better stability. However, skin has only a limited capacity to cleave the unactive esterified forms of vitamin E to the active free tocopherol form, so the antioxidant activity of esters in skin is minimal [59].

Although it is widely used in many topical products, reports of side effects, such as allergic or irritant skin reactions are rare [54]. The question of the pro-oxidant effects of the tocopherols remains a mystery. High levels of tocopherols in vitro induce lipid peroxidation but in vivo evidence is still lacking [46].

1.3.2. Vitamin C

Vitamin C (ascorbic acid, Fig.10), unique in its high reactivity with all aggressive oxygen radicals, is a major – and the only essential – antioxidant in the aqueous cell compartment. Most animals synthesize their own vitamin C, only humans and primates lack the enzyme α-glucono-γ-lactone oxidase, essential for vitamin C synthesis. The animals capable of synthesizing it can increase its production for more than 10 times their normal level when under the stress [59].
Humans obtain vitamin C from citrus fruits, black currants, red peppers and other green leafy vegetables. Because active transport from the gastrointestinal tract is limited, even massive oral doses do not increase its concentration to optimal levels in the skin [59].

Skin contains relatively low amounts of ascorbic acid, about 41 ng/mg dry weight for whole skin with the stratum corneum containing only 7 ng/mg dry weight [46].

There is evidence that skin’s vitamin C levels can be severely depleted by UV radiation and environmental pollutants [59]. Even minimal ultraviolet exposure (1.6 minimal erythema dose) decreases the level of vitamin C to 70% of the normal level and exposure to 10 ppm of ozone in city pollution decreases its epidermal level by 55% [59].

Topical application of vitamin C has been shown to significantly elevate its cutaneous levels. Vitamin C has been touted as important in treating skin pathologies ranging from mild inflammations to skin cancer. Topical vitamin C treatment can significantly retard UVA-mediated damage to the skin. Vitamin C itself is not a sunscreen, however, as an antioxidant it deactivates UV-induced free radicals. In the skin, vitamin C plays a vital role in the metabolism of collagen, where it is necessary for the hydroxylation of lysine and proline in procollagen [62]. It has been further proven as anti-inflammatory, since it decreases the activation of the transcription factor NF-κβ (nuclear factor kappa beta) responsible for many preinflammatory cytokines such as tumor necrosis factor alpha and interleukins (IL1, IL6, IL8). By directly reducing inflammation, post-inflammatory hyperpigmentation is reduced. It is also an excellent depigmentating agent because it inhibits the enzyme tyrosinase and consequently decreases melanin production. All these proved actions of vitamin C contribute to reversal of photoageing: the synthesis of collagen and inhibition of metalloproteinase I (enzyme responsible for UV-induced collagen breakdown) were proven to reduce wrinkles and inhibition of tyrosinase and antinflammatory activity result in depigmenting solar lentigines. Another important action of vitamin C is that it increases the synthesis of several specific lipids on skin surface [59, 64-66].

Despite interesting laboratory research, topical vitamin C preparations have often been disappointing for several reasons. Most preparations are very unstable on exposure to light.
and air. Oxidation occurs very rapidly, and once oxidised these preparations are useless. Even when products are stable, many of them do not penetrate \textit{stratum corneum} [59]. At high concentrations vitamin C can act as a prooxidant, but the prooxidant activity is dependant on the availability of free metal ions, which are usually kept at a low level in the cells. As result, the antioxidant activity will dominate under normal circumstances [47].

\subsection*{1.3.3. Interactions of vitamins C and E}

There is increasing evidence that vitamin E and C, even though present in different compartments of the cell, act synergistically. Vitamin C potentiates the protective efficacy of vitamin E probably by regenerating its radical. \textit{In vitro} lipid oxidation studies showed that by combining vitamins C and E the free radical activity of each vitamin is enhanced [67-69]. The \textit{in vivo} photoprotective effect of topical combination of vitamin C, vitamin E and melatonin was investigated in randomised, double-blind study. After the exposure to UV radiation, vitamin C or E alone had only small effect on erythema observed, whereas the combination of both vitamins enhanced photoprotective response. Even better protection was obtained by using the combination of melatonin with both vitamins [70]. Topical vitamin C with vitamin E was also found to give four-fold protection against UV-induced erythema, compared to two-fold protection by either vitamin alone [71]. In a clinical study it was also confirmed that oral intake of vitamins C and E can reduce the solar simulated irradiation induced skin inflammation, contrarily to either vitamin alone that failed to protect skin [72]. Several double-blind, placebo controlled clinical studies have shown that systemic intake of vitamin C and E combination act synergistically to reduce sunburn reaction and increase minimal erythema dose as reviewed by Bialy et al [73].
1.4. Antioxidant delivery approaches

Presently, there are many antioxidant products on market which have been formulated into conventional dosage forms, with vitamins leading the group. Vitamins have been formulated mainly into tablets and capsules. These conventional forms are easy to formulate and are relatively less expensive. Generally, antioxidants are found in combinations rather than individual products. Many of plant derived antioxidants are not formulated in the pure forms; instead the dry extracts are formulated and marketed. However, antioxidants which have been used in diet or formulated in classical drug delivery systems have some common problems in their efficacy which are related to their physicochemical and biopharmaceutical properties, like low solubility, poor permeability, high instability and/or biotransformation before they reach the site of action [35]. Some examples are listed below.

- **Stability.** Many of the antioxidants are unstable in aqueous solutions. *Epigallocatechin-3-gallate (EGCG)* and *ellagic acid* are unstable in alkaline medium. EGCG is highly unstable in sodium phosphate buffer pH 7.4 at room temperature and ellagic acid degrades quickly in phosphate buffer pH 7.2. The stability of EGCG is dependent on temperature and pH and at higher temperatures the pH effect appears to be enhanced. Many of these agents are vulnerable to photo degradation, too; examples include *coenzyme Q₁₀* and *carotenoids*. The instability of carotenoids is due to their unsaturated bonds which make them susceptible to oxidation [35, 52].

- **Solubility.** Solubility of antioxidants is very diverse depending on the class, source and type of conjugation. *EGCG* is considered to be the least soluble compound among green tea catechins. The poor solubility of *ellagic acid*, which is below 10 μg/ml, poses challenge in its delivery. *Coenzyme Q₁₀* is practically insoluble in water due to its lipophilic carbon chain which can be related to its poor oral bioavailability. Isoflavones like *genistein* are freely soluble at neutral and basic pH but their solubility is below 5% of the original solubility at gastric pH which results in rapid precipitation. The *glucosides of the isoflavones* have higher solubility when compared with their aglycone counterparts. *Vitamin C* is an example of highly soluble antioxidant [35, 36, 49].

- **Permeability.** There are very few reports on the permeability of antioxidants. The apparent permeability value of *EGCG* is $(0.83 \pm 0.24) \times 10^{-7}$. This could be one of the contributors for its poor bioavailability [35].

- **Absorption, distribution, storage and binding.** Absorption of *EGCG* was poor from the Caco2 monolayer. In addition to poor permeability it is also actively excreted by
efflux system. Its plasma protein binding was also reported to be high. Lipoproteins play an important role in the absorption, transportation, and distribution of \textit{coenzyme} \textit{Q}_{10} and other lipophilic antioxidants like \textit{carotenoids} and \textit{vitamin E}. These antioxidants are taken up by the enterocytes, incorporated into the chylomicrons, which are taken up by the liver and antioxidants are released into other lipoproteins like LDL that can enter the cells. There have been suggestions that this transport mechanism is saturable because non proportional increase in the pharmacokinetic parameters has been observed with the increase of the dose. \textit{Ascorbic acid} is well absorbed at lower doses, but absorption decreases as the dose increases due to saturation of sodium-dependent vitamin C transporters that are found in almost all tissues and are necessary for the distribution of vitamin C in the tissues [35, 74, 75].

- \textbf{Metabolism and excretion}. $\beta$-glucosidases play important role in the metabolism of many \textit{polyphenolic glucosides} before absorption; e.g. they separate genistein (aglycone part) from the genestin (glucoside). The bioavailability of \textit{vitamin C} decreases with increase in the dose. It is not protein bound, so it is filtered and almost completely reabsorbed by the kidneys at lower doses (e.g. 200 mg), but with higher doses (e.g. 1250 mg), the urinary excretion is increased which contributes (apart from effect of saturable absorption) to decreased bioavailability [35, 75].

Addressing these problems with the help of conventional dosage forms is difficult and help of advanced delivery systems is a must to maximize the potential roles of antioxidants in prophylaxis and therapy. Different types of strategies were implicated by different groups to achieve successful antioxidant delivery. The major approaches employed in the development of antioxidant delivery are chemical modifications of the drug molecules and novel drug delivery systems.

\textbf{1.4.1. Chemical modifications}

Numerous modifications of endogenous as well as exogenous antioxidants have been attempted in order to get more stable derivatives, increase their solubility, prolong their half life \textit{in vivo}, protect them from degradation/inactivation in GIT, improve skin penetration or attain targeting to the tissues or cells. Some of the major chemical modifications of are discussed briefly in this section.

\textit{Coenzyme} \textit{Q}_{10} is extremely insoluble in water and a possible approach to increase its solubility is to derive ubiquinone analogs with a reduced number of carbons in the side chain compared with \textit{coenzyme} \textit{Q}_{10}. The clinically used synthetic compound idebenone is derived
in this process. However, it has been indicated that many short-chain analogs, including idebenone, enhance superoxide formation by respiratory complex I [76].

Chemical modification of ascorbic acid has led to more stable derivatives such as ascorbyl esters with C_6 to C_{18} fatty acids or ascorbyl phosphate salts [77]. In case of vitamin E, the acetate and acid succinate esters are commonly used clinically for their high stability. The hydrochloride salt of d-α-tocopheryl N,N-dimethylaminoacetate is another prodrug of vitamin E which has high solubility and stability. Several other α-tocopherol derivatives have been shown to be effective prodrugs against lipid peroxidation in the liver, e.g. α-tocopherol-hydroquinone, α-tocopherol-quinone and α-tocopherol-succinate [78].

GSH skin levels may be increased by supplying substrate for the enzymes involved in its synthesis. The most common approach is to provide a source of cysteine which is usually the limiting factor. However, due to its zwitterionic structure, cysteine has difficulty penetrating the skin, so treatment with cysteine is not very effective. Therefore, cysteine derivatives are often used as delivery systems to increase cysteine uptake. Cysteine esters, N- and S-acetyl derivatives, 2-oxo-4-thiazolidine-carboxylic acid and many others were investigated. Inside the cell, these derivatives are metabolized back to cysteine. Treatment with GSH itself is also not successful. Just like cysteine, it has a zwitterionic structure, and is not absorbed. Better results have been obtained with GSH derivatives, such as GSH-methylester, GSH-ethylester and GSH-isopropylester [47].

Coupling of polyethylene glycol (PEG) to the antioxidant enzymes increases their bioavailability and enhances their protective effect. In particular, coupling of SOD and CAT amino groups with PEG minimizes their elimination by the reticuloendothelial system and prolongs their circulation by increasing their half-lives from few minutes to several hours in rats and mice [35].

Chimeric protein constructs consisting of SOD and heparin-binding peptides have an affinity for charged components of the endothelial glycocalix, similarly putrescine coupled enzymes accumulate in brain, and sugar coupled enzymes are used to target hepatic macrophages [35]. Another important site to be targeted in oxidative stress-related diseases is the mitochondria which is the main source for the production of ROS. Mitochondrially targeted coenzyme Q and vitamin E analogs Mito Q and Mito Vit E were synthesized [79].
1.4.2. Novel drug delivery systems

Delivery systems help antioxidants to be delivered efficiently, rather than altering their chemical nature or biological activity. Novel drug delivery systems can be applied to improve the solubility, permeability and stability of antioxidants [80].

**Topical delivery**

Antiageing has become a key marketing focus for skin care in recent years. Antioxidants are used as active molecules in antiageing products to improve skin appearance by slowing down, stopping or even reversing environmental and age related damage that leads to skin wrinkling, discoloration and loss of suppleness. Some of the commercially available antiageing products containing antioxidants incorporated in novel drug delivery systems are listed in Table 3.

Table 3. Commercially available antiageing products containing antioxidants incorporated in colloidal drug delivery systems (adapted from [80]).

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Name of company</th>
<th>Active ingredient</th>
<th>Delivery system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revitalift</td>
<td>L’Oreal</td>
<td>Pro-retinol</td>
<td>nanosome</td>
</tr>
<tr>
<td>Bioperformance</td>
<td>Lancome</td>
<td>Gamma linoleic acid</td>
<td>nanocapsules</td>
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<tr>
<td>Crème Super</td>
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<tr>
<td>Agera microemulsion</td>
<td>Agera</td>
<td>Vitamin E, vitamin A, beta carotene</td>
<td>microemulsion</td>
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<tr>
<td>Agera antioxidant serum</td>
<td>Agera</td>
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<tr>
<td>Advanced night repair</td>
<td>Estee Lauder</td>
<td>Retinyl palmitate, tocopheryl liposomes</td>
<td>liposomes</td>
</tr>
<tr>
<td>protective</td>
<td></td>
<td>acetate, Acuminata extract</td>
<td></td>
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<tr>
<td>Radical sponge</td>
<td>BioResearch</td>
<td>$\text{C}_60$ nanoparticles</td>
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</table>

- **Liposomes.** A variety of liposome based creams containing antioxidants are available on the market. Liposomes as carriers for sodium ascorbyl phosphate for cutaneous photoprotection were prepared and it was shown that liposomes enhance antioxidant penetration into skin when compared to its aqueous solution [81]. Cationic liposomes consisting of double-chained cationic surfactant, phosphatidylcholine and retinoic acid were found to increase delivery of retinoic acid for about two-fold, suggesting the potential use of cationic liposomes for dermal delivery of lipophilic antioxidants [82]. For SOD, liposomes were proven to act as efficient vehicles, allowing the enzyme to enter the skin and prevent the formation of sunburn cells within 15 min of application [47].
- **Solid lipid nanoparticles.** Protection of labile antioxidants has been reported for *retinol* and *vitamin E* that have been successfully incorporated in solid lipid nanoparticles [83, 84].
- **Multiple emulsions.** Casagrande et al. showed the possible usefulness of topical formulations containing quercetin to prevent UVB induced skin damage [85].
- **Microemulsions.** When sodium ascorbyl phosphate was incorporated in the internal phase of microemulsions, sustained release profiles were observed [26].
- **Miscellaneous.** The stability of *ascorbyl palmitate* (AP), an analogue of vitamin C, can be improved by its incorporation in microemulsion, liposomes or solid lipid nanoparticles [86].

**Oral delivery**

- **Microparticles.** Chitosan microparticles produced through spray drying have been evaluated as vehicles for oral delivery of vitamin C in attempt to slow down its release. The release of *vitamin C* from microspheres was sustained and affected by the volume of cross-linking agent added [87-89]. *SOD* and *CAT* have been encapsulated in PLGA microparticles which have been shown to be efficient in releasing drug slowly for extended periods [90].
- **Nanoparticles.** In recent years, the focus is on developing biodegradable polymeric nanoparticles for antioxidant delivery. Nanoparticles can prevent the degradation of *EGCG* and *ellagic acid* in the GIT. Incorporation of *ellagic acid* into suitable delivery system is a very challenging task because of its insufficient solubility in most of the solvents. However, it was successfully incorporated into nanoparticles using using polyvinyl alcohol (PVA), PVA-CS (polyvinyl alcohol-chitosan) blend. *In situ* intestinal permeability studies of these nanoparticles showed increased permeation of ellagic acid in comparison to solution [35]. There are some reports on lipidic and polymeric nanoparticles for *coenzyme Q<sub>10</sub*. It has been demonstrated that the molecule was more stable within polymeric poly(methyl methacrylate) (PMMA) nanoparticles than in oil-based formulation [91, 92].

In another study, the gliadins nanoparticles of *vitamin E* were prepared and shown to sustain the release over a prolonged period of time [93].
- **Self-emulsifying drug delivery systems (SEDDS).** SEDDS offer the potential for enhancing the absorption of poorly soluble and/or poorly permeable compounds
through oral route. SEDDS were shown to improve the delivery of lipophilic compounds such as *coenzyme Q<sub>10</sub>* by the oral route. [94].

- **Gel-based systems.** An alternative and promising research area are hydrogel systems. The hydrogels may be sensitive to environmental stimuli such as pH, ionic strength, electric/magnetic fields, light and temperature depending on the substrate used. Thermoresponsive gels for the controlled delivery of *vitamin E* had been shown to control its release [95].

- **Cyclodextrins.** Aqueous solubility of *coenzyme Q<sub>10</sub>* was increased significantly with the use of an inclusion complex with β-cyclodextrin. When a randomized clinical trial was performed in which a single dose of coenzyme Q<sub>10</sub> was administered orally to healthy human subjects, its absorption and bioavailability increased significantly, probably due to the enhanced water solubility [96].

**Transdermal delivery**
Special mixed lipid carriers in the form of ultradeformable vesicles, transfersomes, may deliver antioxidants transcutaneously into blood circulation. *SOD* administered transdermally in the form of tranferosomes ameliorated the disease symptoms of arthritis in a rat model [97].

**Lung delivery**
The combination of ROS presence and surfactant deficiency promote the development of chronic lung disease that is a major cause of morbidity in premature infants, so it is reasonable to treat it with antioxidants. The term “antioxidant liposomes” is generally used for liposomes containing lipid- or water-soluble LMWA, enzymatic antioxidants or their combinations [98].

*SOD*-loaded liposomes increased its activity of human lung epithelial cells. The highest increase of cellular SOD was observed with anionic liposomes when compared with the neutral as well as cationic liposomes. Similar potential of liposomes for antioxidant delivery was observed, when liposomal *α-tocopherol* was administered intratracheally to the hypoxic rats, where it was able to produce marked antioxidant effect [98].

**Eye delivery**
A possible approach to improve retinal drug delivery is to facilitate localized delivery to the posterior segment of the eye by using nanoporous filter. *Catalase* and *vitamin C* were delivered using these carriers. The results indicated the ability of biocompatible capsules based on nanoporous filters to provide controlled delivery of antioxidant molecules [35].
**Intravenous delivery**
Intravenous administration would primarily be limited to situations in which oxidative stress is a component of acute trauma or disease [98].
*CAT* was successfully incorporated into PEG-PLGA nanoparticles. Nanoparticle-loaded CAT had prolonged activity in proteolytic environment [99].

**Intratumor administration**
The intratumor administration of liposomes is a highly effective approach for the treatment of local solid tumors. Liposomes of *catechin, epicatechin* and *EGCG* were prepared and shown that the liposomal preparations of catechin and epicatechin are retained for longer durations in the tumor in comparison to aqueous solutions of these agents [100].
1.5. Alternative methods for skin toxicity testing

The evaluation of skin toxicity potential is essential step in the development of topical products to ensure the safety of individuals. In the ideal case safety of topical formulations would be tested on human skin \textit{in vivo}. In reality the animal - commonly pig and rat - skin is used. The final preclinical safety assessment of pharmaceutical topical products is largely based on animal experiments. However, ethincal concerns involving the use of laboratory animals, the questions of suitability of animal skin as human skin model and the need for more efficient and cost effective preclinical validation methods have promoted the development of alternative techniques [101].

1.5.1. Skin irritation testing

Several alternative \textit{in vitro} methods for identifying skin irritants have been developed in the last 10 years, the most promising being reconstituted human skin models. In 1998, the European Centre for the Validation of Alternative Methods (ECVAM) commissioned a pre-validation study of five methods: three reconstituted human skin methods (EpiDerm, EpiSkin and Prediskin) and two animal skin model (the mouse skin integrity function test – SIFT and non-perfused pig ear test). Two of three tested reconstituted human skin methods (EpiDerm and EpiSkin) performed well in Phases I and II and proceeded to Phase III, where the predictive ability of both methods was found to be inadequate. However, following refinements to the protocols the predictive ability was improved and, in 2003, ECVAM concluded that they could proceed to a full validation study. In 2007 Episkin model was the first \textit{in vitro} model recognised by ECVAM as a full replacement for the rabbit skin irritation test. In November 2008 two other models (EpiDerm and EpiSkin) were scientifically validated as skin irritation models [102-104]. The pig ear test and SIFT did not perform sufficiently well in phases I and II to progress to phase III; the attempts to improve their reproducibility were unsuccessful [103, 105, 106].

Reconstructed human epidermis models

Even though skin is a heterogeneous membrane that consists of a variety of cells it has been shown that monocultures of human keratinocytes (also called reconstructed human epidermis) can mimic the architecture of the normal human skin and also demonstrate reasonable similarities to the native human tissue in the terms of morphology, lipid composition and biochemical markers. All models consist of primary keratinocytes, seeded on matrices of either dermal components or non-biological origins. Using sophisticated protocols the
keratinocytes fully differentiate and form reconstructed epidermis. The source of keratinocytes can be either neo-natal foreskin or adult skin, obtained from cosmetic surgeries. Currently various companies provide reconstituted human epidermis and the number of distributors is growing [106-108].

In the last decade reconstructed human epidermis became the focus of attention in evaluation of skin irritation by virtue of its ability to produce a wide range of inflammatory mediators. Another reason to favour skin equivalents instead of monolayered keratinocyte cultures is that compounds with a low water-solubility or finished formulations can be tested [107].

The most commonly used parameters to assess skin irritation are measurement of cell viability (e.g. MTT conversion) and the membrane integrity (neutral-red uptake or LDH release). Alternatively several inflammatory mediators can be measured: interleukins (IL1α, IL6, IL8, IL10), tumor necrosis factor α. The same endpoints (most frequently MTT conversion) are also used in skin corrosion and phototoxicity evaluation [107, 109].

**Skin integrity function test (SIFT)**

The *in vitro* mouse skin integrity function test (SIFT) was based on measurement of the transepidermal water loss (TEWL) and electrical resistance (ER) of isolated mouse whole skin before and after application of test material. Skin barrier integrity was calculated as the ratios of TEWL and ER pre- and post-application of test material (the TEWL increasing, and the ER decreasing, with reduced barrier integrity) [105].

In attempt to ameliorate the performance of the test, several other skin models were tested, including human, pig and rat, but mouse performed the best [110].

**Non-perfused pig ear test**

Porcine ear skin is the animal tissue that resembles the most to human skin [111], so attempts were made to develop a pig model for skin irritation studies. The non-perfused pig ear test is based on determination of the absolute increase in transepidermal water loss (TEWL) as the endpoint to distinguish between irritants and non-irritants, following exposure of the pig ear to test material [105].

**1.5.2. Skin corrosion testing**

Skin corrosion is defined as the production of irreversible tissue damage in the skin following the application of a test material. According to current international regulatory requirements,
assessment of skin corrosion is mandatory for all chemicals placed on the market [108]. Skin corrosivity testing in vivo may cause severe discomfort and pain to test animals. Therefore, many attempts have been made to replace the in vivo tests on laboratory animals by in vitro methods for identifying skin corrosives. The rat skin transcutaneous electrical resistance (TER) and a human epidermal models (EpiSkin, EpiDerm) assays were endorsed in 1998 by the ECVAM as scientifically valid for use as replacements for the rabbit test for distinguishing between corrosive and non-corrosive chemicals. A few years latter, the commercial Corrositex assay has been endorsed as being valid for evaluating the corrosivity potential of acids, acid derivatives and bases, followed by EpiSkin reconstructed human epidermis in 2006 [104].

Rat skin transcutaneous electrical resistance (TER) assay
The TER assay has been used successfully as a routine test. Test materials are applied to the epidermal surfaces of skin discs taken from the pelts of humanely-killed young rats. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the TER below a predetermined threshold level. Irritants and non-irritants do not reduce TER below the threshold level [101, 105].

1.5.3. Skin phototoxicity testing
Phototoxicity can be defined as a skin inflammatory reaction, elicited by topical application or systemic administration of chemicals and subsequent exposure to the light, particularly UVA radiation. Over the past years, several in vitro models have been implemented for phototoxicity assessment, most of them based on reconstructed human epidermis models, but only one of them, 3T3 NRU assay, has been scientifically validated as an alternative method for skin phototoxicity [112-115].

3T3 NRU assay
The 3T3 NRU assay is cytotoxicity assay performed on Balb/c 3T3 mouse fibroblast cell line. The basis of this test is a comparison of the cytotoxicity of the chemical when tested with and without exposure to noncytotoxic dose of UVA light. Cytotoxicity is expressed as a concentration-dependant reduction in the uptake of the vital dye, neutral red [101, 116].
Literature


107. website, E. *http://ecvam.jrc.it*.


2. Objectives of the thesis
The thesis focuses on topical ME simultaneously loaded with lipophilic vitamin E, skin’s major membrane antioxidant and hydrophilic vitamin C, the main component of skin water-soluble antioxidant defence. Vitamins C and E have been chosen since there is increasing evidence that they act synergistically and from the viewpoint of controlling the physiological balance between lipid- and water-soluble antioxidants. The work has been divided into several parts. In chapter 1 two issues have been addressed: viscosity modification of liquid ME and stability of vitamins C and E. The viscosity of liquid ME that have been previously developed and characterized in the Department of Pharmaceutical Technology of Faculty of Pharmacy in Ljubljana has been optimized for topical application in order to ensure that vehicle is aesthetically acceptable to patients, easy to use and that it adheres to skin sufficiently. Two different approaches have been used to optimize the rheological behaviour of liquid o/w and w/o ME, both composed of identical pharmaceutically acceptable ingredients (isopropyl myristate as oily phase, water as aqueous phase, Tween 40 as surfactant, Imwitor 308 as cosurfactant) and having same percentage of surfactants. Transparent microemulsion gel that has been formed upon the addition of specific amounts of water to liquid o/w ME has been investigated as a possible solution to viscosity problem. Alternatively, various thickening agents have been screened in attempt to find those which would increase the viscosity of the system without affecting its stability and spontaneous formation. The best thickening agents and their optimal concentrations have been determined. Further, the optimized systems have been characterized and their potential to protect vitamins C and E from oxidative degradation has been evaluated. As vitamin E was found to be reasonably stable under normal storage conditions, its degradation has been accelerated by UVA radiation. Moreover, we have also investigated to what extent the presence of vitamin C increases the stability of vitamin E.

Apart from poor stability, the inefficient skin permeability is another major drawback of antioxidants. In chapter 2 the influence of ME microstructure and thickening agent on the percutaneous absorption of both vitamins following topical application to reconstructed human epidermis as a novel model for human skin has been explored.

In chapters 3 and 4 the properties of two systems - ME thickened with colloidal silica and microemulsion gel formed form o/w ME by extra addition of water - have been investigated in details. In chapter 3 two-fold action of colloidal silica in the ME has been evaluated: first, its ability to affect formulation characteristics (partition coefficient of the vitamins, solubility and release) and second, its direct impact on skin by transepidermal water loss measurements, scanning electron microscopy and keratinocyte viability determination. The influence of
colloidal silica on skin bioavailability of vitamins C and E has been further examined on isolated pig ear skin as the most relevant *in vitro* animal model for human skin.

In *chapter 4* we have studied temperature-driven changes of microemulsion gel in comparison to liquid o/w ME and conventionally thickened o/w ME using rheological and droplet size measurements. The effectiveness of all three formulations as a vehicle for simultaneous topical delivery of vitamins C and E has been investigated using *in vitro* skin permeation test.

Since the topical vehicles should be not only efficient, but also safe, *chapter 5* describes an assessment of toxicity potential of ME. Their dermal irritation and phototoxicity potential has been tested in comparison to commercially available topical ME using several *in vitro* methods. MTT test was used as an endpoint to evaluate cell viability of reconstructed human epidermis and keratinocyte cell line. Results have been correlated with pig ear transepidermal water loss and changes in cell morphology visualised by fluorescence microscopy.
3. Results and Discussion
3.1. Chapter 1

Stability of vitamins C and E in topical microemulsions for combined antioxidant therapy

*Stabilnost vitaminov C in E v dermalnih mikroemulzijah za kombinirano antioksidantno terapijo*

Branka Rozman, Mirjana Gašperlin

Abstract
An interesting strategy for protecting skin from excessive exposure to free radicals is to support the skin endogenous antioxidant system. As the balance between different skin antioxidants is very important, a combined therapy using at least two antioxidants is desirable.
In the present work, o/w, w/o and gel-like microemulsions (ME), all composed of the same ingredients, were selected as carrier systems for dermal delivery of vitamins C and E. Gel-like ME was found to offer the best protection for both vitamins, although other ME also significantly increased their stability compared to that solution. In the presence of vitamin C no decrease in vitamin E content occurred. To obtain ME appropriate for dermal use, their viscosity was increased by adding thickening agents. On the basis of visual examination of viscosity and physical stability of thickened systems, several thickeners were selected. The addition of thickener significantly increased the viscosity of ME and changed the behaviour of systems from ideal Newtonian to thixotropic. Finally, the stability of both vitamins was examined as a function of thickening agent and of the location of vitamins in the ME. The addition of thickeners changed the stability of at least one vitamin, but the systems generally still protected vitamins better than solutions. It is very likely that the changes in internal organization of ME resulting from the addition of thickener, that were confirmed by thermal analysis and changes in the solubility of oxygen in the outer phase, were the most important factors that influenced the stability of vitamins in thickened systems.

Key words: stability, vitamins, antioxidants, microemulsion, topical application
**Povzetek**
Zanimiva strategija za zaščito kože pred čezmerno izpostavljenostjo radikalom je podpora koži lastnega antioksidativnega sistema. Ker je ravnovesje med različnimi antioksidantmi v koži zelo pomembno, je zaželena sočasna uporaba najmanj dveh antioksidantov. V tem raziskovalnem delu so bile kot nosilni sistemi za dermalno dostavo vitaminov C in E izbrane o/v, v/o in gelirana mikroemulzija (ME), vseh pripravljene iz istih sestavin. Gelirana ME je najbolje ščitila oba vitamina, čeprav sta tudi drugi dve ME bistveno povečali njuno stabilnost v primerjavi z raztopino. V prisotnosti vitamina C se vsebnost vitamina E ni zmanjšala. Za izboljšanje dermalne aplikacije smo ME zgostili; optimalna zgoščevala smo izbrali na podlagi vizualnega pregleda viskoznosti in fizikalne stabilnosti zgoščenih sistemov. Dodatek zgoščeval je občutno povečal viskoznost ME in spremenil njihovo reološko obnašanje iz Newtonskega v tiksotropnega. Na koncu smo proučevali stabilnost obeh vitaminov v odvisnosti od zgoščevala in njune lokacije v ME. Zgoščevalo je spremenilo stabilnost vsaj enega vitamina, vendar pa so tudi zgoščeni sistemi še vedno bolje ščitili vitamina kot raztopine. Zelo verjetno so spremembe v notranji strukturi ME, ki so posledica dodajanja zgoščeval in ki so bile potrjene s termično analizo, skupaj s spremembami v topnosti kisika v zunanj faze najpomembnejši dejavniki, ki vplivajo na stabilnost vitaminov v zgoščenih ME.

**Ključne besede:** stabilnost, vitamin, antioksidant, mikroemulzija, dermalna dostava
Introduction

It is generally agreed that oxidative stress makes a major contribution to skin ageing, skin disorders and skin diseases. Skin has developed several lines of defence against damaging reactive metabolites, the most important being enzymatic (e.g. SOD, catalase, peroxidase) and non-enzymatic (e.g. glutathione, α-tocopherol - vitamin E, ascorbate - vitamin C, β-carotene, ubiquinone) antioxidant systems. A promising strategy for enhancing skin protection from oxidative stress would be to support the endogenous skin antioxidant system. The most intensively studied antioxidants for preventing skin oxidative damage have been vitamin C, vitamin E and β-carotene (Kohen 1999; Steenvorden and Beijersbergen van Henegoven 1997; Offord et al. 2002).

Vitamin C, unique in its high reactivity with all aggressive oxygen radicals, is a major – and the only essential – antioxidant in the aqueous cell compartment, whereas vitamin E is the most important chain-breaking radical scavenger in the liposoluble compartment, so constituting the major specific defence line against lipid peroxidation. There is increasing evidence that vitamin E and C, even though present in different compartments of the cell, act synergistically. Vitamin C potentiates the protective efficacy of vitamin E by regenerating its radical. Regeneration probably occurs at the interphase region (Fig.1) (Steenvorden and Beijersbergen van Henegoven 1997; Packer and Fuchs 1993).

Fig. 1. Protective effects of vitamin E and vitamin C against lipid peroxidation. LH - lipid molecule; L· - lipid radical, LOO· - lipid peroxyl radical; LOOH – lipid peroxide.
As antioxidant defence mechanisms are linked, the balance between different skin antioxidants is very important. If only one of the antioxidants is supported, another will soon become a limiting factor. Tocopheroxyl radical depletes other antioxidants by its own regeneration (Fig.1), so vitamin E monotherapy could result in an overall negative effect. As a consequence of the synergistic interplay of different antioxidants, a combined therapy of vitamin E with vitamin E recycling antioxidants, such as vitamin C, glutathione, ubiquinone or carotenoids, is desirable (Kohen 1999; Packer and Fuchs 1993; Fuchs 1998; Turunen, Olson and Dallner 2004).

Oral supplementation of ascorbate is not thought to increase its skin concentration sufficiently. In spite of its very low lipophilicity, mouse skin was found to be relatively highly permeable to vitamin C, indicating its possible topical use. Topical use of vitamin C is also recommended because of its de-pigmenting activity and its ability to promote the synthesis of collagen. As vitamin C in solution undergoes rapid oxidation, its use in pharmaceutical products is limited above all by its low stability.

Topical administration of vitamin E was found to be generally more effective in protecting skin against oxidative stress than a systemic treatment. Vitamin E is very slowly oxidised by atmospheric oxygen and is generally considered to be stable. However, it is possible that it could undergo photodegradation when applied to skin that is exposed to UV radiation (Steenvorden and Beijersbergen van Henegoven 1997; Fuchs 1998).

The specific structure of microemulsion (ME) allows the incorporation of lipophilic vitamin E and hydrophilic vitamin C in the same system. ME are clear, thermodynamically stable dispersions of water and oil, stabilized by an interfacial film of surfactant molecules. It was shown that they are able to increase drug solubility, modify drug release and protect labile drugs (Lawrence and Rees 2000). ME are often very low viscosity Newtonian fluids and cannot be applied directly to the skin. However, from phase diagrams constructed for the selected components, areas with lamellar structures and consequently increased viscosity can be formed (Podlogar et al. 2004). The more usual way to solve the viscosity problem is to add a suitable thickening agent that can modify the rheological behaviour without significant influence on other features of ME, such as stability, transparency and spontaneous formation (Osmann-Gardabbou et al. 2000, Spiclin et al. 2003).

The aim of this work was to develop ME that simultaneously contain the lipophilic (vitamin E) and the hydrophilic (vitamin C) antioxidants, and are suitable for dermal use. Since the
formulation can significantly affect drug stability, the potential of w/o and o/w ME to protect vitamins from oxidative degradation will be evaluated. We will investigate the extent to which the ME structure will influence the stability of vitamin C and E and also to what extent vitamin C will increase the stability of vitamin E. As vitamin E was found to be reasonably stable under normal storage conditions, its degradation will be accelerated by UVA radiation. The rheological behaviour of o/w and w/o ME will be modified to be appropriate for dermal application by adding various thickeners. The best thickening agents and their optimal concentrations will be determined. Finally the influence of selected thickeners on the stability of both vitamins will be assessed for both ME types.

Materials and Methods

Isopropyl myristate (IPM) was obtained from Fluka Chemie, Switzerland and used as the lipophilic phase. Tween 40 – polyoxyethylene (20) sorbitan monopalmitate (Fluka Chemie Switzerland) – was used as surfactant and Imwitor 308 – glyceryl caprylate (Condea, Germany) – as cosurfactant. Twice distilled water was used as the hydrophilic phase. α-Tocopherol (vitamin E) and ascorbic acid (vitamin C) were from Fluka, Switzerland. The following thickening agents were used: hydroxypropyl methyl cellulose - HPMC (Methocel K4M, Colorcon, UK), sodium alginate (Protanal LF 120M, FMC BioPolymer, USA), xanthan gum (Sigma Aldrich, Germany), locust bean gum (Sigma Aldrich, Germany), microcrystalline cellulose (Lek, Slovenia), colloidal silica (Aerosil 200, Degussa, Germany), magnesium stearate (Fluka, Germany), cetostearyl alcohol (Lanette O, Cognis, Germany), titanium dioxide (Ridel de Haen, Germany), zinc dioxide (Lex, Slovenia), white wax (Pharmachem, Slovenia), carbomer (Carbopol 974 PNF, BF Goodrich, Belgium) and poloxamer (Lutrol F68, Basf, Germany).

Preparation of microemulsions (ME)

The components of ME are acceptable for dermal use. Both types of non-thickened ME and gel-like ME differ only in quantitative composition (w/w percent), shown in Table 1.

Table 1. Composition of w/o, o/w and gel-like ME (w/w %).

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>ME W/O</th>
<th>ME O/W</th>
<th>gel-like ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>10</td>
<td>45</td>
<td>60</td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td>60</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Tween 40</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Imwitor 308</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>
All ME were prepared in the same way. The surfactant and cosurfactant were blended in a 1:1 mass ratio to obtain the surfactant mixture. IPM and distilled water were then added. Components were mixed with a magnetic stirrer for 5 minutes at room temperature. Vitamin C (0.4% w/w concentration) and/or E (1% w/w concentration) were incorporated in ME by stirring with a magnetic stirrer for 30 minutes. No preservatives were added in order to avoid possible interactions with vitamins.

**Viscosity modification of ME**

Thickening agents were added to ME containing vitamins and stirred with a magnetic stirrer for 30 minutes. When cetostearyl alcohol or white wax were used as thickeners, the system was blended at 55°C. In that case the vitamins were added to ME after thickening. The thickened ME were left covered for at least 24 hours. Afterwards they were checked visually for physical stability.

**Rheological measurements**

A Rheolab MC 100 Paar Physica, equipped with a cone and plate sensor system MK 22, was used for rheological characterization. Rheological measurements were carried out under destructive and non-destructive conditions at 20 ± 1°C. Under non-destructive shear conditions of oscillatory shear, the linear viscoelastic regime was determined at a frequency of 0.5 Hz. The frequency dependence of dynamic moduli was measured under conditions of linear viscoelastic response at a constant strain amplitude 0,1. All measurements were made in triplicate.

The absolute dynamic viscosity of non-thickened ME was determined using a Hoeppler viscometer at 25°C.

**DSC measurements**

A Model Pyris 1 (Perkin Elmer) was used for differential scanning calorimetry (DSC) measurements. Approximately 15 mg of sample was weighed precisely into a small aluminium pan and sealed hermetically. The empty sealed pan was used as a reference. Samples were cooled from 20 to –40°C at a rate of 10K/min.

**Vitamin E (photo)stability**

The stability of vitamin E was determined in samples stored in closed glass flasks at room temperature (22+/−1°C) over a period of two months. Half the samples were stored in the dark and half in the light to study the influence of light.
The amount of non-degraded vitamin E was determined quantitatively by HPLC immediately after the preparation of sample and subsequently on the 1st, 2nd, 3rd, 7th, 14th, 28th and 56th days of storage.

For the studies of photostability 10g of samples were irradiated in test tubes without stirring for 48h. A UVA lamp Spectroline ENF-260C/FE 0,17 ampere, λ=365 nm. The distance of the test tube from the UVA lamp was 5 cm. Approximately 500 mg of sample was accurately weighed into a glass flask, diluted to 50 ml with methanol, and analysed by HPLC. HPLC analysis was carried out with a Knauer HPLC pump 64, a sample injector Rhodyne 7125 with a 20 μL sample loop, and a Knauer variable wavelength detector.

Chromatographic conditions: the stationary phase was a 120x4 mm ID column packed with 5 μm Nucleosil C18; the mobile phase was methanol-acetonitrile 70:30. The flow rate was 1,5 ml/min and UV detection at 291 nm. The method was validated for the determination of vitamin E in ME.

**Vitamin C stability**

The stability of vitamin C was determined in samples stored in well-closed glass flasks at room temperature (22+/− 1°C).

The amount of non-degraded vitamin C was determined quantitatively by HPLC immediately after preparation of the sample and subsequently on the 1st, 2nd, 3rd, 7th, 14th and 28th days.

For HPLC analysis, approximately 500 mg of sample was accurately weighed into a glass flask and diluted to 100 ml with methanol. All samples were prepared in triplicate.

HPLC analysis was carried out with a Knauer HPLC pump 64, a sample injector Rhodyne 7125 with a 20 μL sample loop, and a Knauer variable wavelength detector. Chromatographic conditions: the stationary phase was 250x4 mm ID column packed with 5μm Nucleosil C18-NH2, and the mobile phase methanol – acetonitrile – 0,02M phosphate buffer pH 3,5 (20:30:50). The flow rate was 1 ml/min and UV detection at 243 nm. The method was validated for the determination of vitamin C in ME. Limit of quantification (LOQ) for chromatographic determination of vitamin C was determined from the calibration curve and was 22,7 μM; limit of detection (LOD) was 7,5 μM.

**Statistical analysis**

Fractions of non-degraded vitamin are reported as arithmetic means ± standard deviation. Statistical analysis was performed using a two-tailed Student’s test with the software package SPSS®. Significance was tested at the 0.05 level of probability.
Results and discussion

Viscosity modification of ME

According to the broad definition of ME by Danielsson and Lindmann they are optically clear and thermodynamically stable systems. This definition does not mention the microstructure of ME. However, it is well known that an optically clear system can have a wide range of microstructures, the most frequent being spherical droplets, rod-like micelles, lamellar and hexagonal phases (Lawrence and Rees 2000). The non-thickened ME used in our experiments consisted of fine droplets of inner phase dispersed in continuous medium, as characterized by Podlogar and coworkers (Podlogar et al. 2004; Podlogar, Bester Rogac, and Gasperlin 2005). They are stable, transparent liquids with limited use for dermal application because of their low viscosity. The rheological properties were therefore modified by addition of various thickeners. A wide range of thickeners was tested (Table 2).

Table 2. Thickening agents tested for w/o and o/w ME.

<table>
<thead>
<tr>
<th>Microemulsion type</th>
<th>Thickening agent</th>
<th>Concentration (w/w %)</th>
<th>Stability after 24h</th>
<th>Viscosity after 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>o/w</td>
<td>Hydroxypropyl methyl cellulose</td>
<td>1</td>
<td>stable system</td>
<td>too low</td>
</tr>
<tr>
<td></td>
<td>Microcrystalline cellulose</td>
<td>3</td>
<td>sedimentation</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Xanthan (xan)</td>
<td>2</td>
<td>stable opalescent system</td>
<td>too low</td>
</tr>
<tr>
<td></td>
<td>Locust bean gum (LBG)</td>
<td>1</td>
<td>stable opalescent system</td>
<td>too low</td>
</tr>
<tr>
<td></td>
<td>Sodium alginate (alg)</td>
<td>1</td>
<td>stable opalescent system</td>
<td>too low</td>
</tr>
<tr>
<td></td>
<td>Mixture of xan and lbg</td>
<td>3</td>
<td>inhomogeneous system</td>
<td>sufficient</td>
</tr>
<tr>
<td></td>
<td>Mixture of xan and alg</td>
<td>3 (1:1)</td>
<td>stable opalescent system</td>
<td>sufficient</td>
</tr>
<tr>
<td></td>
<td>Poloxamer 168</td>
<td>5</td>
<td>inhomogeneous system</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Carbomer</td>
<td>2.5</td>
<td>stable opalescent system</td>
<td>sufficient</td>
</tr>
<tr>
<td></td>
<td>Colloidal silica</td>
<td>10</td>
<td>stable system</td>
<td>sufficient</td>
</tr>
<tr>
<td>w/o</td>
<td>White wax</td>
<td>10</td>
<td>stable milky system</td>
<td>sufficient</td>
</tr>
<tr>
<td></td>
<td>Magnesium stearate</td>
<td>2</td>
<td>sedimentation</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Ceteosteryl alcohol</td>
<td>5</td>
<td>macromulsion</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Titanium dioxide</td>
<td>5</td>
<td>sedimentation</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Zinc oxide</td>
<td>5</td>
<td>sedimentation</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Colloidal silica</td>
<td>10</td>
<td>stable system</td>
<td>sufficient</td>
</tr>
</tbody>
</table>

The physical stability and viscosity of thickened systems were checked visually after 24 hours. On that basis colloidal silica, a 1:1 w/w mixture of xanthan and alginate (xan-alg mixture) and carbomer were selected as thickeners for o/w ME. For w/o ME colloidal silica and white wax were the only ones found to be appropriate. The physical stability of thickened ME was checked every week over 2 months and no instability was observed.

The flow characteristics of non-thickened ME and of selected thickened systems were characterized by rheological measurements under destructive (Figs.2, 3) and non-destructive conditions (Fig.4).
Fig. 2. Flow curves of non-thickened, thickened and gel-like o/w ME.

Fig. 3. Flow curves of non-thickened and thickened w/o ME.
Fig. 4. Frequency dependence of $G'$ and $G''$ for selected thickened o/w ME at constant strain amplitude.

Table 3. Absolute dynamic viscosities of non-thickened and gel-like ME determined with a Hoeppler viscometer.

<table>
<thead>
<tr>
<th></th>
<th>Absolute dynamic viscosity (mPas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/O ME</td>
<td>25.8</td>
</tr>
<tr>
<td>O/W ME</td>
<td>27.2</td>
</tr>
<tr>
<td>gel-like ME</td>
<td>79.6</td>
</tr>
</tbody>
</table>

Non-thickened ME of both types are Newtonian fluids with viscosities less than 0.5 Pas (Figs. 2, 3, Table 3). The third investigated structure was gel-like ME. This term was chosen only to distinguish this system from liquid o/w ME. The gel-like ME has a higher content of water, which allows strong hydration of the hydrophilic chains of non-ionic surfactant. Hydrated chains are connected by hydrogen bonds, resulting in strong interactions between droplets (Podlogar et al. 2005), which is expressed by slightly increased dynamic viscosity (Table 3), and can also be seen from Fig. 2 as increased viscosity at higher shear rates and by the rheopexic behaviour. The viscosity of gel-like ME was thus characterized as appropriate for dermal use.

The addition of thickening agent significantly increased the viscosity of o/w and w/o ME and changed the ideal Newtonian to thixotropic behaviour. As clearly seen from Fig. 2, the o/w ME thickened with colloidal silica had a much higher viscosity at lower shear rates and greater hysteresis than the systems thickened with polymers. The increased viscosity of ME
thickened with silica is a consequence of the weak forces between the particles that form agglomerates. As the size of particles is not uniform, there are some empty spaces in the structure they form and the fluid phase is caught inside. When the shear force increases, the structure is destroyed and the fluid is released, the result being lower viscosity (Olhero and Ferreira 2004). Forward and reverse rate sweep tests show a distinct hysteresis loop which indicates the pronounced thixotropic character of the system. Silica was also selected as a thickener for w/o ME. It increased the viscosity, which was comparable to that of the opposite type of ME. The viscosity of the system thickened with white wax was comparable to that of o/w ME thickened with carbomer (Figs. 2, 3).

More information on the network structure of thickened ME can be obtained from oscillatory measurements. Typical curves of the frequency dependence of storage and loss modulus for thickened ME are shown in Fig. 4. ME thickened with silica and carbomer form a weak gel, which, in the latter case, is the consequence of physical entanglement of polymeric chains. The addition of xan-alg mixture to o/w ME resulted in a highly viscous polymeric solution. Because of the inhomogeneous parts in the systems with higher concentration of polymers it was not possible to prepare weak gels with mixed xan-alg.

The exposure of thickened and non-thickened ME to UV light for 48 h did not change their rheological behaviour.

**Thermal analysis**

The microstructure of ME can be examined by thermal analysis. The state of water in a microemulsion system, and consequently its microstructure, can be determined by controlled cooling of the sample (Podlogar et al. 2004; Podlogar, Bester Rogac, and Gasperlin 2005). Fig. 5 shows the cooling curves of o/w ME and water dispersion of a xan–alg mixture. Two peaks are seen on the thermograms of ME. The broad peak at approximately −5°C represents the solidification of IPM. The second peak between −10°C and −20°C represents the freezing of bulk water (Podlogar et al. 2004; Podlogar, Bester Rogac, and Gasperlin 2005). The freezing peaks of water in non-thickened and gel-like o/w ME appear, as expected, at approximately the same temperature and have very similar shapes, indicating the similar structure of the two samples.

The addition of colloidal silica broadened the water peak slightly, indicating interactions between silica particles and water. Thickening of ME with polymers resulted in change of water behaviour. A shift of freezing point towards higher temperatures was observed,
meaning that the bulk water was less tightly bound than in non-thickened ME. When the system was thickened with carbomer the peak remained sharp, which is characteristic of polymer dispersion with well defined structure. In contrast, the freezing peak of water became much broader in the presence of the xan-alg mixture, indicating a less homogenous mixture of natural polymers. The cooling curves were also less reproducible. The control run with water dispersion of xan-alg led to the conclusion that the water is mostly involved in hydration of the polymer chains (Fig. 5).

The cooling curves of non-thickened and thickened w/o ME are shown in Fig. 6. Due to the large amount of IPM, and consequently lower water content, the latter is present in the inner phase. It freezes at approximately −35°C. The addition of colloidal silica broadens both peaks slightly, whereas thickening with white wax almost eliminates the peak of water, and the broadening of the IPM peak is even more pronounced. The observed behaviour indicates that white wax is solubilized in IPM and the surfactant mixture, which influences their lipophilicity and probably perturbs the packing characteristics of the surfactant, thus causing changes in the internal organization of ME.
We first examined the stability of vitamin E, alone and in combination with vitamin C, under normal storage conditions (Fig. 7). Vitamin E dissolved in IPM and also incorporated in both types of ME was found to be stable. Neither carrier nor light had any significant influence on its stability. The addition of vitamin C also did not affect the stability of vitamin E.

Vitamin E may undergo photo-oxidation on exposure to UV light (Steenvorden and Beijersbergen van Henegouwen 1997; Gallarate et al. 2004). After application of vitamin E to skin exposed to sun its photoinstability leads to increased levels of tocopheroxyl radical that can induce lipid peroxidation or deplete other antioxidants (Kohen 1999, Gallarate et al.)
Therefore incorporation into a formulation that would enhance its photostability is desirable. In order to find out to what extent different types of ME influence the photostability of vitamin E, samples were UVA irradiated for 48 hours (Fig. 8). Long exposure time was the consequence of weaker light emission of lamp used in our experiments (3.5 x 10^-4 W/cm²) than the standard solar irradiation (1.1 x 10^-3 W/cm²). UVA light constitutes 90 – 99 % of the UV radiation that reaches the surface of the earth and is particularly important in causing oxidative stress in the skin by inducing free radicals (Halliday 2005).

All types of non-thickened ME afford better protection from UVA light than oily solution. Gel-like ME was found to be the best protective system, followed by o/w and w/o ME. When vitamin E was incorporated into water continuous ME it became dispersed in the fine droplets of the oily phase, where it was more protected because water and the interface area can act as a barrier for UV light and oxygen. Nevertheless, even w/o ME protected vitamin E from UV light better than the oily phase alone. Because of the large amount of surfactants in ME, they can also be present in the continuous phase (Podlogar et al. 2004; Podlogar, Bester Rogac, and Gasperlin 2005). Thus, in w/o ME, vitamin E can be dissolved in the outer phase as well as being incorporated into the surfactant micelles.
Another factor that influences the stability of vitamin E in ME is its partition coefficient between the oily and surfactant phases. The molecule of vitamin E has two distinct domains; a chromanol nucleus terminated by a polar OH group and a long hydrophobic phytyl side chain (Fig.9) (Bongiorno et al. 2006, Avellone et al. 2004). We confirmed that vitamin E is freely soluble in the oily phase as well as in the surfactant mixture of ME, so we assume that, in the ME, it partitions between the oily phase and the surfactant mixture. Avellone et al. (Avellone et al. 2004) showed that the chromanol ring of vitamin E competes with water molecules for binding sites on hydrophilic regions of surfactant. When the content of water is increased, the concentration of vitamin E in the surfactant mixture decreases. In o/w ME, strong hydration of the surfactant mixture is thus desirable, because it hinders the incorporation of vitamin E into surfactant mixture, leaving a higher fraction dispersed in the inner phase, in which it is more protected. This explains why the gel-like ME are found to be the best protective vehicles. In ME of opposite type the incorporation of vitamin E into the surfactant film is desirable as the chromanol ring is oriented towards the inner phase and so less sensitive to photodegradation than when present in the outer phase. This results in vitamin E being more stable than in oily solution.

The addition of vitamin C to ME in the same molar concentration as vitamin E significantly protected the latter from UV degradation. Vitamin C is mainly present in the aqueous phase of ME but its good solubility in the surfactant mixture and hydration of the surfactant film indicates that it is also present in the interfacial area, contributing to the regeneration of vitamin E.

We further evaluated the stability of vitamin C incorporated into non-thickened ME. Aerobic degradation of vitamin C was studied in ME stored in the dark, to assess whether the partition influences its resistance to oxidation (Fig.10).
Fig. 10. Fractions of non-degraded vitamin C in non-thickened ME and in aqueous solution during one-month storage.

After 14 days, w/o ME was found to protect vitamin C significantly better than o/w ME. This was expected for at least two reasons:

- The presence of continuous oily phase, in which vitamin C is insoluble, provides protection, despite the fact that oxygen solubility in oils is roughly one-order of magnitude higher than in water (Gallarate et al. 1999). The interface may act as a barrier for oxygen diffusion into the inner aqueous phase.

- The degradation of vitamin C is highly concentration dependent. Although both ME contained the same total amount of vitamin C, the latter is soluble only in the aqueous phase and in the surfactant mixture, which means that its local concentration in the hydrophilic compartments of ME was higher in the case of w/o ME. The higher the concentration of vitamin C, the more stable it is.

According to above explanation one would expect that vitamin C would be least stable when incorporated into gel-like ME that has water as continuous phase and the highest percent of hydrophilic components, but the opposite was in fact the case. In the gel-like ME the surfactants are highly hydrated, so less water is available for dissolving vitamin C. As a consequence, vitamin C is located to a higher degree in the surfactant film. The increased viscosity also reduces the rate of diffusion of oxygen.

*Stability of vitamins E and C in thickened ME*
The majority of thickened ME did not offer the same level of protection for vitamins as non-thickened ME although, compared to solutions, the stability of vitamins was in most cases improved (Figs. 11-13).

![Fig. 11. Fraction of non-degraded vitamin E in different ME after 48 hours of exposure to UVA light.](image)

![Fig. 12. Fractions of non-degraded vitamin C in thickened and non-thickened o/w ME, and in solution stored in the dark for one month.](image)
When the xan-alg mixture was used as thickening agent, the stability of both vitamins decreased significantly. After 28 days no vitamin C could be detected (Fig. 12). Its instability is most probably the consequence of the pro-oxidative effect of alginate (Trommer and Neubert 2005). Thickening of o/w ME with carbomer had no significant influence on the stability of vitamin C, but it significantly decreased that of vitamin E. Vitamin E was significantly less stable in o/w ME thickened with polymers (carbomer, xan-alg mixture) than in IPM. As seen from the thermograms of ME thickened with polymers (Fig. 5), the water was used predominantly for hydrating the polymer, so less free water was available for hydration of surfactants. The dehydrated surfactant film could not shield vitamin E from UV light so efficiently and, at the same time, incorporated more vitamin E, where it was more exposed to light.

O/w ME thickened with colloidal silica lowered the stability of both vitamins, but not significantly (Figs. 11, 12). In w/o ME it significantly enhanced the stability of vitamin E and accelerated the degradation of vitamin C (Figs. 11, 13). The better protection of vitamin E could be explained by the ability of silica dispersion to absorb UV light (Fig. 14). As the samples with vitamin C were stored in the dark, UV absorption was not important. Another factor that could influence vitamin stability is the increased oxygen transport in gels formed with colloidal silica (http://freepatentsonline.com/5091275.html). As colloidal silica is soluble in all three phases of ME, the reduced stability of vitamin C was probably due to increased oxygen transport in the sample.
Fig. 14. Absorbance as a function of wavelength for w/o ME thickened with colloidal silica, non-thickened ME and oily phase.

Both vitamins in w/o ME thickened with white wax are less stable than in non-thickened ME. The oxygen solubility in lipophilic vehicles depends on the polarity of the vehicle (Akoh, C. C., Min and Akoh, A. C. 2002). The more polar the vehicle, the lower the oxygen solubility. When white wax is added to the oily phase of ME, the oxygen solubility increases and accelerates the degradation of vitamins. As white wax produced by bleaching was used, it is also possible that it contained traces of impurities that were oxidants. Further, the addition of white wax also influences the structure of ME; this is probably more important for the stability of vitamin C, which is incorporated into the inner phase (Fig.6).

**Conclusions**

The incorporation of vitamins E and C into non-thickened o/w and w/o ME, and into gel-like o/w ME, enhanced their stability. Gel-like o/w ME was found to be the best protective system for both vitamins. The presence of vitamin C at the same time improved the UVA stability of vitamin E. Addition of a selected thickener (carbomer, xan-alg mixture, colloidal silica and white wax), that suitably modified the rheological behaviour of ME, changed either oxygen solubility in the outer phase or structural organization of ME and consequently influenced the stability of vitamins. As non-thickened and thickened ME, with the exception of those thickened with xan-alg mixture, increased the stability of vitamin C and E compared to that in water and oily solution respectively, we can confirm the tested ME to be suitable carrier systems for combined antioxidant topical therapy.
References


http://freepatentsonline.com/5091275.html


Trommer, H., and Neubert, R. H. H. 2005. The examination of polysaccharides as potential antioxidative compounds for topical administration
3.2. Chapter 2

Simultaneous absorption of vitamins C and E from topical microemulsions using reconstructed human epidermis as a skin model

Študija istočasne absorpcije vitaminov C in E iz dermalnih mikroemulzij na celičnih kulturah keratinocitov kot modelu kože

Branka Rozman, Mirjana Gašperlin, Estelle Tinois-Tessoneaud, Fabrice Pirot and Françoise Falson

Abstract
Antioxidants provide the mainstay for skin protection against free radical damage. The structure of microemulsions (ME), colloidal thermodynamically stable dispersions of water, oil and surfactant, allows the incorporation of both lipophilic (vitamin E) and hydrophilic (vitamin C) antioxidants in the same system. The objective of this work was to investigate the potential of non-thickened (o/w, w/o and gel-like) and thickened (with colloidal silica) ME as carriers for the two vitamins using reconstructed human epidermis (RHE). The amounts of these vitamins accumulated in and permeated across the RHE were determined, together with factors affecting skin deposition and permeation. Notable differences were observed between formulations. The absorption of vitamins C and E in RHE layers was in general enhanced by ME compared to solutions. The incorporation of vitamins in the outer phase of ME resulted in greater absorption than that when vitamins were in the inner phase. The location of the antioxidants in the ME and affinity for the vehicle appear to be crucial in the case of non-thickened ME. Addition of thickener enhanced the deposition of vitamins E and C in the RHE. By varying the composition of ME, RHE absorption of the two vitamins can be significantly modulated.

Key words: Microemulsion, reconstructed human epidermis, percutaneous absorption, antioxidant, vitamin C, vitamin E
Povzetek
Antioksidanti zagotavljajo osnovno zaščito kože pred poškodbami radikalov. Struktura mikroemulzij (ME), koloidnih termodinamsko stabilnih disperzije vode, olja in površinsko aktivnih snovi, omogoča vgradnjo lipofilnega (vitamin E) in hidrofilnega (vitamin C), antioksidanta v isti sistem. Cilj tega dela je bil raziskati potencial nezgoščenih (o/v, v/o in gelirana) in zgoščenih (s koloidnim silicijevim dioksidom) ME kot dostavnih sistemov za oba vitaminja z uporabo kultur človeških keratinocitov (RHE). Preučevali smo perkutano absorpcijo obeh vitaminov, z namenom določiti dejavnike, ki vplivajo na njuno porazdelitev v RHE. Nalaganje vitaminov v RHE je bilo večje iz ME kot iz raztopin, opazili pa smo tudi znatne razlike med različnimi ME. Če sta bila vitamina vgrajena v zunanj fazo ME je bila njuna absorpcija večja kot če sta se nahajala pretežno v notranji fazi. Ugotovili smo, da je v primeru nezgoščenih ME za absorpcijo vitaminov ključna njuna lokacija v ME in njuna afiniteta do nosilnega sistema. Dodatek zgoščevala je znatno povečal nalaganje vitaminov E in C v RHE. S spreminjanjem sestave ME je torej mogoče znatno spremeniti absorpcijo vitaminov v RHE.

Ključne besede: mikroemulzija, celične kulture keratinocitov, perkutana absorpcija, antioksidant, vitamin C, vitamin E
Introduction
Skin is a tissue subjected to a high degree of oxidative stress from both endogenous and exogenous sources. As excessive levels of free radicals in skin can induce deleterious reactions such as skin ageing, skin disorders, and skin diseases, several lines of antioxidant defence against damaging reactive metabolites have evolved, the most important being enzymatic (such as superoxide dismutase, catalase, peroxidase), and non-enzymatic (such as glutathione, α-tocopherol - vitamin E, ascorbate - vitamin C, β-carotene and ubiquinone). A promising strategy for enhancing skin protection from oxidative stress would be to support the endogenous skin antioxidant system. The most intensively studied antioxidants for preventing skin oxidative damage have been vitamin C, vitamin E and β-carotene (1-3).

Vitamin C, unique in its high reactivity with all aggressive oxygen radicals, is a major – and the only essential – antioxidant in the aqueous cell compartment, whereas vitamin E is the most important chain-breaking radical scavenger in the liposoluble compartment, thus constituting the major specific defence line against lipid peroxidation. There is increasing evidence that vitamins E and C, even though present in different compartments of the cell, act synergistically (2, 3). In membranes, vitamin E is oxidised when it quenches peroxyl free radicals. The neighbouring intracellular vitamin C reduces the oxidized vitamin E to regenerate its activity. It has been shown that a topically applied combination of vitamin C with vitamin E gives four-fold protection against UV-induced erythema, compared to two-fold protection by either vitamin alone (4-6).

In order to protect skin from antioxidative damage the antioxidant must first be able to penetrate the effective permeation barrier offered by the skin. An effective way to enhance cutaneous bioavailability of drugs is to choose an appropriate delivery system. Topically applied microemulsions (ME) significantly increase skin absorption of drugs and, in some cases, provide its sustained release (7, 8). ME are clear, thermodynamically stable dispersions of water and oil, stabilized by an interfacial film of surfactant molecules (7). Moreover, their specific structure allows the incorporation of both lipophilic and hydrophilic drugs in the same system.

Skin is a heterogeneous membrane composed of a variety of cell types, but the upper layer stratum corneum is the main barrier for percutaneous absorption. Reconstructed human epidermis (RHE) models have a well developed stratum corneum and demonstrate reasonable similarity to the native human tissue in terms of morphology, lipid composition and biochemical markers. EpiSkin®’s model used in our study shows all epidermal layers seen in human skin, but cells of viable parts are organised differently than in native epidermis. All
major classes of epidermal lipids are present; the phospholipids content is very close to that of human epidermis, but EpiSkin® model has higher content of di- and triglycerides. The potential of RHE has been evaluated in permeation studies using model lipophilic and hydrophilic drugs (9-11). However, there are not many studies dealing with the influence of the vehicle on drug permeation through RHE (12-14).

We have demonstrated that MEs provide enhanced stability for vitamins C and E (15). The aim of the present research was to study the influence of formulation on the percutaneous absorption of hydrophilic (vitamin C) and lipophilic (vitamin E) antioxidants following topical application to reconstructed human epidermis. Both antioxidants were simultaneously incorporated in thickened and non-thickened ME.

**Materials and methods**

Isopropyl myristate (IPM) was obtained from Fluka Chemie, Buch, Switzerland and used as the lipophilic phase. Tween 40 – polyoxyethylene (20) sorbitan monopalmitate (Fluka Chemie, Buch, Switzerland) – was used as surfactant and Imwitor 308 – glyceryl caprylate (Condea, Hamburg, Germany) – as cosurfactant. Purified water was used as the hydrophilic phase. α-Tocopherol (vitamin E) and ascorbic acid (vitamin C) were from Fluka, Buch, Switzerland. Colloidal silica (Aerosil 200, Degussa, Dusseldolf, Germany) was used as the thickening agent.

NaCl and orthophosphoric acid were from Prolabo (Fontenay-sous-Bois, France), chicken egg albumin, phosphate buffer saline tablets (pH 7.4) and potassium monobasic phosphate from Sigma Aldrich (Munchen, Germany) and Chremaphor EL from BASF, Ludwigshafen, Germany. Analytical grade acetonitrile and methanol were from Fisher Scientific (Illkirch, France).

Episkin® kits containing 12 cell cultures with 1.07cm² surface area on the nutritive gelatin gel were a gift of Episkin (Lyon, France).

*Preparation of microemulsions (ME)*

The components of ME are acceptable for topical use. Non-thickened ME of both type and gel-like ME differ only in quantitative composition (w/w percent) (Table 1).
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Table 1. Composition of tested formulations (w/w %).

All ME were prepared in the same way. The surfactant and cosurfactant were mixed in a 1:1 mass ratio with a magnetic stirrer for 5 minutes at room temperature. IPM and water were then added. Vitamin C (0.4% w/w concentration) and/or E (1% w/w concentration) were incorporated in ME by stirring with a magnetic stirrer for 30 minutes. No preservatives were added in order to avoid possible interaction with the vitamins. When colloidal silica was used it was added (10% w/w) to ME containing vitamins and stirred with a magnetic stirrer for 30 minutes. The ME were left covered in the dark for at least 24 hours. The final concentrations of the vitamins in the formulation were checked by HPLC at the time of dosing. The final vitamin content was always in the range 95-105% of the amount incorporated.
For comparison the solutions of vitamins were tested (Table 1).

**Rheological measurements**

A Rheolab MC 100 Paar Physica, equipped with a cone and plate sensor system MK 22, was used for rheological characterization. Rheological measurements were carried out under destructive conditions at 20 ± 1°C and 37 ± 1°C. Prior to analysis, the samples were exposed to the temperature of the experiment for 30 min. The glass cover was used in order to prevent water evaporation. All measurements were made in triplicate.

**In vitro skin permeation experiments**

Upon receipt of the Episkin® kit, cell cultures were removed from the nutritive gel and transferred under aseptic conditions into a sterile 12 well culture dish containing 2 ml of the maintenance medium per well. Maintenance medium was provided by Episkin. The cultures were incubated at 37°C, 5% CO₂ and saturated humidity until their use. The RHE were mounted on static Franz diffusional cells (diameter 12 mm, receptor volume 8 mL). The receptor compartment was filled with 8 ml of 0.9% water solution of NaCl to which 3% of albumin was added. Solubilities of vitamin C and E in receptor phase were 652.2+/−44.7 mg/mL and 40.25 mg/mL respectively. The receptor solution was stirred continuously at 100 rpm and the water bath kept at 37 ± 1°C, resulting in RHE surface temperature of 32°C. Donor compartment contained 1 g of formulation (ME simultaneously containing 0.4% of vitamin C and 1% of vitamin E, 0.4% aqueous solution of vitamin C or 1% oily solution of vitamin E). At time intervals of 1, 2, 3, 4 and 6 h, 1 ml of the receptor medium was removed and replaced by fresh, preheated medium. Extraction of vitamins from receptor fluid was done with 1 ml of methanolic solution of Chremaphor EL (1mg/ml). Samples were vortexed with glass bills (3x 2 min with 5 min stops), centrifuged (at 6000 rpm for 15 min) and after filtration (0.45μm filter) analysed by HPLC. For recovery determination a known amount of vitamin C (250 μg/ml, 125 μg/ml and 50 μg/ml) or vitamin E (20 μg/ml) was added to blank receptor solution. After 6h of contact the receptor fluid was submitted to above described assay of extraction and analysis. Recovery test were done in triplicate. The recovery of vitamin C was always in range 95-105% and that of vitamin E was 72+/−8%. After 6 hours the formulation remaining on the surface of the RHE was collected and analyzed for the content of the two vitamins. The RHE samples were cleaned three times with cotton swab soaked in methanol and then dried with fresh cotton
swab. Epidermis was separated from collagen, put into eppendorf tube containing 0.75 ml of methanol and cut into small pieces. The tubes were occasionally vortexed. After 45 min the extract was removed and extraction was repeated with 0.75 ml of fresh methanol until no vitamin was detected in extract. For collagen the same procedure as for epidermis was used, but volume of methanol used was 1.5 ml. Extracts were after filtration analyzed by HPLC. Specificity and recovery of vitamins’ extraction from RHE was proved. RHE that were not in contact with vitamins were submitted to extraction procedure and the retention time of endogenous compounds did not overlap with those of either vitamin. For recovery determination a known amount of each vitamin (10μl of 5mg/ml aqueous or oily solution of vitamin C and E respectively) was added to either epidermis or collagen that were separated prior to experiment. After 6 hours of contact the extractions were done as described previously and recovery was calculated as ratio between the amount of vitamin extracted from tissue and the amount of vitamin added. Recovery of both vitamins from collagen and epidermis is reported in Table 2.

All experiments were performed in 6 parallels.

Table 2. Recovery of vitamins C and E from epidermis and collagen.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td>8.5</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>9.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

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Branka Rozman, Doctoral dissertation

Results and Discussion, Chapter 2

94
HPLC analysis

HPLC analysis was carried out with an Agilent 1200 series HPLC system. The injection volume was 20 μl for the analysis of standards and for determination of vitamin content in the formulations and in extracts of the donor compartment; for all other samples 100 μl was used. As external standards methanolic solution of vitamins C (50 μg/mL) and E (50 μg/mL) were used.

For vitamin E analysis, the stationary phase was a 120x4 mm ID column packed with 5 μm Nucleosil C18 and the mobile phase methanol-acetonitrile 70:30. The flow rate was 1.5 ml/min, and UV detection at 291 nm. The method was validated for the determination of vitamin E in ME. The limit of quantification (LOQ) for chromatographic determination of vitamin E was determined from the calibration curve to be 0.54 μg/mL. The limit of detection (LOD) was 0.18 μg/mL. Intra- and interday precision according to ICH guidelines for three relevant concentrations (50, 100 and 200 μg/ml) were <0.15% and <0.60% respectively.

For vitamin C, the stationary phase was a 250x4 mm ID column packed with 5 μm Nucleosil C18-NH2, and the mobile phase methanol – acetonitrile – 0.02 M phosphate buffer pH 3.5 (20:30:50). The flow rate was 1 ml/min, with UV detection at 243 nm. The method was validated for the determination of vitamin C in ME. The limit of quantification (LOQ) for chromatographic determination of vitamin C was determined from the calibration curve to be 4.0 μg/mL. The limit of detection (LOD) was 1.3 μg/mL. Intra- and interday precision according to ICH guidelines for three relevant concentrations (50, 100 and 200 μg/ml) were <1.0 % and <1.4% respectively.

Measurement of Transepidermal Water Loss (TEWL)

ME containing no vitamins were applied on the RHE in the same quantity as for permeation tests. After 6 hours the formulations were removed and the surface cleaned with 2x 300 μl of PBS buffer pH 7.4 and dried with a cotton swab. The cell cultures were then placed on the nutritive gelatin gel at room temperature for 4 hours. The TEWL was measured with an MPA 5 Tewameter®, Courage Khazaka, Germany. The measurements were done in triplicate for epidermis treated with thickened and non-thickened ME o/w, and in duplicate when treated with gel-like ME and aqueous solution of colloidal silica, and when non-treated.

Data analysis
Influence of formulation on vitamins’ retention in epidermis and collagen as well as on their permeation was evaluated by one way ANOVA. Bonferroni’s test was used for post-hoc comparisons. Significance was tested at the 0.05 level of probability.

**Results**

**Rheological measurements**

At 20°C non-thickened ME of both types are low viscosity Newtonian fluids with viscosities less than 0.5 Pas (Fig. 1). Although the difference in the initial viscosities of non-thickened ME and gel-like ME cannot be clearly seen from Fig. 1, a slightly increased viscosity of the latter was proved by absolute viscosity measurement by Høeppler viscosimeter at 20°C (37.2+/−0.1 mPa.s vs. 97.6+/−0.4 mPa.s for o/w ME and gel-like ME respectively). The increased viscosity at higher shear rates and non-Newtonian behaviour of gel-like ME further distinguish gel-like ME from o/w and w/o ME. The addition of colloidal silica significantly increased the viscosity of ME regardless of type, and triggered a transition from ideal Newtonian to pseudoplastic behaviour. The initial viscosities of the thickened o/w and w/o ME were approximately the same. At 37°C the behaviour of gel-like ME was changed to Newtonian and the viscosity decreased relative to that at 20°C. In contrast, slightly elevated temperature did not affect the rheological behaviour of thickened ME.

![Viscosity diagrams of thickened and non-thickened ME at 20 and 37°C.](image)

Fig. 1. Viscosity diagrams of thickened and non-thickened ME at 20 and 37°C.

**In vitro skin permeation experiments**

The permeation of the two vitamins from 6 different formulations was determined – in solution (aqueous or isopropyl myristate), in o/w ME, w/o ME, gel-like ME and o/w and w/o
ME thickened with 10% of colloidal silica. Three parameters were investigated – the amount of vitamin accumulated in the epidermis, in collagen and in the receptor fluid (Figs. 2-4).

Fig. 2. The amounts of vitamins C and E accumulated in epidermis of RHE from different formulations (♣ aqueous solution of vitamin C and isopropyl myristate solution of vitamin E) after 6 hours. The bars indicate the mean value and standard deviation of six experiments for each formulation.

Vitamin C: *s - p<0,05 compared to aqueous solution
  *m - p<0,05 compared to o/w ME
  *c - p<0,05 compared to o/w ME thickened with colloidal silica

Vitamin E: *s - p<0,05 compared to oily solution
  *m - p<0,05 compared to w/o ME
  *c - p<0,05 compared to w/o ME thickened with colloidal silica
Fig. 3. Vitamins C and E absorbed into collagen layer of RHE from different formulations (♣ aqueous solution of vitamin C and isopropyl myristate solution of vitamin E) after 6 hours.

Vitamin C: *s - p<0.05 compared to aqueous solution
  *m - p<0.05 compared to o/w ME
  *c - p<0.05 compared to o/w ME thickened with colloidal silica

Vitamin E: *s - p<0.05 compared to oily solution
  *m - p<0.05 compared to w/o ME
  *c - p<0.05 compared to w/o ME thickened with colloidal silica

Fig. 4. Permeation profiles of vitamin C from different formulations through RHE.
After 6 hours, more than 80% of vitamin E and 60-80% of the vitamin C remained on the surface (data not shown). Thus, about 10-15% of the vitamins penetrated into RHE. The amounts of vitamins penetrated into the epidermis and collagen from different formulations are shown in Figs. 2 and 3. The distribution of the vitamins in the RHE generally showed slightly higher amounts in the keratinocytes than in collagen, but the concentrations are within the same range. Only in the case of thickened o/w ME a significant difference between vitamin C and vitamin E accumulation in epidermis was observed. In the collagen layer, more vitamin E accumulated than vitamin C.

Vitamin C
Vitamin C from aqueous solution accumulated in the epidermis and was also found in the collagen layer, but not in the receptor solution. When vitamin C was incorporated in the MEs, the tendency to lower accumulation in the epidermis was observed. Moreover, the amount of the vitamin in the collagen increased. The type of ME also influenced its accumulation in the collagen. A difference between o/w ME and gel-like ME was observed, but the amount was the highest for w/o ME. The addition of colloidal silica increased the amount of vitamin C in epidermis, regardless of the type of ME. In the collagen layer however the effect was opposite – less vitamin C accumulated, although for o/w ME the difference was not significant.

The amount of vitamin C accumulated in the receptor solution was significantly lower with thickened than with non-thickened ME (Fig. 4) the exception being with w/o ME. In the latter case, vitamin C was found in the receptor solution only after 6 hours (40 μg/cm²) whereas, in the two other types, detectable amounts were found after 1 hour already (Fig. 4). Thickened o/w ME delivered more vitamin C into the receptor solution than thickened w/o ME and its steady-state flux was also higher (Fig.4, Table 3). In contrast, the steady-state flux of thickened systems was significantly lower from that of non-thickened.

Table 3. Steady state flux (J) for vitamin C in different formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>J (μg/hcm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>o/w ME</td>
<td>185°C</td>
</tr>
<tr>
<td>gel-like ME</td>
<td>176°C</td>
</tr>
<tr>
<td>w/o ME</td>
<td>/</td>
</tr>
<tr>
<td>o/w ME colloidal silica</td>
<td>62 *m</td>
</tr>
<tr>
<td>w/o ME colloidal silica</td>
<td>40 *m</td>
</tr>
</tbody>
</table>

* - p<0,05 compared to o/w ME
*m - p<0,05 compared to o/w ME thickened with colloidal silica.
In order to obtain more detailed information on the influence of colloidal silica on the skin absorption of vitamin C, three additional formulations were tested – vitamin C aqueous solution thickened with 10% colloidal silica and o/w ME containing vitamins C and E, each thickened with 5 and 15% of colloidal silica. Results concerning vitamin C are shown in Table 4a.

Table 4a. Compartmental analysis (epidermis, collagen, receptor fluid) of vitamin C concentration from different formulations.

<table>
<thead>
<tr>
<th>FORMULATION</th>
<th>epidermis (µg/mg)</th>
<th>collagen (µg/mg)</th>
<th>receptor (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aqueous solution</td>
<td>2.11+/−0.35</td>
<td>0.23+/−0.05</td>
<td>0.0</td>
</tr>
<tr>
<td>aqueous solution 10% colloidal silica</td>
<td>2.98+/−0.61</td>
<td>0.23+/−0.02</td>
<td>68.9+/−5.3</td>
</tr>
<tr>
<td>o/w ME</td>
<td>0.93+/−0.06</td>
<td>0.51+/−0.10</td>
<td>504+/−141</td>
</tr>
<tr>
<td>o/w ME 5% colloidal silica</td>
<td>1.36+/−0.35</td>
<td>0.37+/−0.19</td>
<td>438+/−118</td>
</tr>
<tr>
<td>o/w ME 10% colloidal silica</td>
<td>2.93+/−0.30</td>
<td>0.47+/−0.04</td>
<td>402+/−32</td>
</tr>
<tr>
<td>o/w ME 15% colloidal silica</td>
<td>1.63+/−0.03</td>
<td>0.23+/−0.07</td>
<td>46.7+/−3.3</td>
</tr>
</tbody>
</table>

* - p<0.05 compared to aqueous solution 10% colloidal silica
s - p<0.05 compared to ME o/w 5% colloidal silica
h - p<0.05 compared to ME o/w 15% colloidal silica

The addition of colloidal silica in the aqueous solution facilitated the passage of vitamin C into the receptor solution, although the quantity was low. There was no significant difference between the vitamin C permeation parameters and accumulation in the collagen for the ME o/w thickened with 5 and 10% of colloidal silica (Table 4a), whereas the addition of 15% of silica resulted (compared to 10% colloidal silica) in lower concentrations of vitamin in the RHE and in the receptor solution, where the vitamin was found only after 6 hours.

Vitamin E

Vitamin E passed into the receptor solution only when incorporated in ME containing colloidal silica (334 and 24 µg/cm² for thickened w/o and o/w ME respectively). Even in this case the vitamin E was detected in the receptor solution only after 6 hours. The addition of colloidal silica also enhanced the epidermal content of vitamin E when compared to other formulations (Fig. 2). The accumulation of vitamin E in the collagen reflects the influence of the vehicle, the accumulation being highest for w/o ME, followed by isopropyl myristate.
solution and thickened w/o ME, and lowest for ME that contained water as the continuous phase, even if the thickener was used (Fig. 3).

The results of an additional experiment with o/w ME thickened with 5 and 15% of colloidal silica showed that, for vitamin E, the addition of 5% colloidal silica enhanced its passage into the receptor solution, but did not significantly modify its retention in the RHE (Table 4b). The addition of 15% colloidal silica was the least favourable of all the concentrations of thickener tested, but still superior to the ability of non-thickened ME of the same type to deliver the vitamin to the RHE.

Table 4b. Compartmental analysis (epidermis, collagen, receptor fluid) of vitamin E concentration from different formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>epidermis mass (µg/mg)</th>
<th>collagen mass (µg/mg)</th>
<th>receptor mass (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>o/w ME</td>
<td>0.65 +/-0.20</td>
<td>0.14 +/-0.04</td>
<td>0</td>
</tr>
<tr>
<td>o/w ME 5% colloidal silica</td>
<td>3.15 +/-2.10</td>
<td>0.14 +/-0.05</td>
<td>1610 +/-16</td>
</tr>
<tr>
<td>o/w ME 10% colloidal silica</td>
<td>7.94 +/-1.10&lt;sup&gt;th&lt;/sup&gt;</td>
<td>0.44 +/-0.06&lt;sup&gt;th&lt;/sup&gt;</td>
<td>88 +/-6</td>
</tr>
<tr>
<td>o/w ME 15% colloidal silica</td>
<td>3.12 +/-0.58</td>
<td>0.28 +/-0.12</td>
<td>0</td>
</tr>
</tbody>
</table>

*l* - *p*<0.05 compared to ME o/w 5% colloidal silica

*"* - *p*<0.05 compared to ME o/w 15% colloidal silica.

**Transepidermal Water Loss**

The measurement of TEWL is a well established method for assessing the integrity of the skin barrier. When skin is damaged, its barrier function is impaired resulting in greater water loss (16). Transepidermal water loss was measured for 4 formulations – thickened and non-thickened o/w ME, gel-like ME, and an aqueous solution of colloidal silica and non-treated keratinocytes. The results are shown as the percentage of the difference at room temperature between the TEWL of treated and non-treated RHE 4 hours after removal of the formulation (Fig. 5). There is no significant difference between the two non-thickened ME, but the addition of thickener significantly raised the TEWL, which was almost 40% higher than in the case of non-treated RHE. The aqueous solution of colloidal silica also enhanced TEWL, by more than 10%, when compared to non-treated RHE, but significantly less than for RHE treated with ME.
Fig. 5. The change (in %, compared to non-treated RHE) of TEWL 4 hours after the removal of the formulation at room temperature. RHE were treated with formulations for 6 hours.

Discussion

Rheological measurements

Non-thickened ME used in our experiments are stable, transparent liquids that consist of fine droplets of inner phase dispersed in continuous medium (17-19). The application of non-thickened ME to the skin is possible but preparations with increased viscosity are more convenient to apply. A possible answer to the viscosity problem is to use systems with lamellar structure and consequently increased viscosity (18), as in the case of gel-like ME. The term gel-like ME was chosen only to distinguish this system from liquid o/w ME. The gel-like ME still consists of droplets of oil in water, but the higher content of water allows strong hydration of the hydrophilic chains of surfactant. Hydrated chains are connected by hydrogen bonds, resulting in strong interactions between droplets (17), which is expressed by slightly increased dynamic viscosity and can also be seen from Fig. 1 as increased viscosity at higher shear rates. On the basis of the rheological behaviour at room temperature gel-like ME was shown to be appropriate for dermal use. When the gel-like ME was exposed to 37°C in order to simulate the experimental conditions of permeation studies its rheological behaviour changed to that of a low-viscosity Newtonian fluid, indicating the destruction of the gel-like structure. Changes in microstructure due to change of temperature are common phenomena in ME (8).
The more usual way to solve the viscosity problem is to add a suitable thickening agent that can modify rheological behaviour without significant influence on other features of ME, such as stability, transparency and spontaneous formation (20, 21). It has been shown that the addition of 10% colloidal silica does not affect the stability and microstructure of our ME (15) and, as can be seen from Fig. 1, elevated temperature (37°C) did not significantly affect the viscosity of the system.

**In vitro skin permeation**

Antioxidants such as vitamin E and C are known to play a significant role in preventing oxidative stress in skin. However, to provide satisfactory protection they must first cross the stratum corneum, which acts as permeation barrier. RHE is known to have a lower barrier function than human skin. The TEWL is higher and the rate of penetration of molecules such as caffeine is 10 times higher in the RHE than in human skin (22). In spite of these limitations, RHE is an useful model in the study of percutaneous absorption of drugs. Even though human skin models have been shown to be more permeable than human skin ex vivo, they can correctly predict the permeability rank order of compounds with different physicochemical properties (11, 12, 23).

As it is generally recognized that penetration of drugs into the skin may be improved by selecting the appropriate vehicle (24-26), we evaluated the influence of different formulations on the percutaneous absorption of a lipophilic and hydrophilic antioxidant. The absorption of vitamins from thickened and non-thickened ME was compared to that from solution.

When considering the penetration of a drug from different formulations into the skin, several factors have to be considered: vehicle-drug interactions, influence of the carrier on the RHE, and influence of RHE and receptor solution on the formulation. During the experiment ME remained clear and transparent. There was no visual indication that water from the receptor compartment influenced the ME, which is not surprising, as a relatively large amount of formulation was used as a donor. On the contrary, our results clearly show that the vehicle plays an important role in the skin absorption of the two vitamins under investigation.

**Vitamin C**

Vitamin C from aqueous solution accumulated in epidermis but only a small quantity was found in collagen, proving the keratinocytes to provide an efficient barrier for this hydrophilic vitamin. The incorporation of vitamin C in the ME resulted in permeation of this vitamin across the RHE, which can be attributed to the large amount of surfactants in the formulation that facilitated the passage of vitamin, mostly by compromising the barrier function of RHE.
as observed as an increased TEWL. As no difference was seen between the two ME tested for
TEWL, it can be concluded that impairment of the barrier function of RHE probably depends
on the quantity of surfactants. However, despite all ME containing the same amount of
surfactant, significant differences in skin deposition and permeation of vitamin C were
observed. In fact, two groups of ME can be distinguished – one that contain water as the
continuous phase (o/w and gel-like ME) and other that contain oil as the continuous phase
(w/o). The absence of differences between o/w and gel-like ME can be explained by the
destruction of the gel structure of the latter when exposed to 37°C, which was proved by
rheological measurements. On the basis of solubility of vitamin C in the ME
(21.79 mg/g in w/o ME, 85.54 mg/g in o/w ME and 170.4 mg/g in gel-like ME) the more
complex partition of vitamin among all the constituents of the vehicle can be suggested,
limiting its transfer through the RHE.

The addition of colloidal silica to the ME enhanced the deposition of vitamin C in epidermis,
but not in the collagen layer. TEWL measurements showed that the RHE barrier was
influenced after the application of formulations containing colloidal silica. The use of
colloidal silica as a thickening agent enhances the release rate of a hydrophilic drug from w/o
ME (21). It was assumed that colloidal silica modifies the physicochemical properties of the
external phase, thus making the drug diffusion faster. The total amount of vitamin passed in
the receptor solution was however smaller in the case of thickened ME, even though the
epidermis was more permeable, probably due to the increased viscosity of the vehicle. The
addition of thickener significantly increased the viscosity of the formulation, which slowed
down permeation of vitamin. Pseudo zero order kinetics was observed in all the permeation
profiles. This was expected because of the surplus vitamin C on the donor side. The
concentration gradient between receptor and donor phase therefore remained constant,
resulting in constant flux (27). The steady-state flux was determined from the linear part of
the permeation curve (Table 3) for all samples except for w/o ME where the duration of
experiment was too short for its determination. As expected, it was higher in the case of non-
thickened ME. No significant difference was observed between gel-like and o/w ME. There is
also a difference between the two thickened systems; when vitamin C is in the inner phase, its
steady-state flux decreases because the external oil phase presents a barrier to the diffusion of
drug molecule.

The permeation experiment with an aqueous solution of colloidal silica revealed the impact of
the selected thickener on the skin absorption of vitamin C. It confirmed that the increased
concentration of vitamin C in the keratinocyte layer is primarily due to the presence of
colloidal silica. The behaviour of vitamin C, which is a weak acid with pKₐ 4.2, is also affected by the pH of the formulation. The addition of colloidal silica to o/w ME changed the pH from 4.98 to 4.34 which could have changed the degree of ionization in the favour of the non-ionised form that can more easily penetrate into the epidermis. The effect of silica also appears to be concentration dependent, as 5% concentration increased the concentration of vitamin C in the RHE, as compared to non-thickened o/w ME, but its concentration in the keratinocytes layer was lower than in the case of ME thickened with 10% silica. However, increasing the concentration of the thickener above 10% did not increase the efficacy of the formulation, probably due to the high viscosity of the formulation thickened with 15% of silica.

Vitamin E
In the in vitro skin permeation studies across RHE, generally no vitamin E was found in the receptor solution, the exception being ME containing colloidal silica. Either vitamin E did not permeate the skin or the amount was below the limit of detection of HPLC. Moreover, the extraction procedure did not allow 100% extraction of vitamin E from the receptor solution. Probably, the interaction of vitamin E with albumin was not completely eliminated by the single extraction with methanol. It is known that, in vivo, dermis is more likely to be a barrier for lipophilic substances than the keratinocyte layers (28). Generally, transport experiments with lipophilic substances through RHE might suggest a higher transport through the skin than it actually is, because of the lack of dermis (11). The Episkin model is an exception, as it has a dermal substitute in the form of a collagen layer. Our results show that vitamin E could pass into the collagen layer, but remained blocked in this layer for the majority of formulations tested (solution and non-thickened ME). The poor solubility of vitamin E in water does not favour its partitioning from collagen to receptor fluid. We can conclude that, in the binding of vitamin E to the collagen, the latter behaves as an additional receptor compartment that results in reduced drug permeation through the model. These results are in accordance with the penetration studies of vitamin E from a lipophilic vehicle into rabbit skin (29). A significant penetration of vitamin E from isopropyl myristate solution into RHE was observed. This is not surprising, since isopropyl esters of fatty acids enhance the skin permeation of drugs (30). The incorporation of vitamin E in the outer phase of ME further increased its deposition in the collagen. This could be due to the impairment of the barrier function of keratinocytes by the surfactants. Surprisingly, the concentration of vitamin E in the collagen did not reach that in the solution, using either o/w ME or gel-like ME. In both cases vitamin E was trapped in droplets of the
inner phase, so it first had to be released from the internal to the external phase. Vitamin E is insoluble in water, so it cannot partition freely from the inner oily phase of ME into the aqueous continuous phase that is in contact with RHE. The vitamin E concentration in the collagen was slightly greater for gel-like ME than for o/w ME. Lipophilic vitamin E can be solubilized in the oily phase of the formulation and probably also in the surfactant film and micelles. As gel-like ME contains a smaller percentage of oil in the formulation (10%) than o/w ME (25%), the affinity of vitamin E for the former is less and its diffusion from the vehicle is thus facilitated (25). The addition of colloidal silica to the formulation resulted in permeation of vitamin E across RHE, which is facilitated by at least two factors. The first is influence on the barrier function of RHE, and the second the changed partition coefficient \((P_K)\) of vitamin E. The log \(P_K\) for isopropyl myristate/water is 4.82 as opposed to that for isopropyl myristate + colloidal silica/water which is 2.46 (unpublished data), indicating the higher affinity for the hydrophilic phase of vitamin E incorporated into oily solution containing colloidal silica.

**Conclusion**

The present study shows that drug-vehicle interactions, as well as properties of RHE, change on the incorporation of drugs into different formulations and that the penetration characteristics and permeation profile of a drug can consequently be altered. The percutaneous absorption of vitamins C and E from ME was, in general, enhanced relative to that from aqueous solutions. The incorporation of vitamin C or E in the outer phase of ME resulted in greater absorption than that when vitamins were in the inner phase, meaning that, depending on the hydrophilicity of the drug, the same formulation can act as a permeation enhancer or retarder. However, according to our results vitamin C was less affected by its location in the vehicle. Consequently, w/o ME appears to be the better vehicle for simultaneous delivery of vitamins C and E than o/w ME into RHE. Colloidal silica enhanced the deposition of vitamins E and C in the RHE, not only by perturbation of its barrier function but also by changing the pH of the vehicle and hence the affinity of vitamin C for vehicle. Moreover, the addition of colloidal silica decreased the steady-state flux of vitamin C, which is probably related to the increased viscosity of the thickened formulation.

**Acknowledgement**

RHE kits were generously donated by Episkin, Lyon, France. The personnel from EA 4169, working in Hospital Eduard Herriot are acknowledged for their technical assistance. This
work was supported by a grant from the French National Educational Ministry, University Lyon1, and by a grant from the Slovenian Research Agency.

References
3.3. Chapter 3

Dual influence of colloidal silica on skin deposition of vitamins C and E simultaneously incorporated in topical microemulsions

Vpliv koloidnega silicijevega dioksida na sočasno prehajanje vitaminov C in E iz dermalnih mikroemulzij v kožo

Branka Rozman, Mirjam Gosenca, Karine Padois, Françoise Falson and Mirjana Gašperlin

Sent for publication.
Abstract

Colloidal silica is thickener of interest for topical ME since it can be used to optimise viscosity of both hydrophilic and lipophilic ME. The aim of this work was to elucidate its influence on efficiency of ME in simultaneous delivery of vitamins C and E to the skin. The results have shown that the addition of colloidal silica to ME increases skin bioavailability of both vitamins. Two different aspects of its possible function were investigated: firstly its effects on formulation characteristics and secondly its direct impact on the skin. Concerning the former the addition of colloidal silica to ME was shown to increase significantly vitamins solubility in ME and their partition to the phase in which they were less soluble. The effects were more pronounced for vitamin E, which also reflected in release studies, where for ME thickened with colloidal silica no difference in its release from oil- and water-continuous ME was observed. Furthermore, we have proved interactions of colloidal silica with excised skin. It decreased TEWL, probably by retaining water in stratum corneum due to its massive accumulation in the upper layers as revealed by SEM pictures. Comparison of cell viability of RHE treated with ME thickened with colloidal silica and aqueous dispersion of colloidal silica showed no considerable difference.

Key words: microemulsion, topical delivery, antioxidants, vitamin, colloidal silica
Povzetek

Koloidni silicijev dioksid je zgoščevalo izbora za dermalne mikroemulzije (ME), saj se lahko uporablja za optimizacijo viskoznosti tako hidrofilnih kot lipofilnih ME. Cilj tega dela je bila pojasnitev njegovega vpliva na učinkovitost ME kot nosilnih sistemov za sočasno dostavo vitaminov C in E v kožo. Rezultati so pokazali, da je dodatek koloidnega silicijevega dioksid povečal kožno absorpcijo obeh vitaminov. Proučevali smo dva različna vidika njegove potencialne vloge v dermalnih farmacevtskih oblikah: njegove učinke na lastnosti nosilnega sistema in njegov neposredni vpliv na kožo. Pri principevovanju prvega smo ugotovili, da znatno poveča topnost vitaminov v ME in njuno porazdeljevanje v fazo, v kateri sta manj topna. Učinki so bili izrazitejši pri vitaminu E, kar se odražalo tudi v študijah sproščanja, kjer nismo opazili razlike med obema tipoma ME, zgoščenima s koloidnim silicijevim dioksidom, čeprav so bile razlike pri njegovem sproščanju iz nezgoščenih ME obeh tipov precejšnje. Dokazali smo tudi interakcije koloidnega silicijevega dioksid s izolirano kožo. Prehod vode preko kože, na katero smo aplicirali ME zgoščeno s koloidnim silicijevim dioksidom, se je zmanjšal, verjetno zato, ker je koloidni silicijev dioksid zadržal vodo v roženi plasti kože. S pomočjo SEM slik smo namreč ugotovili, da se je masovno kopčil v zgornjih plasteh kože. Primerjava celične viabilnosti kultur človeških keratinocitov ni pokazala občutne razlike med toksičnostjo vodne disperzije koloidnega silicijevega dioksid in ME zgoščene s koloidnim silicijevim dioksidom.

Ključne besede: mikroemulzije, dermalna dostava, vitamin, antioksidanti, koloidni silicijev dioksid
Introduction

Microemulsions (ME) are optically isotropic and thermodynamically stable nanosized systems of water, oil and surfactants. Their stability, transparency, simple preparation, coupled with the possibility of solubilizing both water- and oil-soluble drugs, make them a very interesting drug delivery system (1-3). ME have been demonstrated to improve (trans)dermal delivery of several drugs over the conventional topical preparations like emulsions, gels or aqueous solutions (2). Since ME are usually low-viscosity Newtonian fluids, their rheological properties can make effective skin application difficult. A possible solution to the low viscosity lies in ME gels – transparent systems consisting of lamellar phases that retain the advantages of ME, but have higher viscosity. These systems, however, remain a challenging task for formulators since their microstructures are frequently destroyed on application of small shear stresses, by small changes in temperature or even following incorporation of drug (4-6). A more conventional way to optimise the viscosity of topical ME is the addition of thickener to liquid ME (5). Selecting an optimal thickener can be demanding, since it should improve rheological behaviour of ME without significantly modifying other characteristics such as stability and high water-oil interface area (7). Moreover, thickeners appropriate for oil-continuous are rarely compatible with water-continuous ME. An example of a thickener forming a gel in oil-continuous as well as in water-continuous ME is hydrophilic colloidal silica (8).

Colloidal silica (fumed silica, colloidal silicon dioxide) is a fine, white, light and amorphous powder with particle size usually less than 100 nm. Several grades that differ in particle size, surface area, degree of hydrophilicity and density, produced by modifying the manufacturing process, are commercially available. Hydrophilic colloidal silica can convert non-polar liquids such as olive oil, liquid paraffin or isopropyl myristate into transparent gels. It is also used as a thickening agent for polar liquids (9, 10). The increased viscosity of formulations thickened with silica is a consequence of weak interactions between silica particles that form agglomerates. As the size of the particles is not uniform, there are some spaces in the structure inside which the fluid phase is caught (11). Silica gels are distinguished by high viscosity with little temperature dependence, and by pronounced thixotropic behaviour (9, 10). Colloidal silica is generally regarded as an essentially non-toxic and non-irritant excipient. It is GRAS listed and included in the FDA Inactive Ingredients Guide (10) as well as in Ph. Eur. (monograph “Colloidal Anhydrous Silica”) and in USP (monograph “Colloidal Silicone Dioxide”).

Skin is frequently and directly exposed to pro-oxidative environments, including ultraviolet radiation and air pollutants. To counteract the harmful effects of reactive oxygen species
(ROS), skin is equipped with antioxidant systems that prevent oxidative stress. However, these systems can be depleted, and dermal supplementation of skin endogenous antioxidants plays an important role in prophylaxis and treatment of oxidative stress (12). Vitamins C (L-ascorbic acid) and E (α-tocopherol) are the skin’s major water- and lipid-soluble antioxidants, respectively. They can, especially when delivered topically, inhibit acute ultraviolet damage like erythema and sunburn, as well as chronic photoaging and skin cancer (13-15). Both are highly effective depigmenting agents. Topical vitamin C also increases collagen synthesis. Moreover, because vitamin C regenerates oxidised vitamin E, their combination is synergistic. It has been shown that a topical combination of L-ascorbic acid with α-tocopherol gives two-fold greater protection against UV-induced erythema, than either vitamin alone (15-18). ME are promising delivery systems for combined delivery of vitamins C and E to the skin because of their potential to incorporate hydrophilic and lipophilic molecules in the same system (8, 19). Moreover, they provide protection against (photo)oxidation of both vitamins (8), improve the solubilisation of vitamin E and hence enhance their bioavailability.

We have demonstrated that colloidal silica drastically improves epidermal concentration of a hydrophilic vitamin C and a lipophilic vitamin E in reconstructed human epidermis (20). To the best of our knowledge, no other published study has been done to investigate the effect of colloidal silica in topical preparations on skin penetration of drugs. We have focused in the present work on elucidating the influence of colloidal silica on isolated pig ear skin, as the most relevant in vitro animal model for human skin, by studying skin deposition of the same two vitamins. Two aspects of the function of colloidal silica in the ME were evaluated: first, its ability to affect formulation characteristics (partition coefficient of the vitamins, solubility and release) and secondly, its direct impact on skin by transepidermal water loss measurements (TEWL), scanning electron microscopy and keratinocyte viability determination.

**Materials and methods**

**Preparation of formulations**

Colloidal silica (Aerosil 200) was obtained from Degussa, Germany. Isopropyl myristate (IPM) was from Fluka Chemie, Switzerland and used as the lipophilic phase of ME. Tween 40 – polyoxyethylene (20) sorbitan monopalmitate (Fluka Chemie Switzerland) – was used as surfactant and Imwitor 308 – glyceryl caprylate (Condea, Germany) – as cosurfactant. Purified water was used as hydrophilic phase. α-Tocopherol (vitamin E) and ascorbic acid
(vitamin C) were from Fluka, Switzerland. The composition of tested formulations is given in Table 1.

Table 1. Composition of formulations.

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ME components (oil and hydrophilic phases, surfactants) were mixed with a magnetic stirrer for 5 minutes at room temperature. Vitamins C and E were incorporated into the ME by stirring with a magnetic stirrer for 30 minutes. Colloidal silica was added to formulations containing vitamin(s) and stirred with a plastic spoon. The formulations were left covered for at least 24 hours before use.

**Vitamins’ isopropyl myristate/ water partition coefficient determination**

The partition coefficients of the vitamins were determined in the presence or absence of colloidal silica (5 % (w/w)) for isopropyl myristate – water system containing 10 % (w/w) vitamins. The system was vortexed in a centrifuge tube and then shaken overnight. Next day the system was centrifuged at 10000 rpm for 10 min to separate the phases. Concentrations of the vitamins in each phase were determined by HPLC. Experiments were repeated in triplicate at 25±1°C.

**Solubilisation capacity of formulations**

An excess amount of vitamin C (0.5 g) or vitamin E (3 g) was added to 4 g of ME. Systems were allowed to reach equilibrium by stirring at 25±1°C. After 24 hours samples were centrifuged at 28000 rpm for 20 min at 5±1°C to separate excess vitamin from formulation. After dilution and filtration, samples were analysed by HPLC.

**Release studies**

Release rates of vitamins C and E from formulations were measured through 0.45 μm cellulose acetate membrane (Sartorious, Goettingen, Germany) soaked in receptor solution 24 h before experiment. Franz diffusion cells with a diffusion area of 0.785 cm² and 8 ml receptor volume were used. Isopropyl myristate with 0.5 (w/w) % Tween 40 and 0.5 (w/w) % Imwitor 308 was used as receptor fluid for vitamin E in order to obtain sink conditions and miscibility of receptor fluid with ME. 0.9 % NaCl was used as receptor solution for vitamin C. 500 mg of ME was dosed in the donor compartment. The diffusion membrane was kept at 32°C in a water bath and the receptor fluid was stirred continuously. At predetermined time intervals 0.3 ml samples were taken and replaced by the same volume of fresh preheated receptor solution. Vitamin concentrations were determined by HPLC. Each experiment was done in quadruplicate.
The cumulative amount of vitamin released was plotted against square-root of time according to
\[ Q(t) = k \cdot t^{(1/2)}, \]  
(Eq. 1)
where \( Q(t) \) is the cumulative percentage of vitamin released in time \( t \) (<60%), \( k \) is the rate constant and \( t \) is time.

**Permeation studies**

Pig ears were obtained from a local slaughterhouse. The skin was kept frozen until use. Before use, skin was briefly washed under tap water, hairs were removed and skin was sliced (thickness < 1 mm). Skin slices were mounted on Franz diffusion cells and filled with receptor fluid to equilibrate overnight in a temperature-controlled water bath, resulting in a membrane surface temperature of 32°C. Immediately before the experiment the whole receptor compartment was emptied and refilled with fresh preheated medium. 8 ml of 0.9% NaCl containing 3% chicken egg albumin (Sigma Aldrich, Germany) was used as the receptor fluid. The area available for diffusion was 0.785 cm². At predetermined time intervals (30, 60, 120, 180, 240, 360 min) 1ml of sample was taken from the receptor compartment and replaced by fresh preheated medium. Vitamins were extracted from collected samples with methanol and analysed by HPLC. After 6 hours, the formulation was removed and the skin surface was cleaned. Epidermis was separated from dermis by heat treatment. Both were cut into small pieces and vitamins extracted with MeOH. Samples were analysed by HPLC. The experiments were conducted in quadruplicate.

The steady-state flux of vitamin, \( J \), was estimated from the slope of the straight line portion of the cumulative amount of drug permeated per surface area plotted against time.

**HPLC analysis**

HPLC analysis was carried out with an Agilent 1200 series HPLC system.

Chromatographic conditions for vitamin E: the stationary phase was a 120x4 mm i.d. column packed with 5 μm Nucleosil C18; the mobile phase was methanol-acetonitrile 70:30. The flow rate was 1.5 ml/min and UV detection at 291 nm. The limit of quantification (LOQ) for chromatographic determination of vitamin E was determined from the calibration curve to be 1.25 μM; the limit of detection (LOD) was 0.412 μM.

Chromatographic conditions for vitamin C: the stationary phase was a 250x4 mm i.d. column packed with 5 μm Nucleosil C18-NH2, and the mobile phase methanol – acetonitrile – 0.02 M phosphate buffer pH 3.5 (20:30:50). The flow rate was 1 ml/min and UV detection at 243 nm. LOQ for chromatographic determination of vitamin C was 22.7 μM and LOD was 7.5 μM.
SEM imaging of stratum corneum

Pig ear skin was treated as in the permeation experiments. After six hours of contact formulations were removed and the surface was cleaned with 5x 300 μl of MeOH and dried with a cotton swab. Before observation, tape stripping was done with double-sided conductive tape (diameter 12 mm, Oxon, Oxford instruments, UK) that was afterwards fixed onto metallic studs. A Supra 35 VP (Oberkochen, Zeiss, Germany) scanning electron microscope was used with an acceleration voltage of 1.00 kV and a secondary detector.

Transepidermal water loss (TEWL) measurements

The ECVAM recommended procedure was followed (21) with slight modifications. Pig ears were washed under tap water and skin samples were prepared by removing the whole skin carefully from the underlying cartilage. The skin was mounted on Franz cells containing 8 ml of 0.9% aqueous solution of NaCl. During the experiments the temperature was kept at 25+/−1°C. The Franz cells were placed in a water bath overnight and the next morning TEWL was measured with an MPA 5 Tewameter®, Courage Khazaka, Germany. 50 mg of each formulation tested was placed on the surface of the skin. After four hours, the formulations were removed and the surface was cleaned with 2x 300 μl of PBS buffer pH 7.4 and dried with a cotton swab. The TEWL was measured again 4 hours after the removal of the formulation. Measurements were made in triplicate.

MTT assay

Episkin® kits containing 12 cell cultures with 1.07 cm² surface area on the nutritive gelatin gel were a gift of Episkin (Lyon, France). Upon receipt of the kit, cell cultures were removed from the nutritive gel and transferred under aseptic conditions into a sterile 12 well culture dish containing 2 ml of the maintenance medium per well. Maintenance medium was provided by Episkin. The cultures were incubated at 37°C, 5% CO₂ and saturated humidity until their use.

MTT ((3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide) assay was performed according to the method of Mosmann (22) with modifications proposed by Fentem et al (21). Cell cultures were incubated over the night in the maintenance medium at 37°C, 5% CO₂ and saturated humidity. The next day 50 mg of aqueous dispersion of 10% colloidal silica or distilled water (negative control) was applied to the cell culture and cells were incubated for 4 hours in 2 ml of assay medium. The surface of the culture was rinsed several times with a gentle stream of PBS. 2 ml of MTT dissolved in the assay medium (2mg/ml) were put in each
plate and cells were incubated at 37°C and 5% CO2 during 3h. MTT was extracted from the cells by placing the culture into 2.6 ml of isopropanol overnight at room temperature. The next day optical densities were read on an UV/visible spectrophotometer Shimadru Brukard Instrumente AG, Swiss, at 540 nm. All tests were done in triplicate. The direct reduction of MTT by test product was checked as follows: 25 mg of 10% colloidal silica aqueous dispersion was added to 1 ml of MTT solution (1mg/1ml of medium). The solution was incubated in the dark for 60 min at 37°C. As the solution remained yellow, it was assumed that the formulation cannot reduce the MTT.

Data analysis
Two-tailed Student t-test was used to compare the partition coefficients determined with and without colloidal silica. The influence of the formulation on the vitamins’ solubility in formulation and on their retention in skin layers was evaluated by one way ANOVA. Bonferroni’s test was used for post-hoc comparisons. Significance was tested at the 0.05 level of probability.

Results and discussion
The influence of colloidal silica on delivery characteristics and skin permeation ability of ME simultaneously loaded with vitamins C and E

Modification of vitamins isopropyl myristate/ water partitioning in the presence of colloidal silica
Colloidal silica influenced the partitioning of both vitamins in isopropyl myristate/water. To test the influence of colloidal silica on vitamin partition coefficient it was added to isopropyl myristate/ water system in 5 (w/w) % since in 10 (w/w) % which was used in ME, Pickering emulsion gel was formed making phase separation difficult. Vitamins C and E were added together to an isopropyl myristate/ water system at 10 % (w/w) to ensure that the concentrations of both vitamins in all phases were always above LOQ (Table 2). On the addition of colloidal silica to the ME, the partitioning of hydrophobic vitamin E into water increased 230 fold and that of the hydrophilic vitamin into isopropyl myristate C 1.2 times (Table 2). Although the change of vitamin C partition was less pronounced than that of vitamin E, it was still statistically significant (p= 0.0011).
Addition of colloidal silica increases the solubilisation of vitamins in ME

The addition of colloidal silica to both types of ME increased their solubilisation of both vitamins C and E (Table 3). This effect was more pronounced when vitamins were incorporated in the inner phase of ME (vitamin C in w/o ME; vitamin E in o/w ME) and can be explained by increased affinity of vitamins for the continuous phase as a consequence of silica particles with high surface area (200m²/g (10)) as already indicated by partition coefficient results. Interestingly, as for partition coefficient, the improvement in solubility was greater for lipophilic vitamin E, although colloidal silica used in our study was hydrophilic. Vitamin E, even though being lipophilic, has an ether group and a phenol group that can each participate in interactions with a hydrophilic surface (23). Moreover, it has been observed that poorly water soluble drugs can also be adsorbed to the surface of hydrophilic colloidal silica (24, 25).

Table 3. Solubility of vitamins C and E in o/w and w/o ME at 25+/-1°C.

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<td>vitamin C</td>
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* p < 0.05
compared to o/w ME in the absence of colloidal silica.

# p < 0.05 compared to w/o ME in the absence of colloidal silica.

The influence of colloidal silica on the release of vitamins C and E from ME
Release of vitamins C and E was slower and less complete from ME containing colloidal silica than from non-thickened ME (Figs. 1A&B, Table 4). The main reason is the increased viscosity of formulation that was approximately the same for both types of thickened ME (20). The release of vitamin C from non-thickened ME was slower from w/o than from o/w ME, but the differences were small. The same was also observed for ME containing colloidal silica. However, the release of vitamin E from o/w and w/o ME containing colloidal silica was the same, despite the pronounced difference in its release from non-thickened o/w and w/o ME. As expected, in non-thickened ME, vitamin E release rate was higher from w/o ME where it was mainly located in the continuous phase. The unexpectedly high release rate of vitamin E from thickened o/w ME in respect to thickened w/o ME, can be attributed to increased vitamin partition to the aqueous continuous phase of o/w ME in the presence of colloidal silica.

In order to allow comparison with permeation experiments, the vitamins release rate, characterised by k, the rate constant that was calculated from the slope of the linear portion of the plots of cumulative vitamin released (Q(t)) against t1/2 (Eq. 1), was evaluated (Table 4). Pearson’s coefficients, proving good fitting of experimental data to assumed kinetics, are also listed.
Fig. 1A. Vitamin C release from o/w and w/o ME with and without colloidal silica.

![Graph showing Vitamin C release from o/w and w/o ME with and without colloidal silica.]

Fig. 1B. Vitamin E release from o/w and w/o ME with and without colloidal silica.

![Graph showing Vitamin E release from o/w and w/o ME with and without colloidal silica.]

Table 4. Rate constants (k) for vitamin release from ME with and without colloidal silica.

* Coefficient indicating the extent of linear relationship between Q(t) and t(1/2) (Eq.1).

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<td>k (h^{1/2})</td>
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<td>Pearson's coefficient*</td>
<td></td>
</tr>
<tr>
<td>o/w ME</td>
<td>44.2</td>
<td>29.4</td>
<td>0.995</td>
<td>0.997</td>
</tr>
<tr>
<td>o/w ME colloidal silica</td>
<td>14.0</td>
<td>16.4</td>
<td>0.998</td>
<td>0.998</td>
</tr>
<tr>
<td>w/o ME</td>
<td>39.3</td>
<td>38.5</td>
<td>0.988</td>
<td>0.992</td>
</tr>
<tr>
<td>w/o ME colloidal silica</td>
<td>5.8</td>
<td>14.2</td>
<td>0.985</td>
<td>0.999</td>
</tr>
</tbody>
</table>
In vitro skin delivery of vitamins C and E

To prove the efficacy of ME simultaneously loaded with vitamins C and E we performed skin absorption studies. The amounts of vitamins accumulated in epidermis and dermis are shown in Table 5. The results show relatively high concentrations of both vitamins delivered to the tested skin layers from ME. ME thickened with colloidal silica significantly increased the amount of both vitamins in epidermis. Overall molar concentrations of vitamin C in the dermis were negligible compared to those recovered in epidermis and receptor fluid (Table 5, Fig. 2).

In contrast, considerable amounts of vitamin E were found in dermis. Although the presence of colloidal silica enhanced vitamin E deposition in dermis, the differences between ME with or without colloidal silica were not statistically significant. No vitamin E was found in receptor fluid. It probably bound to skin tissue where it formed a very strong reservoir (26) and consequently its partitioning from skin to receptor fluid was not favoured.

Cumulative amounts of vitamin C permeated through the skin following topical application of different ME are shown in Fig 2. In contrast to vitamin E, vitamin C permeated the skin and was found in receptor fluid after only 30 min. Results with ME thickened with colloidal silica are again outstanding; more vitamin C was found in receptor fluid than expected from the release profiles (Figs.1A&B, 2). The permeation rates (steady-state flux) of vitamin C from ME are shown in Table 6. Both release and permeation rates of vitamin C from the two water-continuous ME (Tables 4&6) were higher for o/w ME. However, the differences in its release rates from o/w ME and o/w ME thickened with colloidal silica were pronounced, whereas the differences in the permeation rates were minimal. Furthermore, for oil-continuous ME the release rate of vitamin C was higher from w/o ME, while the permeation rate was higher from w/o ME containing colloidal silica, showing that no simple correlation exists between vitamin release and permeation.
Fig. 2. Cumulative amounts of vitamin C permeated through the skin following topical application of ME with and without colloidal silica.

Table 5. Skin absorption of vitamins C and E from ME with and without colloidal silica.

<table>
<thead>
<tr>
<th>FORMULATION</th>
<th>AMOUNT IN EPIDERMIS (µg)</th>
<th>AMOUNT IN DERMIS (µg)</th>
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<tr>
<td></td>
<td>vitamin</td>
<td>vitamin</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>E</td>
</tr>
<tr>
<td>o/w ME</td>
<td>2.65±/-</td>
<td>16.1±/-</td>
</tr>
<tr>
<td></td>
<td>0.41#</td>
<td>4.0</td>
</tr>
<tr>
<td>o/w ME colloidal silica</td>
<td>4.62±/-</td>
<td>26.0±/-</td>
</tr>
<tr>
<td></td>
<td>0.65±#</td>
<td>4.3*</td>
</tr>
<tr>
<td>w/o ME</td>
<td>0.057±/-</td>
<td>21.8±/-</td>
</tr>
<tr>
<td></td>
<td>0.004±</td>
<td>4.2</td>
</tr>
<tr>
<td>w/o ME colloidal silica</td>
<td>2.12±/-</td>
<td>34.6±/-</td>
</tr>
<tr>
<td></td>
<td>0.66±#</td>
<td>5.2</td>
</tr>
<tr>
<td>aqueous solution</td>
<td>0.006±/</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>0.000*</td>
<td></td>
</tr>
<tr>
<td>aqueous solution</td>
<td>0.188±/-</td>
<td>/</td>
</tr>
<tr>
<td>colloid silica</td>
<td>0.039*</td>
<td></td>
</tr>
</tbody>
</table>
* p< 0.05 compared to o/w ME in the absence of colloidal silica
# p< 0.05 compared to w/o ME in the absence of colloidal silica

<table>
<thead>
<tr>
<th>FORMULATION</th>
<th>Steady-state flux (nmol/cm²*h)</th>
<th>Pearson’s coefficient</th>
<th>nt*</th>
</tr>
</thead>
<tbody>
<tr>
<td>o/w ME</td>
<td>26.2 +/-3.1</td>
<td>0.993</td>
<td></td>
</tr>
<tr>
<td>o/w ME colloidal silica</td>
<td>22.2 +/-0.89</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td>w/o ME</td>
<td>7.31 +/-1.17</td>
<td>0.992</td>
<td></td>
</tr>
<tr>
<td>w/o ME colloidal silica</td>
<td>13.3 +/-0.97</td>
<td>0.988</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Steady-state flux of vitamin C through pig ear skin from ME with and without colloidal silica.

* Pearson’s coefficient indicates the degree of a linear relationship between cumulative amounts of vitamin C permeated and time.

In order to clarify the influence of colloidal silica on skin deposition of vitamin C the experiment was repeated with a) its aqueous solution b) its aqueous solution thickened with 10% colloidal silica. Aqueous solution thickened with colloidal silica delivered 33-fold higher amounts of vitamin C into epidermis than in the absence of silica (Table 5). Furthermore, the amount of vitamin C delivered to epidermis by an aqueous dispersion of colloidal silica was 3-times higher than that delivered in epidermis by non-thickened w/o ME (Table 5). Neither solutions enabled permeation of vitamin C through the skin, but the one containing colloidal silica allowed its penetration into the dermis (Table 5). These results lead to conclusion that colloidal silica alone acts as an enhancing agent for the delivery of hydrophilic vitamin C into epidermis.
The impact of colloidal silica on skin
The permeation experiments have shown that the addition of colloidal silica to ME increased epidermal concentration of both vitamins and also delivered, relative to release studies, disproportionately large amount of vitamins C and E to receptor fluid and dermis respectively. The significant impact of colloidal silica on the skin delivery of both vitamins is therefore confirmed. Moreover, the pronounced enhancement of skin deposition of vitamins from formulations containing colloidal silica implies that, apart from its influence on vitamin-vehicle interactions, demonstrated by partition coefficient and solubility studies, colloidal silica could also affect skin properties. Therefore, two other techniques, TEWL measurement and SEM imaging, were used to clarify its role in the skin permeation of vitamins. Finally, the influence of topically applied colloidal silica gel on the viability of keratinocytes has been tested using reconstructed human epidermis as the ECVAM recommended model for irritancy and corrosion testing of topical products (21, 27, 28).

Transepidermal water loss
Transepidermal water loss (TEWL) is the outward diffusion of water through the skin and is a commonly used technique for assessing skin barrier function, because its increase reflects an impairment of the water barrier (29). Interestingly, TEWL was very considerably lower after exposure of pig skin to ME containing colloidal silica (Fig.3). Since the skin surface was thoroughly cleaned and colloidal silica was the only component that differed from the other two ME tested (which both increased TEWL) the reduction of TEWL suggests the deposition of colloidal silica particles in the stratum corneum. Hydrophilic silica particles that accumulated in stratum corneum could absorb water and consequently decrease its flux across the skin. This hypothesis explains the massive accumulation of vitamins in epidermis from formulations containing colloidal silica.
Fig. 3. Effect of addition of colloidal silica to ME on transepidermal water loss (TEWL).

**SEM images of pig ear skin treated with ME containing colloidal silica**

The accumulation of colloidal silica particles inside the skin was investigated by SEM. The massive accumulation of colloidal silica observed in the upper layers of stratum corneum from ME containing colloidal silica (Fig. 4B) which is in accordance with the TEWL results. With increasing tape strip number the amount of colloidal silica decreased considerably (Fig.4C), and was on some tapes even absent.
Fig. 4. SEM pictures of stratum corneum: A – untreated skin 2nd tape strip; B – skin treated with w/o ME colloidal silica 2nd tape strip; C – skin treated with w/o ME colloidal silica 8th tape strip.

Cytotoxicity of formulations containing colloidal silica
The cell viability of reconstructed human epidermis (Episkin® large model) after exposure to three different vehicles: aqueous dispersion of colloidal silica, o/w ME and o/w ME thickened
with colloidal silica is summarized in Fig. 5. Cell viability was determined by reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT. Purified water and SDS solution were used as negative (non-toxic) and positive controls. The unchanged viability of cell cultures treated with water (98+/−2%) confirmed that cell viability decreased only due to exposure of cell cultures to the test formulation. All formulations performed slightly better than SDS solution, a standard irritant. The viability of cell culture treated with aqueous solution was however unexpectedly low, taking into account the fact that colloidal silica has always been recognised as a safe excipient and is a widely used thickener (10). o/w ME performed similarly to colloidal silica dispersed in water. The addition of colloidal silica to o/w ME lowered cell viability, but less than it would have been estimated from the performance of aqueous dispersion of colloidal silica and non-thickened o/w ME alone.

![Fig. 5. Cell viability measured by the MTT reduction test following topical application of various formulations.](image)

**Conclusion**
In conclusion, the results presented in this article confirmed that the inclusion of colloidal silica in ME simultaneously loaded with vitamins C and E enhanced vitamins’ skin bioavailability. Dual influence of colloidal silica was observed: it changed not only delivery characteristics of ME, but also skin properties.

References


3.4. Chapter 4

Temperature sensitive microemulsion gel: an effective topical delivery system for simultaneous delivery of vitamins C and E

*Temperaturno občutljiv mikroemulzijski gel: učinkovit nosilni sistem za sočasno dostavo vitaminov C in E*

Branka Rozman, Alenka Zvonar, Françoise Falson and Mirjana Gašperlin

AAPS PharmSciTech. Article in press.
Abstract
Microemulsions (ME) – nanostructured systems composed of water, oil and surfactants – have frequently been used in attempts to increase cutaneous drug delivery. The primary objective addressed in this work has been the development of temperature sensitive microemulsion gel (called gel-like ME), as an effective and safe delivery system suitable for simultaneous topical application of a hydraphilic vitamin C and a lipophilic vitamin E. By changing water content of liquid o/w ME (o/w ME) a gel-like ME with temperature sensitive rheological properties was formed. The temperature-driven changes in its microstructure were confirmed by rotational rheometry, viscosity measurements and droplet size determination. The release studies have shown that the vitamins’ release at skin temperature from gel-like ME were comparable to those from o/w ME and were much faster and more complete than from o/w ME conventionally thickened with polymer (o/w ME carbomer). According to effectiveness in skin delivery of both vitamins o/w ME was found the most appropriate, followed by gel-like ME and by o/w ME carbomer, indicating that no simple correlation between vitamins release and skin absorption could be found. The cytotoxicity studies revealed good cell viability after exposure to ME and confirmed all tested microemulsions as non irritant.

Keywords: microemulsion gel, antioxidant, vitamin, rheology, skin permeation
Povzetek
Mikroemulzije (ME) - nanostrukturirani sistemi sestavljeni iz vode, olje in površinsko aktivnih snovi – se pogosto uporabljajo z namenom izboljšanja dostave učinkov v kožo. Poglaviti cilj tega dela je bil razvoj temperaturno občutljivega mikroemulzijskega gela (imenovanega gelirana ME) kot učinkovitega in varnega sistema, primernega za sočasno dostavo hidrofilnega vitamina C in lipofilnega vitamina E v kožo. S spreminjanjem vsebnosti vode v tekoči hidrofilni ME (o/v ME) je bil razvit mikroemulzijski gel s temperaturno odvisnimi reološkimi lastnostmi. Temperaturno pogojene spremembe mikrostruktura so bile potrjene z rotacijsko reometrijo, meritvami absolutne viskoznosti in dolčevanjem velikosti kapljic. Študije sproščanja so pokazale, da je sproščanje vitaminov iz gelirane ME podobno sproščanju iz o/v ME in je veliko hitrejše in popolnejše kot iz ME zgoščene s polimerom (o/v ME karbomer). Glede na učinkovitost dostave obeh vitaminov v kožo je bila najboljša o/v ME, sledila ji je gelirana, najslabša pa je bila o/v ME karbomer, kar kaže, da preprosta primerjava med sproščanjem vitaminov iz formulacije in njihovo absorpcijo v kožo ni mogoča. Študija citotoksičnost je pokazala dobro preživetje celic po izpostavljenosti ME in potrdila vse preskušane mikroemulzije kot primerne za dermalno aplikacijo.

Ključne besede: mikroemulzijski gel, antioksidant, vitamin, reologija, dermalna dostava
Introduction

In attempts to increase cutaneous drug delivery, ME have been more and more frequently employed over recent years. ME are nanosized mixtures of water, oil and surfactants that are transparent, single phase, optically isotropic and thermodynamically stable. They have been shown to be superior to conventional vehicles like emulsions or hydrogels for dermal delivery of hydrophilic and especially lipophilic drugs. The favourable drug delivery properties of ME are attributed mainly to their excellent solubilising properties. They can also act as penetration enhancers, depending on the nature of the oil and surfactant constituents (1).

Besides optimizing the formulation to maximize cutaneous drug bioavailability, it is also important to ensure that it is aesthetically acceptable to patients, is easy to use and adheres to skin sufficiently (2). Optimising rheological behaviour is therefore one of the crucial steps in development of dermal drug delivery systems. Depending on their composition ME exhibit a number of structures, varying from dispersed droplets of different shape and size to liquid lamellar crystals (3). It is generally known that from o/w ME under certain conditions upon the addition of specific amounts of water transparent isotropic gels can be formed (4). Unfortunately, these water-induced gel structures are frequently easily disturbed and the addition of drug molecules can considerably affect their stability. The more usual way to optimise the rheological behaviour of topical ME is addition of thickening agent that increases the viscosity of the system without affecting its stability and spontaneous formation (5). However, finding an appropriate thickener is a time consuming task since the selection is done empirically and numerous thickeners have to be screened. Thickened ME are usually composed of two distinct structural elements, a network formed by thickener in the outer phase that coexists with the microemulsion droplets (4).

Even though sunlight is indispensable for life, it is well known that UV regions of the spectrum are linked to skin disorders ranging from mild inflammatory effects to serious diseases like skin cancer (6). Cells are equipped with a variety of mechanisms that constantly monitor and repair UV-induced damage, the most important being nucleotide excision repair systems and enzymatic and non-enzymatic antioxidants. Nevertheless, excessive exposure to sunlight can still cause depletion of skin repair mechanisms and the development of effective strategies to support the cells’ protection mechanisms is important in achieving protection against and therapy of cutaneous disorders (7).
Because the most important non-enzymatic aqueous- and lipid-phase antioxidants – vitamins C (L-ascorbic acid) and E (α-tocopherol) respectively – can only be provided exogenously, it is beneficial to enhance oral supplementation by topical application for extra protection of the skin (8). In cells, vitamins C and E act synergistically to provide antioxidant protection. It has been shown that a topical combination of L-ascorbic acid with α-tocopherol gives four-fold protection against UV-induced erythema, compared to two-fold protection by either vitamin alone (8-11).

The major challenge for topical delivery of antioxidants is development of formulations that could stabilise them and that provide a sufficient increase in their penetration into the skin. Furthermore, delivery systems that allow simultaneous incorporation of antioxidants with different lipophilicities such as ME or liposomes are desirable (12-14).

The primary objective addressed in this work has been the development of temperature sensitive ME gel (called gel-like ME), composed of uniquely pharmaceutically acceptable components, as an effective and safe delivery system suitable for simultaneous topical application of a hydrophilic and a lipophilic vitamin. We formulated temperature sensitive ME gel with a suitable consistency for topical application at 20°C that changes into liquid ME at skin temperature and thus accelerates vitamins release. First, we studied temperature-driven changes of gel-like ME in comparison to liquid o/w ME (called o/w ME) and conventionally thickened o/w ME (called o/w ME carbomer) using rheological and droplet size measurements. Second, the effectiveness of all three formulations as a vehicle for simultaneous topical delivery of vitamins C and E was investigated using in vitro skin permeation test. Finally, the in vitro toxicity of model cell cultures exposed to ME was evaluated by the MTT assay and by fluorescence microscopy.

**Materials and methods**

*Preparation of ME*

Isopropyl myristate (IPM) was obtained from Fluka Chemie, Switzerland and used as the lipophilic phase. Tween 40 – polyoxyethylene (20) sorbitan monopalmitate (Fluka Chemie, Switzerland) – was used as surfactant and Imwitor 308 – glyceryl caprylate (Condea, Germany) – as cosurfactant. Purified water was used as the hydrophilic phase. α-Tocopherol (vitamin E, viscous liquid, molecular weight 430.72 g/mol) and ascorbic acid (vitamin C,
white crystalline powder, molecular weight 176.12 g/mol) were from Fluka, Switzerland. The composition of tested ME is given in Table 1.

Table 1. Composition of gel-like ME, o/w ME and o/w ME carbomer.

<table>
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<tr>
<th>COMPO</th>
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<td>Purifie</td>
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<td>Vitami</td>
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<tr>
<td>Carbo</td>
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were prepared in the same way. The surfactant and cosurfactant were blended in a 1:1 mass
ratio to give the surfactant mixture. Isopropyl myristate and distilled water were then added and mixed with a magnetic stirrer for 5 minutes at room temperature. Vitamin C and E were incorporated by stirring with a magnetic stirrer for 30 minutes. o/w ME carbomer was prepared by adding carbomer (Carbopol 974 PNF, BF Goodrich, Belgium) to o/w ME containing vitamins and stirred with a magnetic stirrer for 30 minutes. The ME were left covered for at least 24 hours before use.

The final vitamin content was always in the range 95-105% of the amount added to ME.

Rheological measurements

The rheological characteristics were determined with a Rheolab MC 100 Paar Physica, controlled shear rate rheometer at 20°C and 32°C. The geometry used was a stainless steel cone/plate system MK22 (r= 25 mm, θ= 1°). All measurements were made in triplicate.

The absolute dynamic viscosity of ME was determined in triplicate in a temperature range 20-32°C using a SV-10 Vibro Viscosimeter, A&D Company, Japan. The temperature coefficient of viscosity was calculated:

\[
\text{(Viscosity at end – Viscosity at start)} / \text{(Temperature at end – Temperature at start)} \\
\] *1/(Averaged viscosity)* 100

(Eq.1)

and expressed in [%/°C].

Size determination

Dynamic light scattering measurements of the hydrodynamic radius and polydispersity of the ME structures were performed at 20 and 32°C using a Nano ZS, Malvern Instruments. ME samples were thermostated 5 min before measurement. The viscosity of the samples was determined as described in section Rheological measurements and the refractive indexes were measured using refractometer Carl Zeiss.

Solubility of vitamins C and E

Excess vitamin C (0.5 g) or vitamin E (3 g) was added to 4 g of ME or their components. The systems were allowed to reach equilibrium by stirring at 25+/-1°C for 24 hours. Samples were then centrifuged at 28000 rpm for 20 min at 5°C to separate excess vitamin from the formulations. Under test conditions vitamin E dissolved completely in surfactant mixture and isopropyl myristate. Vitamin E was therefore mixed with surfactant mixture to give mass fractions ranging from 10% to 90% in 10% steps, and left to equilibrate as described above. The same procedure was repeated for vitamin E solubility in isopropyl myristate. After
dilution and filtration, samples were analysed by HPLC. All samples were analysed in triplicate.

**Release studies**

Vitamin C and E release rates from formulations were measured through cellulose acetate membrane (pore size: 0.45 μm, Sartorius, Goettingen, Germany) soaked in receptor solution 24 h before experiments. Franz diffusion cells with a diffusion area of 0.785 cm² and 8 ml of receptor volume were used. To take account of the very low solubility of vitamin E and to ensure its stability, isopropyl myristate solution with 0.5 (w/w) % Tween 40 and 0.5 (w/w) % Imwitor 308 was demonstrated to be a suitable receptor phase. For vitamin C 0.9 % NaCl was used as receptor fluid. 500 mg of ME was dosed in the donor compartment. The system was mounted on Franz diffusion cells and filled with 8 ml of 0.9% NaCl with 3% of chicken egg albumin (Sigma Aldrich, Germany) was used as the receptor fluid. 500 mg of ME was dosed in the donor compartment. The system was kept in a temperature-controlled water bath to maintain the temperature in the donor compartment at 32 or 20°C and the receptor phase was continuously stirred. At predetermined time intervals 0.3 ml samples were taken and replaced by the same volume of fresh preheated receptor. Vitamin concentrations were determined by HPLC. Each experiment was done in quadruplicate.

Cumulative amount of vitamin released was plotted against square-root of time:

\[ Q(t) = K \ast t^{(1/2)} \]  

(Eq. 2)

where \( Q(t) \) is the cumulative amount (nmol/cm²) of vitamin released in time \( t \) (<60%), \( K \) (nmol/(h\(^{(1/2)}\) * cm²)) is the kinetic constant indicative of vitamin release rate and \( t^{(1/2)} \) is square-root of time. The degree of linearity \( Q(t) = f(t^{(1/2)}) \) was checked for all formulations for both vitamins and Pearsons coefficient was always above 0.993.

**Permeation studies**

Pigs’ ears were obtained from the local slaughterhouse. The skin was kept frozen until use. Before use, it was briefly washed under tap water, hairs were removed and the skin sliced (thickness < 1 mm). Skin slices were mounted on Franz diffusion cells and filled with receptor fluid the evening before the experiment and left to equilibrate overnight in water bath maintaining skin surface temperature at 32°C. Before experiment the whole receptor compartment was emptied out and refilled with fresh preheated medium. 8 ml of 0.9% NaCl with 3% of chicken egg albumin (Sigma Aldrich, Germany) was used as the receptor fluid. The area available for diffusion was 0.785 cm². 1g of formulation was spread on the skin surface. At predetermined time intervals (30, 60, 120, 180, 240, 360 min) 1ml of sample was
taken from the receptor compartment and replaced by fresh medium. Vitamins were extracted from collected samples with MeOH and analysed by HPLC. After 6 hours, the formulation was removed and the skin surface cleaned. Epidermis was separated from dermis by heating the samples with hair dryer for 15 s and separating analysed skin layers with the help of two pairs of tweezers. After separation the epidermis and dermis were cut into small pieces and vitamins extracted with MeOH. Samples were analysed by HPLC. The experiments were conducted in a temperature-controlled water bath, resulting in a membrane surface temperature of 32°C in quadruplicate.

The skin permeation rate at steady-state (steady state flux $J$; nmol/cm²*h) was calculated from the slope of the linear portion of the plots of cumulative vitamin C permeation per unit of skin surface area against time.

**HPLC analysis**

HPLC analysis was carried out with an Agilent 1200 series HPLC system.

Chromatographic conditions for vitamin E: a 120x4 mm ID column packed with 5 μm Nucleosil C18 as stationary phase; the mobile phase was methanol-acetonitrile 70:30. The flow rate was 1.5 ml/min. UV detection was at 291 nm. The limit of quantification (LOQ) for chromatographic determination of vitamin E was determined from the calibration curve and was 1.25 μM; the limit of detection (LOD) was 0.412 μM.

Chromatographic conditions for vitamin C: a 250x4 mm ID column packed with 5 μm Nucleosil C18-NH2 as stationary phase; the mobile phase was methanol – acetonitrile – 0.02 M phosphate buffer pH 3.5 (20:30:50). The flow rate was 1 ml/min. UV detection was at 243 nm. LOQ for chromatographic determination of vitamin C was determined from the calibration curve to be 22.7 μM; LOD was 7.5 μM.

**Cell cytotoxicity**

Human embryonic kidney cells (HEK293, ATCC) were cultured in Dulbecco’s modified essential medium (DMEM; Sigma, Germany) supplemented with 10% heat inactivated fetal calf serum (Gibco, Invitrogen, Carlsbad, USA), 2mM L-glutamine (Sigma, Germany) and 100U/ml penicillin/streptomycin (Sigma, Germany). All cells were incubated in humidified atmosphere at 37°C in 5% CO₂.

MTT assay was performed according to the method of Mosmann with modifications for HEK293 cells proposed by Kristl et al (15). Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a tetrazole is metabolically reduced to purple formazan. MTT is cleaved to formazan by succinate-tetrazolium reductase system which belongs to the
mitochondrial respiratory chain and is active only in viable cells. Since the amount of formed formazan is directly proportional to the number of living cells in culture, the intensity of produced color is an indication of the viability of the cells. Cells were seeded in 96-well plates (2.5x10^4 cells/well, 100 μl of cell culture medium) and when 40% confluence was reached, 10 μl of test dispersion were added for 24 hours. Test dispersions were prepared by diluting ME in cell culture medium (1:200) to achieve the final concentration of ME 450 μg/ml. Only formulations without vitamins were tested, since vitamins C and E are both strong antioxidants, capable of reducing MTT to formazan. In negative control experiments cells were treated with cell culture medium. SDS solution diluted in cell culture medium to give final concentration of 450 μg/ml was used as a positive control. After 21 hours 11 μl of the MTT dissolved in the assay medium (2 mg/ml) were put in each well and cells were incubated at 37°C and 5% CO2 during 3h. The insoluble purple formazan product was extracted from the cells by acidic isopropanol into a colored solution. The absorbance of this colored solution was quantified by measuring at 570 nm in an automated plate reader (Safire2™, Tecan, Switzerland). Average cell viability of treated cells was expressed as percentage of absorbance of ME-treated cells as follows:

\[
\text{Cell viability} = \frac{(A_S - A_{S0})}{(A_C - A_{C0})};
\]

where \(A_S\) is the absorbance of treated cells, \(A_C\) the absorbance of untreated cells (control), \(A_{S0}\) the absorbance of ME diluted in medium without cells and \(A_{C0}\) the absorbance of the medium alone. All tests were done in triplicate.

Cell growth and morphology were observed using an inverted phase-contrast microscope (Olympus CKX41, Japan). Cells were plated on square glass cover slips and incubated in 6-well plates overnight. Following the incubation with different ME (final concentration 450 μg/ml of cell culture medium), the cells were fixed with ice cold 4% paraformaldehyde in PBS pH 7.4 for 10 min and permeabilized for 10 min in 0.1% Triton X-100 (both Sigma, Germany). Cell nuclei were visualized by staining with a DNA intercalating dye Hoechst 33342 (Riedel de Haen, Germany, 5 μg/ml) for 30 min in the dark. Actin fibers were stained with Phalloidin TRITC (Sigma, USA, 1:40). After staining the cover slips were removed from the wells, mounted on a slide and viewed using 360 nm/420 nm (Hoechst) and 535 nm/635 nm (Phalloidin – TRITC) excitation/emission filter sets.

Data analysis

Influence of formulation on vitamins’ solubility, release rate, skin permeation and on cell viability was evaluated by one way ANOVA with KaleidaGraph® software package.
Bonferroni’s test was used for post-hoc comparisons. Significance was tested at the 0.05 level of probability.

**Results and discussion**

*Viscosity and rheological behaviour*

Rheological analysis is one of the most frequently used techniques for characterisation of ME structure at macroscopic level. Rheological measurements of tested formulations demonstrated that their flow behaviour depends on composition and on surrounding temperature (Figs. 1; 2A&B).

![Graph of viscosity and shear stress vs temperature and shear rate](image)

Fig. 1. Temperature dependence of absolute viscosity for gel-like ME, o/w ME and o/w ME carbomer.
At 20°C gel-like ME exhibit almost 3-fold higher viscosity than o/w ME that was used as a comparison (Fig. 1). Although the topical application of o/w ME is possible, a slightly increased viscosity of the vehicle, like in the case of gel-like ME, is desirable. For comparison we also formulated o/w ME carbomer with approximately the same viscosity at 20°C as that of gel-like ME. This was done conventionally by adding a suitable thickener to o/w ME. The choice of appropriate thickener was done empirically by screening different polymers such as xanthan, hydroxypropyl methyl cellulose, microcrystalline cellulose, locust bean gum, carbomer and sodium alginate. Only with carbomer, a synthetic high-molecular-weight acrylic acid polymer, a stable system with appropriate viscosity could be formed that was called o/w ME carbomer.

As can be seen from Fig. 1 the viscosity of all three systems in the temperature range 20-32°C is decreasing. In the case of o/w ME and o/w ME carbomer the decrease is linear with time. Moreover, their temperature coefficients of viscosity calculated using Eq.1 were approximately the same (-3.5+/−0.1%/°C and -2.8+/−0.2%/°C for o/w ME and o/w ME carbomer respectively) and consequently at skin physiologic temperature viscosity of o/w ME carbomer is still about 3-times higher than that of o/w ME. On the contrary, the viscosity of gel-like ME decreases drastically with temperature (its temperature coefficient of viscosity is -11.8 %/°C) and at 32°C its viscosity is the same as that of o/w ME. The rapid changes in viscosity, reflected in high temperature coefficient of viscosity, are usually associated with the change in microstructure of ME.

The changes in viscosity following the exposure to shear stress shown on Figs. 2A (at 20°C) and B (at 32°C) additionally confirmed the temperature driven changes in rheological
behaviour of gel-like ME that were on contrary not observed either in o/w ME or in o/w ME carbomer.

At 20°C gel-like ME expressed rheopectic behaviour, rarely seen in pharmaceutical systems (Fig. 2A). It has been proven by SAXS measurements that at rest gel-like ME consists of lamellar structures (16) that are the consequence of increased interactions between highly hydrated surfactant chains (17). At higher shear rates the aqueous layer of surfactants is disrupted, which is reflected in increased resistance to flow (Fig. 2A). However, at a skin temperature, the rheological behaviour of gel-like ME changed to that of o/w ME that are characterised as low-viscosity Newtonian fluids, indicating the destruction of the gel-like structure (Fig. 2B). As mentioned above, o/w ME that differs from gel-like ME only in water content is a stable, ideal Newtonian fluid at 32°C as well as at 20°C. The addition of carbomer to o/w ME changed its rheological behaviour from Newtonian to thixotropic (Fig. 2A). The shear stress applied caused the macromolecules of carbomer to align in the direction of the shear stress vector, which resulted, in opposition to gel-like ME, in reduced resistance to flow. Increase in temperature to 32°C did not affect its rheological behaviour (Fig. 2B).

The addition of vitamins did not influence the viscosity of gel-like ME (75.6 mPa.s at 20°C)

Droplets size

Table 2 shows the hydrodynamic radius of the droplets of ME samples obtained from PCS measurements as at 20°C and 32°C. Polydispersity index is listed only for samples that had a single peak in the intensity chart.

Table 2. Droplet size and polydispersity index (PI) for gel-like ME, o/w ME and o/w ME carbomer at 20°C and 32°C.

<table>
<thead>
<tr>
<th></th>
<th>T= 20°C</th>
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<th>T= 32°C</th>
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</table>
structures in the size range 0.1-10000 nm were observed in gel-like ME 20°C, although the scattering of the sample was good. As described previously gel-like ME is composed of double-layer lamellar phases (16) that could not be measured by PCS. However, it is very interesting to note that at 32°C small droplets were detected, confirming our hypothesis that increase in temperature lead to structural reorganisation of gel-like ME into o/w ME consisting of fine oil droplets dispersed in aqueous phase.
As already confirmed by Podlogar (17) o/w ME also has very small size aggregates, which makes this system optically transparent. Increase in temperature to 32°C does not considerably affect droplets size (Table 2). In the case of o/w ME carbomer the additional peak due to the presence of polymer aggregates (451.3+/-105 at 20°C and 872.7+/-132 at 32°C) was noticed. Nevertheless the droplets size was comparable to that of o/w ME.

**Solubility studies**

The solubility of vitamins C and E was determined in tested ME and in particular components (Table 3).

Table 3. Solubility of vitamins C and E at 25+/-1°C.

<table>
<thead>
<tr>
<th>FORMULATION</th>
<th>vitamin C</th>
<th>vitamin E</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SOLUBILITY (mg/g)+/-SD</td>
<td></td>
</tr>
<tr>
<td>o/w ME</td>
<td>85.5+/-2.6</td>
<td>134+/-5 ♣</td>
</tr>
<tr>
<td>o/w ME carbomer</td>
<td>96.9+/-1.7</td>
<td>107+/-3 ♣</td>
</tr>
<tr>
<td>gel-like ME</td>
<td>170+/-4</td>
<td>74.6+/-0.1 ♣</td>
</tr>
<tr>
<td>water</td>
<td>335+/-2</td>
<td>20.9 ♣</td>
</tr>
<tr>
<td>isopropyl myristate</td>
<td>0.0113+/-0.0009</td>
<td>Completely miscible</td>
</tr>
<tr>
<td>emulsifiers</td>
<td>10.9+/-0.7</td>
<td>Completely miscible</td>
</tr>
</tbody>
</table>

* p<0.05 compared to o/w ME

Significant differences (p<0.05) in solubility of both vitamins in gel-like ME, o/w ME and o/w ME carbomer were seen, that were mostly induced by different composition of the vehicles. Vitamin C is a freely water soluble drug. Its solubility in other components of o/w ME is very limited (Table 3), leading to conclusion that it must be located mainly in the aqueous phase of ME. Accordingly, its solubility in ME is decreased with lower water content (gel-like ME vs. o/w ME – Table 3). On the other hand, vitamin E is a poorly water soluble drug, but completely miscible with the oily and surfactant phases. Consequently, in contrast to vitamin C, its solubility in ME decreases with increased water content. Addition of carbomer slightly lowered vitamin E solubility, probably due to its creation of a more hydrophilic
environment [148]. This would also account for the small increase of vitamin C solubility in o/w ME carbomer.

Release studies
Several parameters can influence the mechanism of drug release from ME, such as type of ME, its internal structure, viscosity, vitamin solubility and vitamin hydrophilicity/lipophilicity. In our study all formulations were of the same type (o/w), but they differed in their rheological properties and microstructure (Figs. 1&2, Table 2). Moreover, they were simultaneously loaded with hydrophilic vitamin C and lipophilic vitamin E whose solubility in the vehicle depended on formulation.

The release profiles of vitamins C and E from tested ME (Figs. 3A-C) were determined at 20 and 32°C. The release rate constant (K, Eq.2) was calculated from the slope of the linear portion of the plots of cumulative drug released against t^{1/2} and expressed in nmol/(cm²*h^{1/2}) in order to facilitate comparison between vitamin E and C release since their molar concentration was the same in all vehicles (23 μmol/ g of formulation).
From gel-like ME at 32°C 75% of vitamin C was released in 6 hours, characteristic time elapsing between two subsequent topical applications. The amount of vitamin E released (Fig. 3A) and its release rate (Table 4) was considerably lower.

Table 4. Release rate constants (K) of both vitamins from different ME calculated from the linear part of release profiles using the time square root model (Eq.1) at 32°C and 20°C.
<table>
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<tr>
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<th>E</th>
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<tbody>
<tr>
<td>o/w ME</td>
<td>C</td>
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<td>32°C</td>
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<td>gel-like ME</td>
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<td>o/w ME</td>
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<td>carbomer</td>
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<td>32°C</td>
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<td>gel-like ME 20°C</td>
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<td>o/w ME 20°C</td>
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</table>

Branka Rozman, Doctoral dissertation

Results and Discussion, Chapter 4
Vitamin E, being lipophilic, is incorporated in the inner phase of ME and has to partition from oily droplets into the continuous phase before being released to the receptor solution, whereas vitamin C is located mainly in the outer aqueous phase. At 20°C no difference between vitamin E and C release from gel-like ME was observed (Fig. 3A, Table 4), which is consistent with our previous conclusions that gel-like ME at 20°C does not have a typical microstructure of oily droplets dispersed in aqueous medium. In contrast to gel-like ME at 20°C the difference in vitamin C and E release from o/w ME was clearly seen (Fig. 3B, Table 4). The comparison of release profiles and release rates of vitamin C at 32°C (Figs. 3A&B, Table 4) revealed no difference between the gel-like and o/w ME, since the structure of the former has been shown to be destroyed at physiologic temperature (Figs. 2, Table 2). However, at 32°C vitamin E was released more slowly from gel-like ME than from o/w ME. The possible explanation is that the former has higher percentage of water, which constitutes a diffusion barrier for vitamin E.

Release of both vitamins from o/w ME carbomer at 32°C was incomplete and very slow in comparison to o/w ME and gel-like ME (Figs. 3A-C, Table 4). As for other two tested ME with decrease of temperature to 20°C the release of both vitamins from o/w ME carbomer was additionally slowed down (Fig. 3C, Table 4).

Permeation studies

• p<0.05 compared to o/w ME at 32°C
• p<0.05 compared to o/w ME at 20°C
The permeation experiments were performed using pig ear skin as barrier. When considering the penetration of a vitamin from different formulations into the skin apart from vehicle-vitamin interactions (proven by solubility and release experiments) influence of the carrier on the skin barrier has to be considered. In the case of ME especially surfactants may alter the structure of the skin and modify the skin absorption of vitamins. Although in the present study all tested ME contained approximately the same amount of surface active agents (Table 1) the differences among tested ME were observed (Table 5).

Table 5. Vitamins’ amounts in epidermis and dermis after 6 hours of contact with gel-like ME, o/w ME and o/w ME carbomer.

<table>
<thead>
<tr>
<th>FORMULATION</th>
<th>epidermis (nmol)</th>
<th>dermis (nmol)</th>
<th>epidermis (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gel-like ME</td>
<td>14.9 +/- 1.5</td>
<td>1.14 +/- 0.03</td>
<td>33.3 +/- 7.7</td>
</tr>
<tr>
<td>o/w ME</td>
<td>15.1 +/- 2.9</td>
<td>2.48 +/- 0.27</td>
<td>37.4 +/- 9.2</td>
</tr>
<tr>
<td>o/w ME carbomer</td>
<td>15.7 +/- 4.2</td>
<td>1.09 +/- 0.20</td>
<td>40.1 +/- 1.8</td>
</tr>
</tbody>
</table>

* p<0.05 compared to o/w ME

Two permeation parameters were evaluated: vitamin accumulation in the skin layers and their permeation through the skin into receptor fluid. Vitamin contents in epidermis and dermis were analysed separately. Gel-like ME delivered in the epidermis approximately the same amount of both vitamins than other two tested ME (p= 0.98 and 0.58 for vitamin C and E respectively), but molar concentrations of vitamin E were on average two-fold higher than those of vitamin C (Table 5).

Concerning delivery into dermis and receptor fluid, gel-like ME delivered fewer vitamins than o/w ME, but more than o/w ME carbomer (Table 5, Fig.4). Significant amounts of vitamin E were found in dermis (Table 5), and none in the receptor fluid. Vitamin E can bind to skin tissue where it forms a very strong reservoir (19) and although the solubility of vitamin E in receptor fluid was improved by adding albumin, its partitioning from skin to receptor fluid was still not favoured. Overall molar concentrations of vitamin C in the dermis were up to 200-fold lower than those of vitamin E (Table 5). It seems that vitamin C, in contrast to vitamin E, does not favour accumulation in dermis.
o/w ME enabled a higher delivery of vitamin E in dermis than gel-like ME, although the differences were not statistically significant. This could be, apart from lower amount of isopropyl myristate, a well-known penetration enhancer (20), also attributed to sustained release of vitamin E from gel-like ME (Figs. 3A&B). o/w ME carbomer also delivered less vitamin E in dermis than o/w ME. Again, this effect can be attributed mainly to unfavourable partitioning of vitamin E from the dispersed oily phase to the continuous aqueous phase.

In all cases vitamin C was found in the receptor solution after 30 min (Fig. 4). Steady-state fluxes were obtained from the slope of the linear part of permeation profiles (Table 6).

![Vitamin C permeation profiles from different ME](image)

**Fig. 4.** Vitamin C permeation profiles from gel-like ME, o/w ME and o/w ME carbomer. Lines: zero-order kinetics. Pearson’s coefficients are given next to the symbols in the legend.

* p<0.05 compared to o/w ME

**Table 6.** Steady-state flux (J) of vitamin C through pig ear skin from different ME.

<table>
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<tr>
<th>ME Type</th>
<th>Steady-state Flux (J)</th>
<th>Pearson’s Coefficient</th>
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<tr>
<td>Gel-like ME</td>
<td>0.995</td>
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<tr>
<td>o/w ME</td>
<td>0.993</td>
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<tr>
<td>o/w ME carbomer</td>
<td>0.993</td>
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</tbody>
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156
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| E | 8 |
|   | 2 |
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|   | - |
|   | 0 |
| e | 9 |
| l- | 0 |
| lik | . |
| e | 2 |
| M | 8 |
| E | 6 |
|   | + |
|   | / |
|   | - |
|   | 0 |
| o/ | 0 |
| w | 0 |
| M | 0 |
| E | 7 |
| ca | 6 |
| rb + | |
| o | / |
| m | - |
| er | 0 |
|   | 5 |
Less vitamin C permeated the skin from gel-like ME than from o/w ME (p<0.001). Moreover, its permeation rate was lower from gel-like ME than from o/w ME although the respective release rates across artificial membranes were the same (Tables 4&6). This phenomenon could be explained by lower amount of penetration enhancer isopropyl myristate in gel-like ME. o/w ME carbomer delivered the lowest amount of vitamin C across the skin (p= 0.002 and <0.001 compared to gel-like and o/w ME respectively) and its permeation rate was also the lowest.

**Cell cytotoxicity**

Fig 5. summarizes the cell viability after 24-hour-exposure of HEK293 cells to different ME that contained no vitamin. HEK293 cells were chosen for cytotoxicity experiments since they are, like normal human keratinocytes, epithelial adherent, but they allow simple manipulation and suitable visualisation. Moreover, this cell culture has already been used to study the photoprotective effects of antioxidants in liposomes (21).

![Figure 5. Cytotoxicity of gel-like ME, liquid o/w ME and o/w ME thickened with carbomer according to MTT assay. SDS was used as a positive control.](image)

* p<0.05 compared to control

All ME show good cell viabilities (larger than 75%). No significant difference (p<0.05) among formulations was seen, which was expected as all systems contained approximately
the same amount of surfactants, the components of ME that are usually associated with irritation. All tested ME performed considerably better (p< 0.001) than the positive control (SDS solution in the same final concentration as ME), a standard irritant in cell culture models. The results indicate that by selecting pharmaceutically acceptable ingredients it is possible to produce low toxicity ME.

The effect of ME on cell morphology was examined by fluorescence microscopy. On Fig. 6 representative pictures of control cells and cells treated with ME are shown.

![Fig 6. Fluorescent transmission micrographs of A) control HEK293 cells B) HEK293 cells treated with o/w ME.](image)

As no difference among different ME was observed only a picture of cell culture treated with o/w ME is represented. No difference in morphology of cell nuclei was observed. However, changes in cytoskeletal architecture reflected in different organisation of actin fibres that could have as a consequence changes in membrane integrity has been observed, probably due to a presence of surfactant molecules. This changes in cell morphology helps to explain the ≈25% loss of cell viability in cell culture after the exposure to ME.

**Conclusion**

Microemulsion gel with temperature sensitive rheological behaviour has been proved an effective and non-irritant vehicle with functionally suitable consistency for simultaneous topical delivery of a hydrophilic vitamin C and a lipophilic vitamin E.

**Acknowledgement**

Authors would like to thank prof. Hans E. Junginger for helpful discussion. We are grateful to assist. Karmen Teskač for her assistance with fluorescence microscope.
This work was supported by a grant of Slovenian Research Agency.

References
3.5. Chapter 5

Key role of structure in microemulsion skin irritation and phototoxicity potential

*Vpliv strukture mikroemulzij na njihovo sposobnost draženja kože in fototoksičnost*

Branka Rozman, Camille Migdal, Françoise Falson and Mirjana Gašperlin

Sent for publication.
Abstract

Skin irritation and phototoxicity potentials of several microemulsions (ME), all composed of approximately 30% Tween 40/ Imwitor 308 surfactant mixture, but having different structure - either droplet-like (o/w ME, o/w ME carbomer, w/o ME and w/o ME white wax) or lamellar (gel-like ME) - were examined. Two different *in vitro* methods were used: MTT test (performed either on reconstructed human epidermis (RHE) or NCTC 2544 cells) and pig ear test. All tested formulations were less irritant than commercial ME. Neither test showed the difference among ME with droplet-like structure. Pig ear test and MTT test performed on RHE indicated that gel-like ME is more irritant than other tested ME, whereas MTT test on NCTC cells showed no difference among formulations. The latter was explained by destruction of ME structure upon its dilution in cell medium. The results of phototoxicity test again showed the increased potential of gel-like ME to cause adverse effects on skin. It can be concluded that ME consisting of the same amount of identical surfactants but having different structures can exhibit different dermal toxicity.

**Key words:** microemulsion, surfactant, structure, skin irritation, keratinocyte cell cultures, reconstructed human epidermis, phototoxicity
Povzetek
Vrednotili smo draženje kože in fototoksičnost mikroemulzij (ME), ki vse vsebujejo približno 30% zmesi površinsko aktivnih snovi Tweena 40 in Imwitorja 308, vendar imajo različno mikrostrukturo - bodisi kapljično (o/v ME, o/v ME karbomer, v/o ME in v/o ME beli vosek) ali lamelarno (gelirana ME). Uporabili smo dve različni in vitro metodi: MTT test (narejen bodisi na EpiSkinovih celičnih kulturah bodisi na NCTC 2544 celični liniji) ter test barijerne funkcije prašičjega ušesa. Vse testirane ME so v primerjavi s komercialno dostopno ME manj dražile kožo. Noben test ni pokazal razlike med ME s kapljičnimi strukturami. Test na prašičjem ušesu in MTT test na EpiSkinovem modelu sta pokazala, da gelirana ME bolj draži kožo kot ostale ME. Nasprotno, MTT test na NCTC 2544 celicah ni zaznal razlike med testiranimi ME, kar je verjetno posledica uničenja strukture ME ob redčenju s celičnim medijem. Rezultati fototoksičnosti so poudarili povečan potencial gelirane ME za povzročanje škodljivih učinkov na koži. Zaključimo lahko, da lahko farmacevtske oblike, ki vsebujejo enake površinsko aktivne snovi v isti koncentracijah, a imajo različne strukture, izkazujejo različne potenciale za draženje kože.

Ključne besede: mikroemulzija, struktura, površinsko aktivne snovi, draženje kože, celične kulture keratinocitov, fototoksičnost
Introduction

Microemulsions (ME) are low viscosity, isotropic, thermodynamically stable, transparent systems of oil, water and surfactant, frequently in combination with cosurfactant, with a droplet size usually in the range of 1-140 nm. They form spontaneously. These versatile systems are currently of great interest in topical drug delivery because of their potential to incorporate a wide range of drugs due to the presence of both hydrophilic and lipophilic domains. They can also protect drugs against oxidation, modify their release and enhance skin deposition. ME exhibit a number of structures, depending on their composition, varying from dispersed droplets of different shape and size to bicontinuous phases [1]. Mixtures of oil, water and surfactants can also form crystalline hexagonal, cubic or lamellar phases that are distinguished by high viscosity and anisotropy [2]. Since these systems do not fulfil the criteria for ME (low viscosity, isotropy), but retain other ME properties such as thermodynamic stability, spontaneous formation and transparency, they are often termed ME gels [3]. The viscosity of ME can also be increased by adding thickening agent, which increases the viscosity of the system without affecting its stability or spontaneous formation [4]. Thickened ME are usually composed of two distinct structural elements, a network formed by thicker in the outer phase that coexists with the microemulsion droplets [5].

The classical in vivo evaluation of the irritancy potential by the Draize test has been widely criticized. Ethical concerns about the use of laboratory animals and the need for more efficient and cost-effective preclinical methods have led to the development of a great number of in vitro alternatives. Since no single in vitro model is able to mimic the visible symptoms of irritation that occur in vivo, more sophisticated parameters that describe skin reaction to topically applied product (e.g. cell viability, interleukin release or transepidermal water loss - TEWL) have been evaluated and a combination of methods is preferred [6, 7].

Human keratinocytes have become the focus of attention in evaluating skin irritation, by virtue of their epidermal location, their importance in maintaining the integrity of the stratum corneum barrier, and their ability to produce a wide range of inflammatory mediators [6].

Three-dimensional cell cultures of normal human keratinocytes (reconstructed human epidermis – RHE), consisting of multilayer differentiating keratinocyte cultures growing on different matrices, constitute the model of choice for skin irritation testing. They imitate the architecture of normal human skin, enabling direct topical application of finished products or compounds with low aqueous solubility [8]. The commercially available Episkin® model is
the first RHE officially validated by the European centre for validation of alternative methods (ECVAM) as a full replacement for the Draize test for skin irritation [9].

Despite the well proven capacity of RHE to distinguish between skin irritants and non-irritants, other less sophisticated skin cell cultures are often used, since they are more economic and simple to grow. The concentrations inducing irritant responses in monolayer keratinocyte cell cultures are usually several orders of magnitude lower than in RHE [7]. HaCat and NCTC 2544 monolayer skin cell cultures have nevertheless been proved useful predictors of skin irritation [10-13].

Other promising *in vitro* models for skin toxicity are the rat skin transcutaneous electrical resistance (TER) assay, the pig ear test and the mouse skin integrity function test (SIFT). These methods have been evaluated independently for identifying skin corrosives or irritants, but only TER was successfully validated for skin corrosion testing [14, 15].

The assessment of photoirritancy of new dosage forms is essential in order to assess possible damage to skin following sun exposure. UVA and visible light are known to be the main wavelength regions involved in the photosensitivity reaction [16]. In the past the test has usually been performed on rabbits after application of the test material and exposure to UV light. Currently available human skin models have been sufficiently developed to allow photocotoxicity testing of pharmaceutical formulations. The Episkin® model has undergone several prevalidation studies that have confirmed its suitability in testing, with very high specificity and accuracy, the photocotoxic potential of chemical substances [16-18].

The purpose of this work was to assess the influence of different ME structures on their potential for skin irritation and photocotoxicity. Five topical ME, all consisting of approx. 30% Tween® 40 / Imwitor® 308 surfactant mixture, but having different structures and consistency, were tested using a set of *in vitro* methods. The MTT test was used as an endpoint to evaluate the cell viability of RHE (Episkin® model) and of the keratinocyte cell line (NCTC 2544). Changes in morphology of NCTC 2544 cells following exposure to ME were visualized by light and fluorescence microscopy. The results obtained on cell cultures were correlated with changes in barrier function of isolated pig ear evaluated by TEWL. Phototoxicity was assessed by exposing ME-treated RHE to a non-cytotoxic UVA dose.
Materials and methods

Preparation of formulations
Isopropyl myristate was obtained from Fluka Chemie, Switzerland and used as the lipophilic phase. Tween 40 – polyoxyethylene (20) sorbitan monopalmitate (Fluka Chemie Switzerland) – was used as surfactant and Imwitor 308 – glyceryl caprylate (Condea, Germany) – as cosurfactant. Purified water was used as the hydrophilic phase.
Both types of non-thickened ME (o/w and w/o) and gel-like ME differed only in quantitative composition (w/w percent), as shown in Table 1. White wax (Pharmachem, Slovenia) and carbomer (Carbopol 974 PNF, BF Goodrich, Belgium) were used as thickening agents for w/o ME and o/w ME, respectively.
All ME were prepared in the same way. The surfactant and cosurfactant were blended in a 1:1 mass ratio to obtain the surfactant mixture. Isopropyl myristate and water were then added. Components were mixed with a magnetic stirrer for 5 minutes at room temperature. In the case of thickened ME, carbomer was added to o/w ME and stirred with a magnetic stirrer for 30 minutes at room temperature, whereas white wax was added to w/o ME and heated to 55°C with continuous stirring. Thickened ME were left covered for at least 24 hours before use.

Table 1. Composition of ME (w/w %).

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>w/o ME</th>
<th>o/w ME</th>
<th>gel-like ME</th>
<th>w/o ME white wax</th>
<th>o/w ME carbomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>10.0</td>
<td>45.0</td>
<td>60.0</td>
<td>9.0</td>
<td>43.9</td>
</tr>
<tr>
<td>Isopropyl myristate</td>
<td>60.0</td>
<td>25.0</td>
<td>10.0</td>
<td>54.0</td>
<td>24.4</td>
</tr>
<tr>
<td>Tween 40</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
<td>13.5</td>
<td>14.6</td>
</tr>
<tr>
<td>Imwitor 308</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
<td>13.5</td>
<td>14.6</td>
</tr>
<tr>
<td>White wax</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>10.0</td>
<td>/</td>
</tr>
<tr>
<td>Carbomer</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Commercial product (Diverin® gel, Lek Pharmaceuticals) was tested for comparison.

Characterization of formulations
Hydrodynamic radius and polydispersity of the ME structures were measured by photon correlation spectroscopy (PCS) at 20°C, using a Nano ZS, Malvern Instruments. Samples were thermostated 5 min before measurement. Viscosity was determined with an SV-10
Vibro Viscosimeter (A&D Company, Japan) and refractive index with a Carl Zeiss refractometer.

Cell cultures
Episkin® kits containing 12 cell cultures with 1.07cm² surface area on the nutrient gelatine gel, maintenance medium, assay medium and 12-well plates were provided by Episkin (Lyon, France).
Normal human undifferentiated keratinocytes, NCTC 2544, were from Interlab Cell line collection (Genoa, Italy) and maintained in Eagle’s minimum essential medium with Earle’s balanced salt solution (Sigma, Germany), supplemented with 10% foetal bovine serum (Gibco, Invitrogen, USA), 100 U/ml penicillin – streptomycin mixture (Sigma, Germany), 2mM L-glutamine (Sigma, Germany) and 1% non-essential amino acids (Sigma, Germany) at 37°C in humidified atmosphere containing 5% CO₂. Cells were subcultured with Trypsin/EDTA (Sigma, Germany) when they reached 80-90% confluence.

Cell toxicity test
The effect of tested formulations on cell viability was evaluated by MTT assay according to the method of Mosmann [19] with slight modifications. In this method yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a tetrazole, is metabolically reduced to purple formazan by succinate-tetrazolium reductase system which belongs to the mitochondrial respiratory chain and is active only in viable cells. Since the amount of formed formazan is directly proportional to the number of living cells in culture, the intensity of produced colour is an indication of cell viability.
RHE were, upon receipt, incubated overnight in the maintenance medium at 37°C, 5% CO₂ and saturated humidity. The next day 50 mg of test product was applied to the RHE surface and the cells incubated for 4h on the 2 ml of assay medium. Afterwards the surface of the culture was rinsed several times with a gentle stream of PBS. 2 ml of MTT (Sigma Aldrich, Switzerland) dissolved in the assay medium (2mg/ml) were put in each plate and the cells incubated at 37°C and 5% CO₂ for 3h. MTT was extracted from the cells by transferring the culture into 2.6 ml of acidic isopropanol (Fisher Scientific, France) overnight at room temperature. The next day absorbances were read at 540 nm. All tests were done in triplicate. Direct reduction of MTT by test products was checked as follows: 25 mg of the test product was added to 1 ml of MTT solution (1mg/ 1ml of medium). The solution was incubated in the dark for 60 min at 37°C. If no solution turned to blue/purple, it was assumed that the test products cannot reduce MTT.
For NCTC 2544 cells, a modified procedure adapted to monolayer immersed cell cultures was followed [20]. Cells were seeded in 96-well plates (2.5x10⁴ cells/well, 100 μl of cell culture medium) and, when appropriate confluence was reached, 10 μl of test dispersion was added for 4 hours. Test dispersions were prepared by diluting ME in PBS to achieve the final concentration of ME (450 μg/ml). In negative control experiments cells were treated with PBS alone. After 1 hour 11 μl of the MTT dissolved in the assay medium (5 mg/ml) were placed in each well and cells were incubated at 37°C and 5% CO₂ during 3h. The insoluble purple formazan product was extracted from the cells by acidic isopropanol as a coloured solution. The absorbance was measured at 540 nm in an automated plate reader (Safire²™ Tecan, Switzerland). Average cell viability of treated cells was expressed as the percentage of absorbance of non-treated cells as follows:

\[
\text{Cell viability} = \frac{(A_S - A_{S0})}{(A_C - A_{C0})};
\]

where \(A_S\) is the absorbance of treated cells, \(A_C\) the absorbance of untreated cells (control), \(A_{S0}\) the absorbance of ME diluted in medium without cells and \(A_{C0}\) the absorbance of the medium alone [21]. All tests were done in sixplicate in three independent assays.

**Morphological examination of NCTC 2544 cells**

The morphology of NCTC 2544 cells was examined by inverted light (Olympus CKX41, Japan) and fluorescence (Olympus IX81, Japan) microscopy. Cells were plated on square glass cover slips and incubated in 6-well plates overnight. Following the incubation with ME (final concentration 450 μg/ml of cell culture medium), cells were examined under the inverted microscope.

For fluorescence microscopy, cells were fixed with ice cold 4% paraformaldehyde in PBS pH 7.4 for 10 min and permeabilized for 10 min in 0.1% Triton X-100 (both Sigma, Germany). Cell nuclei were visualized by staining with a DNA intercalating dye Hoechst 33342 (Riedel de Haen, Germany, 5 μg/ml) for 30 min in the dark. Actin fibres were stained with Phalloidin TRITC (Sigma, USA, 1:40). After staining, the cover slips were removed from the wells, mounted on a slide and viewed using 360 nm/ 420 nm (Hoechst) and 535 nm/ 635 nm (Phalloidin – TRITC) excitation/emission filter sets.

**The non-perfused pig ear test for skin irritance**

Pig ears (obtained from a local abattoir) were washed under tap water and whole skin was carefully removed from the underlying cartilage. The skin was mounted on Franz cells containing 8 ml of 0.9% aqueous solution of NaCl (Prolabo, France). During the experiments
the temperature was kept at 25±1°C. The Franz cells were placed in a water bath overnight and the next morning TEWL was measured with an MPA 5 Tewameter®, Courage Khazaka, Germany. Approximately 50 mg of the formulations tested were then placed on the surface of the skin. Following a 4-hour exposure of the pig ear to test material, formulations were removed and the surface was cleaned with 2x 300 μl of PBS buffer pH 7.4 and dried with a cotton swab. The TEWL was again measured 4 hours after the removal of formulation.

**Phototoxicity test**

Keratinocytes were incubated overnight. The next morning the maintenance medium was replaced by test medium. Three formulations were tested: w/o ME, o/w ME and gel-like ME, all in triplicate. Approximately 50 mg of tested product was applied to each well. The keratinocytes with formulations were incubated at 37°C and 5% of CO2 for 15 minutes and then immediately exposed to a dose of 27 Jcm² UVA light for 15 minutes at room temperature without covers. A Spectroline ENF-260C/FE 0.17 ampere UVA lamp, λ=365 nm was used. The non-irradiated cultures treated with formulations were first incubated at 37°C and 5% CO2, and then placed in the dark at room temperature for 15 min. Control RHE were not exposed either to formulation or to UVA light.

The formulations were then removed from the keratinocytes and the cells rinsed 6x with 200 μl of PBS. The cells were fed with fresh culture medium, placed in the incubator and cell viability (as described in section Cell cytotoxicity test) measured after 24 hours.

**Data analysis**

The influence of formulation on cell viability and pig ear TEWL was evaluated by one way ANOVA with KaleidaGraph® software package. Bonferroni’s test was used for post-hoc comparisons. Significance was tested at the 0.05 level of probability.

**Results and discussion**

**Characterization of ME**

All the ME ingredients are widely used in pharmaceutical formulations and are generally regarded as non-toxic and non-irritant excipients. They all exhibit low acute oral toxicity in animals (Table 2) and are included in the FDA inactive ingredient data base [22]. Data for topical toxicity were found only for isopropyl myristate (Table 2).
Table 2. Toxicological information and regulatory status for the ingredients of ME

<table>
<thead>
<tr>
<th>INGREDIENT</th>
<th>TOXICITY DATA*</th>
</tr>
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<tbody>
<tr>
<td>Isopropyl myristate</td>
<td>Oral, mouse, ( LD_{50} ) 49700 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Skin, rabbit, ( LD_{50} ) 5000 mg/kg</td>
</tr>
<tr>
<td>Tween 40</td>
<td>Oral, rat, ( LD_{50} ) 38400 mg/kg</td>
</tr>
<tr>
<td>Imwitor 308</td>
<td>No toxicology data available</td>
</tr>
<tr>
<td>Carbomer</td>
<td>Oral, mouse, ( LD_{50} ) 4600 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Oral, rat, ( LD_{50} ) 4100 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Oral, guinea pig, ( LD_{50} ) 2500 mg/kg</td>
</tr>
<tr>
<td>White wax</td>
<td>Oral, rat, ( LD_{50} ) &gt;5000 mg/kg</td>
</tr>
</tbody>
</table>

*\( LD_{50} \) (median lethal dose) were obtained from the MSDS of each component provided by the manufacturer.

ME were characterized physically by absolute viscosity and droplet size. o/w ME and w/o ME have relatively low viscosity, making proper skin application difficult (Table 3). Both systems were therefore thickened, o/w ME with carbomer and w/o ME with white wax, increasing the viscosities by 2.5 and 4-fold, respectively. Gel-like ME exhibited a viscosity comparable to those of w/o ME white wax and o/w ME carbomer. The viscosity of the commercial product was the highest, approaching the semisolid state.

Table 3. Absolute viscosities of tested formulations at 20°C.
The size of droplets was measured by PCS. Table 4 shows the hydrodynamic radius and polydispersity index of the ME. The latter is listed only for samples that had a single peak in the intensity chart.

Table 4. Droplet size and polydispersity index (PI) of ME at 20°C.

<table>
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<th>FO</th>
<th>D</th>
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<tr>
<td>RM</td>
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<td>E</td>
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</tbody>
</table>

(\( n \)
\( m \))

w/o 5 0
M   8 1
E   6 7
+   0
-   
0
. 0 2
o/ 1 0
w . .
M 2 2
E 3 4 + 2 /
- 0 . 0 7
ge / /

I-
lik
e
M
E
o/ 0 /
w .
M 8
E 7
car
rb +
o /
m -
er 0 .
0 4
1 4
5
1 +
/ /
- 8
The very small size of the o/w and w/o ME aggregates (Table 4) is optical. o/w ME was carborner an additional peak due to the presence of carbomer an al transparency. When thickened with M polymer aggregates M additional peak due to the presence of E was observed. However, droplet size was comparable to that of o/w ME. The addition of white droplet size of w/o E wax increased the remained stable / [23]. Interestingly, no structures in the 10000 nm were observed in gel-like ME, although the scattering of the sample was good. As described previously, a gel-like ME that differs from o/w and w/o ME only in the water/oil ratio is composed of double-layer lamellar phases that cannot be measured by PCS [24]. Podlogar and co-workers hypothesized that lamellar phases are the consequence of strong hydration of surfactant chains that allow formation of hydrogen bonds, resulting in increased interactions between surfactants [25, 26]. The commercial product also exhibited two peaks, the smaller belonging to droplets of inner phase and the larger to polymer that was added to increase the viscosity of the system.

Cytotoxicity of formulations on RHE

\[
\begin{array}{ccc}
\text{w/o} & 2 & 0 \\
\text{o} & 9 & . \\
\text{ME} & 0 & 4 \\
\text{w} & / & 2 \\
\text{hit} & - & \\
\text{e} & 1.7 & \\
\text{w} & 7 & \\
\text{ax} & 6 & /
\end{array}
\]
The direct effect of the formulations on the keratinocytes was investigated by the MTT test. Surfactants can disrupt cell membranes resulting in the release of cytoplasm and consequent cell death [7].

None of the tested formulations were able to reduce the MTT solution directly. Non-treated cell cultures were tested at time zero and were set to represent 100% viability. All other results are expressed as a percentage of that cell viability (Fig. 1).

![Graph showing cell viability of different ME formulations](image)

* p<0.05 compared to o/w ME
# p<0.05 compared to w/o ME

All the formulated ME were considerably less toxic than commercial ME. No difference between o/w and w/o ME was observed, which is not surprising, since both systems contained the same amount of surfactants, that are usually associated with skin irritation. However, gel-like ME also contained the same amount of the same surfactant mixture, but was more cytotoxic. The differences in cell viability between these three formulations could not be attributed to cytotoxicity of their other ingredients, since neither isopropyl myristate (oily phase of ME, cell viability 87.3%) nor water (aqueous phase of ME, cell viability 102%) is cytotoxic. The only possible explanation is the different structure of the vehicles – o/w ME and w/o ME that both had droplet-like ME had the same cytotoxicity, whereas gel-like ME, consisting of lamellar phases, was more cytotoxic.

It is generally known that skin irritation is related not simply to the total concentration of the surfactants but rather to the combination of the surfactants used [27]. However, to the best of
our knowledge, it has been never reported that even the same total concentration of identical surfactants can trigger a different cellular response.

The addition of carbomer and white wax to o/w and w/o ME respectively did not influence the cell viability when compared to non-thickened ME. It can be concluded that these two thickeners do not potentiate the irritancy of the formulations.

**Cytotoxicity of formulations on NCTC 2544 cells**

The MTT experiment was repeated on NCTC 2544, a human keratinocyte cell culture. Results of cell viability after the exposure to NCTC 2544 cells are shown on Fig.2.

![Percent NCTC 2544 cell viability determined with MTT test after 4-hour exposure of cell cultures to tested formulations.](image)

* p<0.05 compared to o/w ME  
# p<0.05 compared to w/o ME  

Commercial ME was again shown to be significantly more toxic than other tested ME. However, in contrast to the results obtained on RHE there was no difference between the other formulations tested, indicating that gel-like ME has the same skin irritation potential as o/w and w/o ME. This difference between the results obtained on the two different skin cell culture models can be explained by their different morphology. RHE, having a multilayered differentiated epidermis produced by exposure of keratinocytes to air, allows the application of non-diluted formulation to its surface, mimicking perfectly the application of topical vehicle on skin *in vivo*. NCTC 2544, on the other hand, are typical submerged cell cultures
and the formulation can be dosed only if diluted in cell medium. Upon dilution the structure of ME is destroyed, as proved by droplet size measurements. When ME were diluted in water to the same final concentration as the one used in experiments on NCTC 2544 cell cultures, o/w ME and gel-like ME consisted of small oily droplets dispersed in water and their sizes were approximately the same (45.7+/−1.6 nm for o/w ME and 48.5+/−3.6 nm for gel-like ME), leading to the same effects on cell cultures and confirming our theory that ME structure is the factor that influences skin irritation potential of vehicle.

The addition of thickeners (carbomer, white wax) to ME was again confirmed not to be significant.

**Cell morphology**

One of the advantages of NCTC 2544 cells is that, since they are monolayer epithelial adherent cells, they are easy to visualize. No differences were observed between gel-like ME, o/w ME, w/o ME, w/o ME white wax and o/w ME carbomer; a representative picture of cell culture treated with gel-like ME is therefore presented (Fig. 3B). Alterations in cellular morphology following exposure of cells to commercial ME at the same concentration as gel-like ME were evident (Fig. 3C), compared with PBS-treated cells used as control (Fig. 3A). Many of the cells treated with the commercial ME were detached.

Fig. 3. Representative live-cell light transmission micrographs of NCTC 2544 cells showing their morphology (phase annulus of 10x). Cells were treated for 4 hours with the addition of: A – PBS (control) B – gel-like ME; C – commercial ME.

Further, alterations in nuclear morphology were visualized by staining DNA with intercalating dye Hoechst 33342 and actin fibres were examined by staining with Phalloidin-TRITC (Fig. 4).
Again, no differences between control cells (Fig.4A) and ME consisting of Tween 40/Imwitor 308 surfactants were observed. A representative micrograph of NCTC 2544 cells treated with gel-like ME is shown (Fig.4B). Striking differences were seen in cells treated with commercial ME. Many cells detached and, after washing, large areas of the cover slip contained no cells. The cells that remained had totally different morphology from non-treated cells. Cell nuclei were smaller, although no differences in their shapes were seen. Moreover, alterations in cytoskeletal architecture, reflected in different organization of actin fibres, were observed, indicating the potential cytotoxicity of commercial ME, already confirmed by MTT assays.

**Pig ear test**

TEWL provides a sensitive measure of skin barrier function [28]. Irritants entering the *stratum corneum* can lead to delipidation and protein denaturation, resulting in loss of barrier function and consequently higher TEWL [7]. The results are expressed as an absolute increase in TEWL following the exposure of pig skin to the formulation. We focused on ME containing Tween 40/Imwitor 308 surfactant mixture in order to determine whether the outstanding properties of gel-like ME could be detected using this model. Significant differences in effects of different formulations were observed (Fig.5).
Fig. 5. The change in TEWL of pig ear skin on exposure to ME formulations for 4 hours.

* p<0.05 compared to o/w ME

# p<0.05 compared to w/o ME

When comparing the three formulations that contained no thickener (w/o, o/w and gel-like ME) no significant difference was observed between o/w and w/o ME, whereas gel-like ME provoked a considerable increase in TEWL. The results confirm those from the MTT test in the RHE model, and indicate that gel-like ME has a higher irritation potential than o/w or w/o ME. The differences are due to the different structures of the formulations, which influence not only their ability to disrupt the stratum corneum barrier (increased TEWL) but also their tendency to cause cell death (decreased cell viability, measured by MTT test).

Thickened ME (o/w ME carbomer and w/o ME white wax) induced increases in TEWL than non-thickened o/w and w/o ME, but in all cases the TEWL was higher than before treatment. Thickened formulations induced smaller levels of TEWL than non-thickened, which could indicate that the washing of the skin surface did not completely remove the occlusive thickeners [28] from the skin.

**UVA phototoxicity test**

The results of the phototoxicity assay using RHE are given in Table 5 as the mean % of control MTT conversion. The control was exposed neither to UVA light nor to formulation.
Table 5. Results of the phototoxicity assay. Cell viability is expressed as % of control MTT conversion in the presence and absence of UVA radiation. The exposure time was 0.5 h and UVA dose 27 J/cm².

<table>
<thead>
<tr>
<th>FORMULATION</th>
<th>% OF CELL VIABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UVA- exposed RHE</td>
</tr>
<tr>
<td>w/o ME</td>
<td>74.2 +/- 5.9</td>
</tr>
<tr>
<td>o/w ME</td>
<td>70.8 +/- 7.3</td>
</tr>
<tr>
<td>gel-like ME</td>
<td>28.2 +/- 4.6*</td>
</tr>
</tbody>
</table>

*p<0.05 compared to non UVA-exposed RHE.

We tested only the formulations (o/w ME, w/o ME and gel-like ME) that had been shown to be the most interesting on the basis of irritation studies. Exposure of the non-treated cell cultures to UVA dose of 27 J/cm² did not decrease cell viability (105% viability compared to non exposed control), indicating that the UVA dose was non-cytotoxic, which is in agreement with published statements that the Episkin® model can be exposed to physiological UVA doses [16]. Thus the measured cytotoxicity reflects either the effects of formulation alone or the combined effects of formulation and UVA light on RHE viability. A test formulation is considered to be phototoxic if there is an increase in its toxicity in the presence of UV light of more than 25% [17]. Taking into account this criterion, the results demonstrate the absence of phototoxic potential for non-thickened o/w ME and w/o ME. However, for gel-like ME, the difference in cell viability between irradiated and non-irradiated epidermis exceeded 25%, classifying gel-like ME as photoirritant. It can be concluded that the structure of the vehicle also influences its skin phototoxicity potential.

Conclusions
The dermal irritation predicted from the cytotoxic effects in RHE and NCTC 2544 cells has demonstrated that all ME based on a Tween 40/ Imwitor 308 surfactant mixture are significantly less toxic than commercial ME. RHE is shown to be a better model for skin irritation than NCTC 2544 cells, since the former allows topical application of undiluted product, and hence the possibility of studying the
influence of the structure of the vehicle on its irritation potential. On the basis of the RHE irritation potential it was shown that formulations consisting of the same amounts of identical surfactants can differ in their cytotoxicity; ME consisting of lamellar structures were more irritant than ME composed of droplets. Results of the pig ear test correlated with RHE toxicity tests for ME without thickener. The results of the phototoxicity test further stressed the increased tendency of ME with lamellar structures to cause adverse effects on the skin. Isotropic liquid and thickened o/w and w/o ME were confirmed as suitable vehicles for skin application.

Acknowledgements

Kits of reconstructed human epidermis were partially donated by Episkin, Lyon, France. This work was supported by grants from the French National Education Ministry, Université Lyon 1 and the Slovenian Research Agency. Asist. Karmen Teskač is acknowledged for her assistance with fluorescence microscopy.

Literature

4. Conclusion
This work focused on development and characterisation of topical ME as vehicles for simultaneous delivery of vitamins C and E.

Viscosity of liquid hydrophilic (o/w) and lipophilic (w/o) ME, both composed of the same pharmaceutically acceptable ingredients, was optimised for topical application by addition of suitable thickener. Carbomer and mixture of xanthan and alginate revealed as suitable thickeners for o/w ME and white wax for w/o ME. Interestingly, hydrophilic colloidal silica could be used as a thickening agent for both types of ME. Addition of thickener in concentrations that led to a consistency appropriate for topical application changed the rheological behaviour of ME from ideal Newtonian to thixotropic and in some cases (white wax, mixture of xanthan and alginate) influenced the structure of a system. Transparent microemulsion gel (called gel-like ME) was developed by increasing water content of o/w ME as an alternative and innovative solution for viscosity problem. Gel-like ME exhibited at room temperature rheoplectic rheological behaviour, rarely seen in pharmaceutical systems. The analysis of its microstructure has revealed that it consists of lamellar phases.

The incorporation of vitamins E and C into o/w, w/o and gel-like ME enhanced their stability. Gel-like ME was found to be the best protective system for both vitamins. The presence of vitamin C improved UVA stability of vitamin E. Addition of a thickener to ME influenced the stability of vitamins by changing either oxygen solubility in the outer phase or structural organization of ME. Nevertheless thickened ME, the exception being ME thickened with xanthan-alginate mixture, increased the stability of vitamins compared to solutions.

When percutaneous absorption of vitamins C and E from non-thickened ME, ME thickened with colloidal silica and from vitamin solutions was studied using reconstructed human epidermis as an alternative model for skin absorption studies, ME revealed as more efficient vehicles than solutions. If vitamins were loaded in outer phase of ME (vitamin C in o/w ME and vitamin E in w/o ME) greater absorption was observed compared to their absorption from ME where they were incorporated in inner phase (vitamin C in w/o ME and vitamin E in o/w ME), meaning that, depending on the hydrophilicity of the drug, the same formulation can act as a permeation enhancer or retarder. However, vitamin C was less affected by its location in the vehicle. Consequently, w/o ME was confirmed better vehicle for simultaneous skin delivery of vitamins C and E than o/w ME. Colloidal silica enhanced the deposition both
vitamins in the RHE, not only by perturbation of its barrier function but also by changing the pH of the vehicle and hence vitamin C partition from ME in RHE.

The effects of colloidal silica that drastically enhanced the deposition of vitamins in reconstructed human epidermis were further studied on isolated pig ear skin as established model for skin absorption studies. The enhancing effect of colloidal silica on vitamins’ skin bioavailability was confirmed. Dual influence of colloidal silica in ME was observed: it changed not only delivery characteristics of ME (vitamin solubility, partition and release) but also skin properties, mainly due to its massive accumulation in stratum corneum. Comparison of cell viability of RHE treated with ME thickened with colloidal silica and aqueous dispersion of colloidal silica showed no considerable difference.

The absorption studies have also indicated that gel-like ME possesses temperature sensitive rheological properties. When this phenomenon was examined in detail, the temperature-driven changes in its microstructure were confirmed by using rotational rheometry, viscosity measurements and droplet size determination. The release studies have shown that the vitamins’ release rates at skin temperature from gel-like ME were comparable to those from o/w ME and were much faster and more complete than from o/w ME conventionally thickened with polymer (carbomer). However, concerning the effectiveness in isolated pig skin delivery of both vitamins o/w ME was found the most appropriate, followed by gel-like ME and by o/w ME thickened with carbomer.

Finally, the irritation potential of ME was assessed in comparison to commercially available ME. Two different models were used: reconstructed human epidermis as ECVAM validated model for skin irritation testing and NCTC 2544 human keratinocyte cell cultures. Both models confirmed our ME less irritant than commercial product. Surprisingly, using reconstructed human epidermis gel-like ME revealed to be more irritant than other ME despite the fact that all ME without thickener contained the same amount of surfactants, substances usually associated with skin irritation. On the contrary NCTC 2544 cells did not distinguish the difference among o/w, w/o and gel-like ME. Since ME were applied directly on surface of reconstructed human epidermis, but had to be diluted prior the application on NCTC 2544 cells, it was concluded that apart from surfactant content the structure of vehicle is very important for skin acceptability of final product. The results obtained on reconstructed human epidermis correlated well with pig ear TEWL.
In summary, ME have been proved effective and non-irritant vehicles with functionally suitable consistency for simultaneous topical delivery of a hydrophilic vitamin C and a lipophilic vitamin E.
III. Appendix
Bibliography

Articles

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Curriculum vitae

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International mobility
2006-2007 Master programe M2 Recherche: Physiologie, Biodisponibilite, Pharmacologie cutanee; University of Lyon, Faculty of Pharmacy, Lyon, France
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Conference attendance and in-depth scientific training and education

- Galenos – towards an European Euro PhD in advanced Drug Delivery, Galenos workshop, March 10-12, 2005, Ljubljana, Slovenia
- PAT – Process Analytical Technologies in Pharmaceutical and Chemical Industry, June 9, 2005, Ljubljana
- From gene to recombinant drug delivery, June 23, 2005, Ljubljana
- Socrates Intensive Programme "Cell Culture and in vitro Models for Drug Absorption and Delivery ", March 1-10, 2006, Saarbrucken, Germany - active participation
- New excipients and novelties in their application, June 8, 2006, Ljubljana
- Socrates Intensive Programme "Skin barrier function: Pharmaceutical and Cosmetic applications", September 15-29, 2006, Lyon, France
- 12eme Rencontre Pharmapeptides "Vaccins", November 23, 2006, Archamps, France
- Congres annuel de recherche dermatologique – CARD, June, 21-23, 2007, Lyon, France – active participation
- European Intensive Program - Galenos Course, Skin Barrier Function : "Cutaneous Absorption and Environmental Factors", September, 16 – October, 2, 2007, Lyon, France – active participation
- Innovation in Drug Delivery: From Biomaterials to Devices, September, 30 – October, 3, 2007, Naples, Italy – active participation
- 6th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, April 7-10, Barcelone, Spain – active participation
- Simpozij ob 33. skupščini Slovenskega farmacevtskega društva, May, 15-17, 2008 Portorož, Slovenija
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