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Assessment of Nano-enabled Technologies in Cosmetics

Part of the “Better control of nano” initiative 2012-2015

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Title:

Assessment if Nano-enabled Technologies in Cosmetics

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Abbreviations List

Abbreviation	Definition
AES	Auger Electron Spectroscopy
BCOP	Bovine corneal opacity and permeability
BSA	Bovine Serum Albumin
BSA	Bovine Serum Albumin
BSI	British Standards Institution
CEN	European Committee For Standardization
CLSM	Confocal Laser Scanning Microscopy
CM	centimetre
CoQ10	Coenzyme Q10
Danish EPA	Danish Environmental Protection Agency
DNCB	Dinitrochlorobenzene
EDX	Energy Dispersive X-Ray Spectroscopy
EELS	Electron Energy Loss Spectroscopy
ENP	Engineered Nanoparticles
EPA	Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
FDC	Franz-type Diffusion Cell
FITC	Fluorescein Isothiocyanate
FLIM	Fluorescence Lifetime Imaging Microscopy
H_2O	Water
HEK	Human epidermal keratinocytes
HEMA	Poly-2-hydroxyl methacrylate
HPLC	High-performance liquid chromatography
HRS	Hours
ICC	Indocarbocyanine
ICP	Inductively Coupled Plasma
ID	Intradermal
IP	Intraperitoneal
IR	Infrared
ISO	International Organization For Standardization
JRC	Joint Research Centre
LD_{50}	Median Lethal Dose
LLNA	local lymph node assay
M	Meter
MG	Milligram

Abbreviation	Definition
MS	Mass Spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Not ascertained
NIOSH	National Institute Of Occupational Safety And Health
NIR	Near Infrared
NLC	Nanostructured Lipid Carrier
NM	Nanometre
NMSC	Non-melanoma Skin Cancer
NP	Nanoparticle
NR	Nile Red
NRU	Neutral Red Uptake
O/W	Oil in Water
OECD	Organisation For Economic Cooperation And Development
OECD	Organisation for Economic Co-operation and Development
OECD WPMN	Organisation for Economic Co-operation and Development Working Party On Manufactured Nanomaterials
OES	Optical Emission Spectrophotometry
OMC	Octyl Methoxycinnamate
PBPK	Physiologically based pharmacokinetic
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Pol(lactic-co-glycolic) acid
PSD	autosomal recessive generalized Peeling Skin Disease
RBC	Red Blood Cell
RHE	Reconstituted Human Epidermis
ROS	Reactive oxygen species
RP	Retinyl Palmitate
RSV	Resveratrol
RSV	Resveratrol
SAP	Sodium Ascorbyl Phosphate
SB	*Stratum Basale*
SC	*Stratum corneum*
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SD	*Stratum Disjunctum*
SEM	Scanning Electron Microscope
SG	*Stratum Granulosum*
SGV	*Stratum Germinativum*
SLN	Solid Lipid Nanoparticle
SPIONS	Superparamagnetic Iron Oxide Nanoparticles
SPM	Scanning Probe Microscopy
SR	*Stratum Reticulare*
SS	*Stratum Spinosum*
STIM	Scanning Transmission Ion Microscopy

Abbreviation	Definition
TEM	Transmission Electron Microscopy
TEWL	Transepidermal Water Loss
TG	Test Guideline
TiO_2	Titanium Dioxide
UV	Ultraviolet
UVR	Ultraviolet Radiation
wt.	Weight
ZnO	Zinc Oxide

Executive Summary

This report constitutes the culmination of a comprehensive review of the available literature on nano-enabled technologies for cosmetic products, specifically addressing soluble nano-transporters. The project is part of the Danish Environmental Protection Agency's (EPA) efforts on further clarifying possible risks to consumers and the environment from the use of nanomaterials in products on the Danish market. Accompanying this report is an appraised database (web link can be found in Appendix 1) summarising the literature which formed the basis of this review which sought to address the following areas:

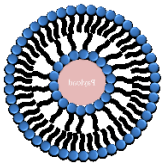
- types and uses of soluble nano-transporters in cosmetic applications;
- assessment of the extent of dermal absorption/ penetration of nano-transporters;
- evidence of dermal/ systemic toxicity arising from interactions with nano-transporters;
- identification of nano-specific characteristics that may influence dermal absorption/ toxicity of nano-transporters;
- assessment of the specific research areas that require more knowledge.
- to discuss to which extent provisions on nanomaterials in the cosmetic regulation are to be applied for nano-transporters

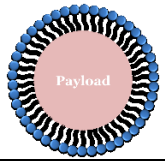
The literature search strategy is further described in chapter 4 and a web link to the database and a list of the relevant literature are presented in Appendix 1.

Description of Nano-transporters

Nano-transporters are a diverse array of vehicles which serve the dual purpose of both protecting active ingredient payloads and improving their delivery to the skin. Compositionally they can differ markedly and can include polymers and lipids as well as a diverse range of surfactants. The different forms of nano-transporters such as *liposomes, niosomes, SLN (Solid Lipid Nanoparticles), NLC (Nanostructured Lipid Carriers), nanoemulsions, nanospheres/nanocapsules* etc. are classified to an extent on structure and within these different groupings, may differ in terms of their specific compositions meaning that, for example, not all nanoemulsions have exactly the same mix of lipids and surfactants.

OVERVIEW OF NANO-TRANSPORTER TYPES AND SIZE RANGES

Nano-Transporters	Size (nm)*	Description
Liposomes 	25 - few hundred microns	Spherical, artificial vesicles composed of a bilayer cholesterol and other natural phospholipids
Niosomes Similar structure as liposomes	100 - 2000	Closed bilayer of non-ionic surfactant in lamellar phase surrounding and aqueous cavity

Nano-Transporters	Size (nm)*	Description
Solid Lipid Nanoparticle (SLN) and Nanostructured Lipid Carrier (NLC) 	150 - 300 (SLN) 80 - 300 (NLC)	SLNs are lipid nanoparticles derived from an oil-in-water (o/w) emulsion where the oil phase has been substituted by a solid lipid. In NLC the oil phase is a blend of liquid and solid lipids.
Nanoemulsions	10 - 100	Colloid oil droplets in e.g. water phase
Nanoparticulates (nanocapsules, nanospheres,)	3 - 1000	Nanocapsules are vesicles in which a polymeric membrane surrounds and encapsulates a liquid core (e.g. oil). Nanospheres are solid polymeric particles.

**Note that the upper size level for nearly all nano-transporters are above 100 nm which in general is used as the upper level for nanomaterials*

Use of nano-transporters

Based on the literature search cosmetic products representing nano-enabled technologies were found in a wide range of cosmetic products. Thus, 165 patents were found to have direct applications in cosmetics. Such uses may cover:

- UVR protection
- moisturisers for skin, hair and nails,
- antioxidants,
- skin whitening,
- prevention of aging and wrinkles,
- promotion of collagen synthesis,
- anti-cellulite,
- treatment of skin allergy and
- repairing skin injury

Specifically concerning nano-transporters, these are seen as particularly attractive for the cosmetic industry since they present many advantages including:

- The protection of sensitive agents
- Enhanced dermal penetration of active ingredients
- The controlled release
- A reduction in the amount of agents and additives
- Longer shelf life and hence greater product effectiveness

Characterisation

The Scientific Committee for Consumer Safety in its guidance on the safety assessment on nanomaterials in cosmetics SCCS highlights that characterisation of a nanomaterial in a cosmetic formulation is more difficult compared to characterisation in a raw material. Whilst it may be easier to characterise a particle as produced, the actual properties and the characteristics may be modified during incorporation into a product, during storage, during application, during residence in the skin or other environment and thus the characterisation may change throughout the particle life cycle. From a toxicological perspective, it is the physicochemical characteristics at the point of application

and during residence in or on the skin that is most relevant. This is because this is the point of biological interaction and it is the properties at this time, which may drive a biological effect.

Even though the characterisation of biodurable and solid nanoparticles in the biological environment is highly challenging, the situation is likely to be even more difficult for biosoluble nano-transporters. This is because such transporters are somewhat labile, for example due to lipids fusing with the skin or size potentially changing through fusion (as opposed to aggregation). Whilst these labile properties' can be beneficial to the action of the nano-transporters (e.g. causing release of the active payload) they also provide additional challenges to the identification and characterisation of the nano-transporters after application. Indeed, this becomes apparent when considering that in the assessment of dermal penetration, *in vitro* studies tend to measure the penetration of the active payload into the receptor fluid rather than measure the nano-transporters directly. This provides ambiguity as to i) if the nano-transporters themselves can penetrate the skin and ii) how long they can persist in the skin/ body.

It is clear to see that there are significant challenges for proper characterisation of nano-transporters but as with any toxicological evaluation, proper characterisation of the test material is essential for correct interpretation. Given the specific nature of nano-transporters (the labile and transient nature), further tools to detect and characterise these particles in the biological environment are likely to be needed.

Dermal absorption

The literature provides a convincing picture of effective dermal penetration achieved through the use of nano-transporters with both lipid and polymer based transporters allowing the passage of active substances such as drugs, anti-oxidants or labels through the outer skin layer, s*tratum corneum* which is the principal diffusion barrier of the skin. The ability of these materials to penetrate efficiently through the s*tratum corneum* marks a considerable difference between these soluble nano-transporters and more conventional solid nanoparticles such as TiO2, and ZnO for which the penetration is extremely low.

The result of improved penetration over that of solid, insoluble nanoparticles into the dermal layers could mean that the relative dose of soluble nano-transporters received by these lower layers is greater than that of conventional solid nanoparticles. In terms of full transdermal penetration resulting in systemic absorption, the evidence suggests to the most part nano-transporters are retained within the skin. Specific localisation and retention within the skin limits the potential unforeseen and unintended impact of nano-transporters themselves (rather than their payload, which may dissociate and travel further) and therefore provides a more localised focus on health impact. However whilst the evidence does point towards a relatively high level of dermal retention of nano-transporters, these findings are not unanimous and more evidence would be needed to rule out absorption and systemic availability of nano-transporters.

One aspect of nano-transporter fate, which has great relevance to the comparison to insoluble nanoparticles is their biodegradation. A key aspect of this is in understanding the rate of degradation, which in turn may affect the level of penetration, which can be achieved before the nano-transporters lose their integrity, releasing its payload. Differing compositions and differing types of nano-transporters (e.g. polymeric nanocapsules vs. liposomes) will degrade at different rates with some persisting longer and offering greater protection to their payloads whilst others may be more susceptible to the dermal environment. The rate of biodegradability and the effect this has on potential risks is very much subject to debate as some nanoparticles such as ZnO are indeed soluble and do release zinc ions systemically. Similarly, through modification nano-transporters could be produced to display sufficient resilience to degradation but the point at which they may be classified as a *persistent* nanoparticle currently is unclear.

In relation to the physicochemical determinants of dermal absorption, the literature very much lacks systematic evaluations and comparative analyses of nano-transporters differing in specific physicochemical attributes. However, some studies do occur and similar to what is noted for conventional nanoparticles, size does appear to have an effect on the ability of particles to penetrate with smaller particles able to penetrate.

Another physicochemical property to receive attention within the literature is surface charge. The net charge of the skin is negative owing to its composition and so particles with a strong negative charge would be subject to repulsive forces whilst particles with a strong positive charge would be subject to attractive forces, possibly increasing interaction with the skin cells. Evidence for nano-transporters such as nanoemulsions suggests that a positive surface charge results in enhanced dermal accumulation of actives although this does not necessarily translate to dermal absorption of nano-transporters. Instead, it may be the case that a positive charge facilitates improved release of the active payload within the skin (with or without improved penetration into the skin).

Overall the findings can be summarised as follows:

- Nano-transporters can effectively and efficiently penetrate the skin and locate in and beyond the *stratum corneum*
- Where penetration has occurred, evidence suggests that nano-transporters are to a significant extent retained within the dermal layer
- Nano-transporters interact with the skin cells and degrade, releasing their payload into the skin where it may diffuse further including transdermally
- Size can influence penetration with smaller particles enhancing drug permeation and very large particles (tens of microns) being excluded from penetration into the SC
- Damage to the barrier quality of the SC can lead to faster penetration of the dermal layer
- Surface charge can have an effect on dermal penetration and positively charged particles show enhanced dermal accumulation of actives although this does not necessarily translate to dermal absorption of nano-transporters
- Nanoparticles (i.e. nanospheres/nanocapsules) seems as the type of nano-transporters that may obtain the highest resistance towards degradation in the dermal tissue.

Dermal toxicity

The toxicity of nano-transporters has been assessed through a number of different approaches ranging from *in vitro* assays using unrelated cell types to dermal toxicity (e.g. red blood cells and hepatocytes) to more closely related *in vitro* models including keratinocytes and multi-cell modes such as reconstructed human epidermis. In addition, *in vivo* models have been used to assess toxicity including both animal models as well as human volunteers. The latter represents the gold standard in terms of relevance but offers limitations in the degree of investigations that can be performed to assess toxicity (i.e. typically non-invasive).

In vitro analysis using liposomes across a range of 3 single cell models showed that whilst both the highest and lowest surface charged particles caused significant cell death, it was much more rapid in the case of the highly positively charged particles. This in turn could suggest that highly charged particles may cause dermal toxicity over a short period of time. However, a study in humans also evaluated the role of surface charge with nano-emulsions. Here the application of positively charged particles to human volunteers showed no signs of toxicity (e.g. erythema) and instead showed

significant improvement in skin moisture levels and elasticity. Therefore, based on these results it could be concluded that rather than a negative impact on dermal health, a positive charge may have a beneficial effect. It is of course difficult to make direct comparisons between these studies as the same nano-transporter was not employed, the nano-particles applied to humans were not as positively charged as those used in cell culture etc. However, this comparison does serve the purpose of demonstrating that validation of *in vitro* test results is important in drawing solid conclusions.

Studies have indicated that components such as lauroylcholine, stearic acid, polylactic acid, and sodium dodecyl sulphate can all have a negative impact on biocompatibility. Data from *in vitro* and *in vivo* studies suggest that with respect to irritation potential cationic surfactantia can be the most detrimental followed by anionic surfactantia with non-ionic surfactantia being the least problematic.

In terms of endpoints evaluated, the majority of *in vitro* analyses focus on an assessment of cell death as a measure of toxicity, which in itself, is a relatively rudimentary assessment of toxicity and says very little about possible sub-lethal effects (e.g. inflammation) or mechanisms. Indeed, detailed assessment of cellular interactions and effects is very much missing from the literature pertaining to the cosmetic applications of these materials. Assessment of inflammatory potential has been assessed *in vivo* using methods such as the patch or draize test and typically shown no effect and this is also supported by measures of blood cytokines after systemic application of nano-transporters. Genotoxicity is also an area of concern and this has been assessed both *in vitro* and *in vivo* with negative effects in both systems.

Taking nano-transporters collectively, the literature provides an impression of relative low toxicity and biocompatibility with these materials although there are exceptions. In relation to toxicity, there does not appear to be a clear relationship between size or surface charge and observed toxicity. Indeed as toxicity with many of these materials lacks a specific trend and considering the present stage of knowledge, it seems that the composition of substances used for generating the nano-transporters may determine the cytotoxicity profiles instead of size.

In relation to allergic responses; nano-transporters themselves do not appear to be allergenic, however, the improved penetration and controlled release of actives does bring with it potential issues. It has been noted in several studies using well established contact allergens that encapsulation, for example into ethosomes, can lead to an enhanced and in some cases more rapid allergic response than would normally be seen had the active not been encapsulated. This is possibly due to an increased dose of the allergenic substance being presented to the responsive, epidermis and dermis; possibly over a greater period of time (sustained release).

Overall, the findings can be summarised as follows:

- The level of toxicological evaluation of nano-transporters for cosmetic applications is sporadic and not seen as comprehensive, particularly around sub-lethal endpoints in dermal cells such as inflammation, genotoxicity and sensitisation

- Nano-transporters on balance appear to be of relative low toxicity although there is a spectrum of toxicity

- Typical adverse effects noted are cell death within *in vitro* models which are more sensitive than *in vivo* models

- *In vivo*, nano-transporters are well tolerated and even high dose systemic administration causes minimal toxicity

- Nano-transporters do not appear to be genotoxic

- Nano-transporters may enhance the sensitising potency and allergic response of contact allergens

- No clear correlations between size or surface charge and toxicity are apparent

- Within observed toxicity, composition appears to be of primary importance with several substances showing less than optimal biocompatibility

Knowledge gaps and Research Needs

Based on the review in this project, several knowledge gaps and research needs have been identified including:

- lack of knowledge on the actual level of penetration/ systemic absorption of the actual carrier (as opposed to the payload).

- It is not clear if polymer based nanocapsules etc. degrade to the same extent as other nano-transportes and therefore, if these are more likely to become systemically available.

- Further use of flexion systems as more realistic models for dermal penetration of nano-transporters as compared to the use of static models e.g. the Franz-type diffusion cell.

- The impact of the various compositions on the activity of nano-transporters is not fully apparent. This in itself hinders progressive understanding about the role physicochemical properties (as well as composition) plays in toxicity which in turn, reduces the ability to read-across between and/or group materials based on shared properties relevant to their toxicity (or absence thereof).

- When considering the level of characterisation in relation to nano-transporters the following should be reported as a minimum dataset:
    - Composition (including sources)
    - Production method
    - Size (often including particle size distribution)
    - Zeta-potential

  and information regarding stability and persistency of the nano-transporter.

- Also, it needs to be further clarified how the various types of nano-transporters may affect the sensitizing properties of contact allergens.

Regulatory aspects

It has been discussed to which extent the provisions towards nanomaterials in the cosmetic regulation would also address the nano-transporters and in the cosmetic regulation (EC No 1223/2009) a nanomaterial is defined as:

"an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm".

From this it can be seen that a nano-transporter has to be a biopersistent material within the size range of 1 – 100 nm in order to be included in this definition. Considering biopersistency alone it appears from the description of the various nano-transporters in this report that only the nanoparticulates may come into consideration as the described properties of the other transporters

(liposomes; niosomes; Solid Lipid Nanoparticles; Nanostructured Lipid Carriers; and nanoemulsions) indicate that these nano-transporters are rather labile with a structure that rapidly degrade upon dermal application. With respect to nanoparticles (i.e. nanospheres/nanocapsules), some members in this group may have a higher degree of persistency, and thus, it would be up to a definition (or a criterion) regarding the word "biopersistency" that would determine whether these (or some of these) nano-transporters should be covered by the nanomaterial provisions.

Also, it has to be noted that liquid and labile nanoparticles in general not have been considered to be within the scope of the nanomaterial definition (neither the overall EU definition recommended by the EU Commission in 2011 or the definition as given in the cosmetic regulation). One reason may be less concern for nano-specific properties and toxicity. Thus, SCCP (2007) in its "Opinion on Safety of Nanomaterials in Cosmetic Products" considered that soluble and/or biodegradable nanoparticles which disintegrate upon application to skin into their molecular species (e.g. liposomes, microemulsions, nanoemulsions) would be of less concern, and most probably be comparable to ordinary chemicals in terms of risk assessment.

Currently, the overall EU nanomaterial definition is subject to a revision (planned to be in place in 2016), and a revision in the context of the cosmetic regulation is also under discussion. Therefore, an option for covering the most persistent of the nano-transporters (belonging to the category of nanocapsules/ nanospheres) by the provisions for nanomaterials would be to obtain specific criteria for persistency at a level that would include these nano-transporters.

Sammenfatning

Denne rapport er resultatet af en detaljeret gennemgang af den tilgængelige litteratur om nanoteknologibaserede kosmetiske produkter, som især omhandler opløselige nanotransportører. Projektet er en del af Miljøstyrelsens målsætning for at beskrive eventuelle risici for forbrugerne og miljøet ved anvendelse af nanoteknologibaserede produkter på det danske marked. I forbindelse med denne rapport er der angivet web link til en database (web link kan findes i bilag 1), der opsummerer den fundne litteratur, der har dannet grundlag for vurderingen vedrørende:

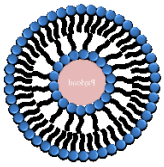
- typer og anvendelser af opløselige nanotransportører i kosmetisk brug;
- vurdering af omfanget af dermal absorption/penetration af nanotransportører;
- dokumentation for dermal/ systemisk toksicitet som følge af interaktioner med nanotransportører;
- identifikation af nanospecifikke egenskaber, der kan påvirke nanotransportørers dermale absorption/ toksicitet;
- vurdering af de specifikke forskningsområder, der kræver mere viden;
- diskussion af i hvilken udstrækning de regulatoriske krav for nanomaterialer i den kosmetiske lovgivning også bør/kan anvendes for nanotransportørerne.

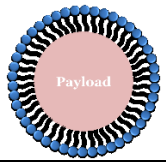
Selve litteratursøgningsstrategien er yderligere beskrevet i kapitel 4 og et web link til databasen samt en liste over den relevante litteratur fremgår af bilag 1.

Beskrivelse af nanotransportører

Nanotransportører er en bred vifte af bærestoffer/ bærematerialer, der tjener det dobbelte formål at beskytte aktivstoffet og forbedre levering til huden. Sammensætningerne af transportørerne kan være markant forskellige og kan omfatte polymerer og lipider samt en lang række overfladeaktive stoffer. De forskellige former for nanotransportører, såsom *liposomer*, *niosomer*, *SLN* (*Solid Lipid Nanoparticles*), *NLC* (*Nanostructured Lipid Carriers*), *nanoemulsioner*, *nanosfærer/ nanokapsler* mv. kan i et vist omfang indeles ud fra struktur, men kan inden for disse forskellige strukturer variere i sammensætning, hvilket betyder, at for eksempel ikke alle nanoemulsioner har præcis den samme blanding af lipider og overfladeaktive stoffer.

OVERSIGT OVER TYPER OG STØRRELSESINTERVALLER AF NANOTRANSPORTØRER

Nanotransportører	Størrelse (nm)*	Beskrivelse
Liposomer 	25 - få hundrede mikrometer	Sfæriske, kunstigt frembragte vesikler sammensat af et dobbeltlag af kolesterol og andre naturlige phospholipider
Niosomer Struktur tilsvarende liposomer	100 - 2000	Lukket dobbeltlag af ikke-ionisk overfladeaktivt stof der omgiver et vandigt hulrum

Nanotransportører	Størrelse (nm)*	Beskrivelse
Solid Lipid Nanoparticle (SLN) and Nanostructured Lipid Carrier (NLC)  	150 - 300 (SLN) 80 - 300 (NLC)	SLN er lipid nanopartikler afledt af en olie-i-vand (o/w) emulsion, hvor oliefasen er blevet byttet ud fast lipider. I NLC er oliefasen en blanding af flydende og faste lipider.
Nanoemulsioner	10 - 100	Kolloide oliedråber i fx vandfase
Nanopartikler (nanokapsler, nanosfærer)	3 - 1000	Nanokapsler er vesikler, hvori en polymermembran omgiver og indkapsler en flydende kerne (fx olie). Nanosfærer er faste polymerpartikler.

** Bemærk at den øvre størrelsesgrænse for næsten alle nanotransportører er over 100 nm, hvilket generelt anvendes som den øvre afgrænsning for nanomaterialer.*

Anvendelse af nanotransportører

Baseret på litteratursøgning blev der fundet en lang række kosmetiske produkter, som kan betegnes som nanoteknologibaserede. Der blev fundet 165 nanobaserede patenter i kosmetik, som omfattede følgende områder:

- UVR beskyttelse
- Fugtighedsbevarende præparater til hud, hår og negle
- Antioksidanter
- Hudblegning
- Forebyggelse af aldring og rynker,
- Fremme af kollagen syntese,
- Anti-cellulitis (mod appelsinhud),
- Bbehandling af hudallergi
- Reparation af hudlæsioner

Nanotransportører anses som særligt attraktive for den kosmetiske industri, da de giver mange fordele, herunder:

- Beskyttelse af mindre stabile aktivstoffer
- Forbedret dermal penetration af aktivstoffer
- Kontrolleret frigivelse
- Reduktion i mængden af anvendelse af aktivstoffer og additiver
- Længere holdbarhed og bevarelse af produkteffektivitet

Karakterisering

Den Videnskabelige Komité for Forbrugersikkerhed (SCCS) fremhæver i sin vejledning om sikkerhedsvurdering af nanomaterialer i kosmetik, at karakterisering af et nanomateriale i kosmetisk formuleringer er vanskeligere i forhold til karakterisering af de rene råvare. Mens det kan være lettere at karakterisere en partikel som fremstillet, kan de faktiske egenskaber og karakteristika af partiklen ændres ved indarbejdelse i et produkt, under opbevaring, under påføring, under ophold på huden eller i andet miljø. Dermed kan partiklen ændre karakteristika i løbet af partiklens livscyklus. Ud fra et toksikologisk perspektiv er det de fysisk-kemiske egenskaber ved

påføringspunktet og under ophold i eller på huden, der er mest relevante. Det skyldes, at det er egenskaberne på tidspunktet for biologisk interaktion som er afgørende for en biologisk virkning.

Selvom karakterisering af de mere biopersistente og de faste nanopartikler i det biologiske miljø er meget udfordrende, er situationen sandsynligvis endnu vanskeligere for bioopløselige nanotransportører. Dette skyldes, at sådanne transportører er noget labile, for eksempel på grund af at lipider kan fusionere med huden, hvorved strukturen og størrelsen kan ændre sig (i modsætning til aggregering). Selv om disse labile egenskaber kan være til gavn for virkningen af nanotransportørerne (fx forårsager de frigivelse af det aktive stof), giver dette imidlertid yderligere udfordringer for identifikation og karakterisering af nanotransportørerne efter påføring. Ved vurderingen af dermal penetration har man i de hidtil udførte *in vitro* undersøgelser haft en tendens til at måle udbredelsen og skæbnen af selve aktivstoffet, der transporteres, i stedet for at undersøge nanotransportøren, hvorfor viden vedrørende skæbnen af nanotransportøren er underbelyst. Det giver uklarhed med hensyn til, i) om nanotransportører kan trænge igennem huden, og ii) hvor længe de kan forblive i huden/ kroppen.

Det er tydeligt at se, at der er betydelige udfordringer for opnå korrekt karakterisering af nanotransportører, men som med enhver toksikologisk vurdering er korrekt karakterisering af testmaterialet en forudsætning for en korrekt fortolkning af de toksikologiske data. I betragtning af nanotransportørers særlige karakter (labile og med omskiftelige karakteristika), vil yderligere metoder til analyse og karakterisering af disse partikler i det biologiske miljø være nødvendige.

Dermal absorption

Litteraturen giver et veldokumenteret billede af at anvendelse af nanotransportører (både lipid- og polymer-baserede transportører) sikrer en effektiv dermal penetration gennem det ydeste hudlag, *stratum corneum (*hornlaget, som er hudens vigtigste diffusionsbarriere), og dermed tillader passage af aktivstoffer såsom lægemidler og antioxidanter. Disse materialers evne til at trænge effektivt gennem *stratum corneum* udgør en betydelig forskel mellem disse opløselige nanotransportører og mere konventionelle faste nanopartikler, såsom TiO2 og ZnO, for hvilke hudpenetrationen er meget lav.

Resultatet af øget penetration i forhold til de faste, uopløselige nanopartikler i de dermale lag kan betyde, at dosis af opløselige nanotransportører i de dybere hudlag er forholdsvis større end for konventionelle faste nanopartikler. Hvad angår fuld transdermal penetration som medfører systemisk absorption, tyder det på, at de fleste nanotransportører forbliver i hudelagene. Lokalisering og fastholdelse i huden begrænser potentielt uforudsete og utilsigtede virkninger af nanotransportørerne (snarere end deres last, som ofte kan dissociere og udbredes yderligere), hvilket medfører større fokus på evt. sundhedsmæssige konsekvenser lokalt i vævet. Selvom de fleste undersøgelser peger i retning af en relativt stor dermal tilbageholdelse af nanotransportører, er fundene dog ikke entydige, og der vil være behov for flere beviser for at udelukke absorption og systemisk tilgængelighed af nanotransportører, generelt.

Et aspekt af nanotransportørers skæbne, som har stor relevans for sammenligning med uopløselige nanopartikler, er deres bionedbrydelighed. Her er det vigtigt at kende nedbrydningshastigheden, da denne vil påvirke graden af penetration, som opnås, før nanotransportørerne nedbrydes og frigiver deres last. Forskellige sammensætninger og forskellige typer nanotransportører (fx polymere nanokapsler vs. liposomer) vil nedbrydes med forskellige hastigheder, hvor nogle vil være mere persistente og dermed i højere grad beskytte deres last, mens andre kan være mere følsomme over for nedbrydning i det dermale miljø. Hastigheden af bionedbrydelighed og effekten det har på potentielle risici er genstand for megen debat, da visse faste nanopartikler såsom ZnO faktisk er opløselige og dermed frigiver zink-ioner for systemisk optagelse. For de mere opløselige nanotransportører kan det tænkes, at disse gennem modifikation kan gøres mere modstandsdygtige

over for nedbrydning, men punktet, hvor de kan klassificeres som *persistente* nanopartikler er i øjeblikket uklart.

I forbindelse med at beskrive de fysisk-kemiske egenskabers betydning for dermal absorption er der i litteraturen stor mangel på systematiske vurderinger og sammenlignende analyser af nanotransportører med forskellige fysisk-kemiske egenskaber. Men fra de undersøgelser, der foreligger, tyder det på at størrelse har en indvirkning på partiklers evne til at trænge ind i huden, idet det - ligesom for de konventionelle nanopartikler - gælder, at de mindste partikler har størst penetrationsevne.
En anden fysisk-kemisk egenskab som får opmærksomhed i litteraturen er overfladeladningen af transportøren. Huden nettoladning er negativ på grund af dens sammensætning, og derfor vil partikler med en stærk negativ ladning blive frastødt på huden, mens partikler med en stærk positiv ladning vil blive tiltrukket, hvilket muligvis øger interaktionen med hudcellerne. Beviser fra nanotransportører såsom nanoemulsioner tyder på, at en positiv overfladeladning resulterer i forøget dermal akkumulering af aktivstoffer, selv om dette ikke nødvendigvis medfører dermal absorption af nanotransportørerne. I stedet kan det være tilfældet, at en positiv ladning fremmer frigivelse af den aktive last i huden (med eller uden forbedret penetration ind i huden).

Samlet set kan resultaterne opsummeres som følger:

- Nanotransportører kan effektivt trænge ind i huden og placere sig i *stratum corneum* eller i dybereliggende dermale cellelag

- Hvor der er påvist penetration, tyder data på, at nanotransportørerne i væsentligt omfang tilbageholdes i de dermale lag

- Nanotransportører interagerer med hudceller og nedbrydes, idet de frigiver deres last i selve hudlaget, hvorfra de kan diffundere yderligere igennem huden

- Størrelsen af nanotransportøren kan påvirke penetrationen, hvor de mindre partikelstørrelser har bedre penetration, mens de større partikelstørrelse har nedsat (evt. udelukker) penetration ind i *stratum corneum*

- Skader på *stratum corneums* barrierelag kan medføre hurtigere/øget penetration til de underliggende dermale lag

- Overfladeladning kan have en effekt på dermal penetration, og positivt ladede partikler viser forøget dermal akkumulering af aktivstoffer, selvom dette ikke nødvendigvis fører til dermal absorption af nanotransportører

- Nanopartikler (dvs. nanosfærer/ nanokapsler) synes at være den type nanotransportører, der besidder størst persistens i dermalt væv

Dermal toksicitet
Nanotransportørers toksicitet er blevet vurderet ved en række forskellige metoder, der spænder fra *in vitro* tests med anvendelse af celletyper, som er ubeslægtede med dermal toksicitet (fx røde blodlegemer og hepatocytter), til mere nært beslægtede *in vitro* modeller med anvendelse af keratinocytter og multicelle systemer, såsom rekonstrueret human epidermis. Desuden er *in vivo* metoder blevet anvendt til vurdering af toksiciteten, bla. dyreeksperimentelle undersøgelser samt undersøgelser med frivillige forsøgspersoner. Sidstnævnte repræsenterer højeste standard hvad angår relevans, men der er selvsagt begrænsninger i typen af undersøger, der kan udføres for at vurdere toksicitet (typisk anvendes her ikke-invasive undersøgelsesmetoder).

In vitro tests med anvendelse af liposomer i 3 forskellige cellemodeller viste, at partikler med højeste og lavest elektrisk overfaldeladning medførte celledød, og at hurtigt response især blev opnået med stærkt positivt ladede partikler. Dette kunne antyde, at mest ladede partikler kan forårsage dermal toksicitet i løbet af meget kort tid. En anden undersøgelse med mennesker vurderede effekten af overfladeladningen i nanoemulsioner. Her viste anvendelsen af positivt ladede partikler på humane frivillige ingen tegn på toksicitet (fx hududslæt/rødme), men viste i stedet signifikant forbedring af hudens fugtniveau og elasticitet. Baseret på disse resultater kan det derfor konkluderes, at i stedet for en negativ indvirkning på dermal sundhed, kan en positiv ladning have en gavnlig virkning. Det er naturligvis vanskeligt at foretage direkte sammenligninger mellem disse studier, da de samme nanotransportører ikke blev anvendt. De nanopartikler, der anvendtes til mennesker, var ikke så positivt ladede som dem, der anvendtes i cellekultur osv. Men denne sammenligning tjener det formål at påvise, at validering af *in vitro* testresultater er vigtig, før der drages konklusioner herudfra.
Undersøgelser har vist, at komponenter såsom lauroylcholin, stearinsyre, polylaktat og natriumdodecylsulfat alle kan have en negativ virkning på bioforligeligheden. Data fra *in vitro* og *in vivo* undersøgelser peger på, at med hensyn til irritationspotentiale kan positivt ladede overfladeaktive stoffer være de mest skadelige, efterfulgt af de negativt ladede overfladeaktive stoffer, mens non-ioniske overfladeaktive stoffer (dvs. uden ladning) synes at være mindst problematiske.

Hvad angår de vurderede effekter, fokuserer størstedelen af *in vitro* undersøgelserne på en vurdering af celledød som et mål for toksicitet, hvilket i sig selv er en forholdsvis grov vurdering af toksicitet, og ikke siger meget om mulige subletale virkninger på vævet (fx inflammation) eller mekanismerne for toksiciteten. Faktisk er detaljeret vurdering af cellulære interaktioner og effekter en stor mangel i litteraturen vedrørende kosmetisk brug af disse materialer. Vurdering af det inflammatoriske potentiale er blevet foretaget *in vivo* ved hjælp af metoder som lappe- eller draize test, og har typisk ikke vist nogen virkning. Dette understøttes også af målinger af cytokiner frigivet til blodet efter systemisk udsættelse for nanotransportører. Genotoksicitet er også et fokusområde, som er blevet vurderet både i *in vitro* og *in vivo* undersøgelser, men med fravær af effekter i begge typer testsystemer.

Ved vurdering af nanotransportører samlet set giver litteraturen indtryk af relativ lav toksicitet og høj grad af bioforligelighed for disse materialer, selv om der er undtagelser. I relation til toksicitet synes der ikke at være en entydig sammenhæng mellem størrelse eller overfladeladning og den observerede toksicitet. Så ud fra den nuværende viden ser det ud til at det snarere er *sammensætningen* af nanotransportørerne frem for fx størrelsen og ladningen, der er af størst betydning for toksiciteten.

Nanotransportører anses ikke i sig selv at være allergifremkaldende, men den øgede penetration og kontrollerede frigivelse af aktivstoffer kan medføre utilsigtede følgevirkninger. Det er i flere undersøgelser blevet vist at indkapsling af allergifremkaldende stoffer i nanotransportører (fx ethosomer der er liposomer med et vist indhold af ethanol) kan medføre en øget og i nogle tilfælde hurtigere allergisk reaktion, end der normalt ville ses, hvis ikke aktivstoffet var blevet indkapslet. Dette skyldes sandsynligvis at nanotransportøren medfører frigivelse af en øget dosis af det allergene stof efter penetration til de mest følsomme hudlag (epidermis og dermis), samt eventuelt frigivelse over en længere periode.

Samlet set kan resultaterne sammenfattes således:

- Omfanget af toksikologisk vurderinger af nanotransportører til kosmetiske anvendelser er sporadisk og ikke særligt omfattende. Især savnes data vedrørende subletale effekter i det dermale væv, såsom inflammation, genotoksicitet og sensibilisering

- Celledød er beskrevet som typiske skadelige virkninger i forbindelse med testning i *in vitro*-modeller. Disse modeller er imidlertid mere følsomme end *in vivo*-modeller

- *In vivo* besidder nanotransportører lav toksicitet, og selv høj systemisk dosis medfører minimale effekter

- Nanotransportører anses ikke at være genotoksiske

- Nanotransportører kan forstærke kontaktallergeners sensibiliserende virkning og den allergiske reaktion

- Overordnet anses nanotransportører at besidde relativ ringe toksicitet, selvom der er fundet varierende grader af toksicitet

- Der ses ingen klar sammenhæng mellem størrelse, overfladens elektriske ladning og toksicitet
- I forhold til de toksiske effekter der er set, vurderes sammensætningen af nanotransportørerne at have størst betydning for det toksikologiske response.

Manglende viden og forskningsbehov

På baggrund af gennemgangen i dette projekt er der udpeget flere områder med manglende viden og forskningsbehov, herunder:

- Viden mht. det faktiske niveau af penetration/ systemisk absorption af nanotransportøren (i modsætning til nyttelasten).

- Viden om hvorvidt polymerbaserede nanokapsler osv. nedbrydes i samme omfang som andre nanotransportører og derfor, om disse er mere tilbøjelige til at blive systemisk tilgængelige.

- Viden genereret under anvendelse af fleksible test-systemer (dvs. med fysisk påvirkning af huden fx bøjning) som mere realistiske modeller for dermal penetration af nanotransportører sammenlignet med brugen af statiske modeller (fx diffusionsceller af Franz-typen).

- Større viden om sammenhængen mellem nanotransportørernes sammensætning og toksiske effekter samt øget forståelse vedrørende betydningen af de fysisk-kemiske egenskaber. Denne viden vil øge muligheden for at foretage analogislutninger og/ eller gruppere materialer baseret på fælles egenskaber, som er relevante for deres toksicitet (eller fravær heraf).

- Med hensyn til karakteriseringen af nanotransportører, bør denne som minimum omfatte:
    - Sammensætning (herunder kilder)
    - Produktionsmetode
    - Størrelse (samt med partikelstørrelsesfordeling)
    - Zeta-potentiale (elektrisk ladning)

  Samt oplysninger om stabilitet og persistens af nanotransportøren.

- Bedre afklaring af hvordan de forskellige typer nanotransportører kan påvirke sensibiliserende egenskaber af kontaktallergener.

Lovgivningsmæssige aspekter

Indledningsvist skal det bemærkes, at flydende og labile nanopartikler generelt ikke er blevet anset for at falde inden for rammerne af definitionen nanomateriale (hverken den overordnede EU-definition anbefalet af EU-Kommissionen i 2011 eller definitionen som givet i kosmetikforordningen). En årsag kan være mindre betænkelighed i forhold til nanospecifikke egenskaber og toksicitet. Således mente SCCP (2007) i sin "Udtalelse om sikkerheden ved nanomaterialer i kosmetiske produkter", at opløselige og/ eller biologisk nedbrydelige nanopartikler, som nedbrydes ved påføring på hud til deres molekylære arter (fx liposomer, mikroemulsioner, nanoemulsioner), ville være mindre betænkelige og sandsynligvis ville være sammenlignelige med almindelige kemikalier med hensyn til risikovurdering.

Der har imidlertid været diskussion om, i hvilket omfang bestemmelserne om nanomaterialer i kosmetikforordningen også omfatter nanotransportørerne. I kosmetiskeforordningen (EF-nr. 1223/2009) defineres et nanomateriale som:

"et uopløseligt eller biopersistent og forsætligt fremstillet materiale med en eller flere eksterne dimensioner, eller en intern struktur, på en skala fra 1 til 100 nm".

Fra dette kan det ses at for at blive omfattet af den nuværende definition af nanomaterialer, skal en nanotransportør være opbygget af et biopersistent materiale og i størrelsesordenen 1 - 100 nm..
Vedrørende biopersistens alene fremgår det af beskrivelsen af de forskellige nanotransportører i denne rapport, at kun nanopartikler har mulighed for at komme i betragtning, da de beskrevne egenskaber af de øvrige transportører (liposomer, niosomer, faste lipid nanopartikler, nanostrukturerede lipidtransportører og nanoemulsioner) indikerer, at disse nanotransportører alle er forholdsvis labile, med en struktur der hurtigt nedbrydes efter dermal applikation.
Med hensyn til nanopartikler (dvs. nanosfærer/ nanokapsler) kan den kemiske sammensætning, der anvendes til disse transportører, have en højere grad af persistens, og det vil således være op til en definition (eller et kriterie) for "biopersistens" der afgør, om disse (eller nogle af disse) nanotransportører kan være omfattet af bestemmelserne for nanomaterialer.

I øjeblikket er den overordnede EU-definition af nanomaterialer under revision af EU-Kommissionen (er planlagt til offentliggørelse i løbet af 2016), og en revision i forbindelse med kosmetikforordningen er ligeledes under diskussion.
Der kan således være en mulighed for at få inkluderet de mest biopersistente nanotransportører (tilhørende kategorien nanokapsler / nanosfærer) under bestemmelserne for nanomaterialer ved at få fastsat specifikke kriterier for persistens på et niveau, der tager hensyn til at disse nanotransportører også kan omfattes.

1 Introduction

1.1 Danish initiative for "Better control of nanomaterials"

The Danish government and the Red-Green Alliance (a.k.a. Enhedslisten) have signed an agreement called "Bedre styr på nanomaterialer" ("Better control of nanomaterials") for four years (2012-2015) that focuses on the use of nanomaterials in products on the Danish market and their consequences on consumers and the environment. The Danish Environmental Protection Agency (EPA) has initiated a series of projects with the aim of further clarifying possible risks to consumers and the environment. The current project and accompanying literature database (web link can be found in Appendix 1) is part of this series and address a form of nanomaterials, namely soluble 'nano-transporters, which are used in consumer applications yet are not currently encompassed by the definition of a nanomaterial.

1.2 Project Outline

The Danish EPA has previously commissioned a literary study on the systemic absorption of nanomaterials *via* dermal exposure (Environmental Project No. 1504, 2013) (Poland, Read et al. 2013) which specifically dealt with nanoparticles meeting the current definition for nanomaterials in the EU Cosmetics Regulation (Regulation (EC) No 1223/2009). Within this definition, a "nanomaterial" means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm". Whilst this definition encompasses a significant proportion of nanomaterials used in cosmetic preparations such as sunscreens (either now or in the future); it does not cover all. Specifically, soluble nanomaterials such as liposomes or solid lipid nanoparticles (SLN) are not covered yet these materials are increasingly being used within cosmetic preparations (see chapter 5 for a full discussion) as 'nano-transporters'.

The aim of this project is to collect and assess information from the available literature on nano-enabled technologies for cosmetic products specially addressing these soluble nano-transporters. In doing so, the following areas will be addressed:

- types and uses of soluble nano-transporters in cosmetic applications;
- assessment of the extent of dermal absorption/ penetration of nano-transporters;
- evidence of dermal/ systemic toxicity arising from interactions with nano-transporters;
- identification of nano-specific characteristics that may influence dermal absorption/ toxicity of nano-transporters;
- assessment of the specific research areas that require more knowledge.

The overall purposes of this assessment is to provide a comprehensive and contemporary summary of the dermal interaction of these materials to enable an informed consideration of how these materials should be viewed with cosmetics regulation. As with any new technology, information gaps are likely and a further aim of this project is to evaluate the specific areas in which more knowledge on soluble nano transporters is needed.

1.3 Nanoparticle Exposures

The term 'nano' is a unit prefix meaning one billionth and the term 'nano-meter' obviously refer to the size metric of one billionth of a meter and nanoparticles or nanomaterials are substances within this size metric. The specific definition of nanomaterials varies with different organisations but

these typically they refer to materials with at least one dimension in the range of 1 to 100 nanometres. Some definitions, require that the nano-scale confers them specific properties (Yokel and Macphail 2011), whereas other consider the size distribution of the particles in a material (Commission 2011). It is worth considering that whilst many definitions specify a cut off at 100nm, this size does not necessarily convey a step change in material properties nor does it reflect a step change in material toxicity (Donaldson and Poland 2013). Indeed in terms of properties and toxicity, there is unlikely to be a significant difference between particles which are 90nm in size from an identical particle composition which is 110nm in size. Instead, from the point of view of this report it is perhaps more useful to consider the nano-range as a continuum rather than a threshold.

The body is exposed to particulates and indeed any exogenous substances through a variety of routes including the respiratory tree (via inhalation), the gut (via ingestion) and the skin via interactions with the external environment to which the skin forms our most effective barrier.

In terms of surface area, the skin covers a relatively small surface area of 1-2 m^2 (Tobin 2006, Elder, Vidyasagar et al. 2009) whilst in comparison, the gut has a much larger surface area of 32m^2 (smaller than previously thought) (Helander and Fändriks 2014) and the alveolar (gas exchange) region of the lungs have an enormous surface area of 102 m^2 (Stone, Mercer et al. 1992). Of the 3 main exposure routes, whilst the lung has the largest relative surface area, this surface is protected both by the fundamental anatomy of the respiratory system limiting the size of materials that can penetrate into the body and also the action of clearance mechanisms (e.g. alveolar macrophages) to keep the surface of the lung clean and free from infection. However, the skin is in constant contact with the external environment either from the clothes we wear, the surroundings we touch or the products we apply to the skin. Therefore, the affect exposures can have on the skin are of obvious concern for the effect on health.

As mentioned, skin exposure to materials can occur in a number of scenarios from the unintentional such as dermal exposure to a solvent during a manufacturing process to the intentional such as the application of a cosmetic cream to the skin. In both these scenarios, exposures can occur to nanomaterials, for example as emissions from a process in terms of unintentional exposure or where nanomaterials have been incorporated into a product such as sunscreen (Osmond and Mccall 2010, Beani 2012).

Human exposure to nanomaterials is not a new thing as nano-sized particles can be produced by a variety of processes with combustion (e.g. wood burning (Kocbach Bølling, Pagels et al. 2009) or vehicle emissions (Donaldson, Duffin et al. 2013)) being one of the most ubiquitous. However in the context of this review, manufactured or engineered nanomaterials are the focus and their development, production and use is a more recent form of potential human exposure.

Materials have been developed and manipulated at the nano-scale because of beneficial properties small size can bring including high surface area, modified spectral properties, altered solubility, improved electrical and thermal conductance as well as other properties. These improved properties of nano-scale materials have given rise to numerous industrial and commercial applications and there are numerous products on the market containing such materials. As a result, exposure to nanomaterials is increasingly likely and can range from being a possibility (e.g. the accidental exposure during production of a nanomaterial or nanomaterial containing product) to a certainty such as through the direct application of a nanomaterial to the body (e.g. intravenous injection of superparamagnetic iron oxide nanoparticles (SPIONs) as a medical contrast agent (Zhang, Rajan et al. 2015)).

1.4 Nano-particles and the Skin

Dermal exposures to engineered nanoparticles (eNP) can occur in numerous ways but in considering consumer exposures to engineered nanomaterials, repeated exposures are more likely to be as a result of intentional application of a nanoparticle to the body rather than unintentional exposures, as may occur in an occupational environment.

An example of the direct application of nanomaterials to the body in a consumer application is the use of a nanoparticle containing cosmetic such as a sunscreen, lotion, powder, or moisturiser. Here the inclusion of nanoparticles confers beneficial properties to the cosmetic such as the ability to form transparent layer on the skin or improved tactile feel of a cream.

It is this repeated exposure to engineered nanoparticles resulting from consumer use of cosmetics (as with repeated exposure to any chemical or substance) that raises some concern as to the potential implications for health. In considering the effects of eNP on the skin and specifically the effect on non-biopersistent nano-transporters; it is useful to first understand the composition of the dermal barrier.

1.4.1 The Skin Structure

The skin is a multifunctional organ forming a barrier across the surface of the body. It is multifunctional in that it serves not only provide a protective barrier against mechanical, thermal and physical injury caused by our interactions with the environment but also is a sensory organ through touch and detecting of thermal changes, reacting to them to regulate body temperature. The barrier qualities of the skin also serve to reduce the harmful effects of UV radiation (as well as producing vitamin D in response to light), prevent moisture loss from the body and detect and mount a response to infections.

Generally the skin is structurally composed of 3 main layers consisting of the outside layer or epidermis, the dermis and the inside layer, the hypodermis, as schematically represented in Figure 1. Whilst structurally, the skin is similar in the different regions of the body, the relative thickness of the various layers can vary dramatically reflecting the relative functions of the different regions. For example the thickness of the *stratum corneum* of the finger tips is much greater than that of other parts of the body such as the abdomen which is subject to far less abrasion and therefore in need of lesser protection than the finger tips.

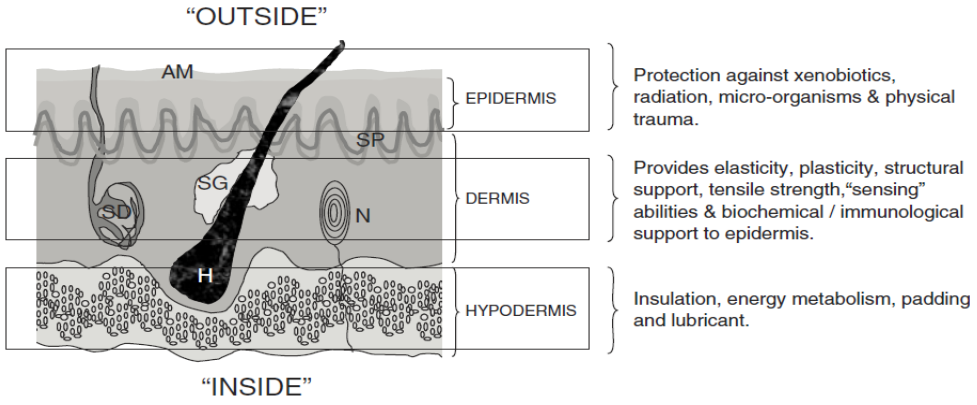


FIGURE 1: SCHEMATIC REPRESENTATION OF SKIN STRUCTURE AND ASSOCIATED FUNCTIONS.

Reproduced from (Chilcott, Price et al. 2008). Note that the relative thickness of each layer is not to scale. Several adnexal structures are shown (SP = Superficial Plexus, SG = Sebaceous Gland, SD = Sweat Duct, N = Pacinian corpuscle, H = Hair). In humans the skin is covered with a thin layer of lipids known as the acid mantle (AM), which comprises sebum, cell debris and sweat residua.

The epidermis is entirely devoid of blood vessels and instead, it is nourished from capillaries in the underlying layers by diffusion through the tissue fluid (Fawcett 1986). The main cell type composing the epidermis is the keratinocyte, and the outermost layer is the *stratum corneum* (SC) which is composed of flat, dead cells, called corneocytes which are continuously worn away and shed (this outmost layer is sometimes referred to as the *stratum disjunctum* (SD)).

This is followed by the underlying *stratum lucidum* (SL) which is a thin clear layer of closely compacted, highly refractile eosinophilic cells and appears in histological sections as a clear wavy stripe between the *stratum granulosum* and *stratum corneum*. The *stratum granulosum* consists of 3 - 5 layers of flattened cells which appear as a dark layer due to the presence of irregularly shaped granules which stain intensely with basic dyes such as hematoxylin (Fawcett 1986). This layer is followed by the *stratum spinosum* (SS) and finally the *stratum basale* (SB) which is composed of dividing cells that proliferate and push the cells toward the outside of the skin where they gradually morphologically and functionally change and flatten and finally die in the outer layer (*stratum corneum*).

As well as keratinocytes, the epidermis is also composed of various other cells types including cells which produce the pigment melanin under exposure to ultra-violet (UV) light (melanocytes) but also cells from the immune system such as Langerhans cells. In addition, the skin contains hair follicles, which are invaginations of the sub-SC that penetrate deep into the epidermis and reach the dermis and the density of such follicles can vary with body location. Skin appendages such as hair follicles, sebaceous glands and sweat glands have been highlighted as possible alternative routes of entry of the skin and have even been targeted for this effect for purposes such as vaccination and drug delivery (Lademann, Richter et al. 2006, Lademann, Richter et al. 2007, Jung, Patzelt et al. 2009, Blume-Peytavi and Vogt 2011, Lee, Shen et al. 2012).

The difference in the relative thickness of the *stratum corneum* can have an obvious effect on the level and rate of penetration of substances thorough the epidermis as well as the ability of substances to be absorbed into the body. Highly thick and well developed *stratum corneum* such as that found on the finger tips and soles of feet provide a substantial barrier, devoid of living cells and vasculature whilst much thinner layers such as those covering more delicate regions of the body such as underarms and mucous membranes provide much less of a barrier. To put this into perspective, the epidermis varies from 70 – 120 μm thick across much of the body but can be as thick as 800 μm on the palms and 1400 μm on the soles of the feet (Fawcett 1986).

Whilst epidermal thickness will have some impact on exposures occurring occupationally as such exposures tend to occur on exposed regions such as the hands, it becomes far more important when we consider cosmetic applications. Cosmetics may be applied to any region of the body with applications to regions of the body such as the underarms (e.g. deodorants), lips (e.g. lip gloss), abdomen (e.g. lotions), and scalp (e.g. haircare products as well as hands and feet (e.g. moisturisers) being daily occurrences. In addition to anatomical region, age and species variations in the skin structure are responsible for significant modifications in the efficiency of skin as a barrier against the environment (Chilcott, Price et al. 2008).

Below the epidermis is the dermis layer, which is mainly composed of extra-cellular matrix such as collagen and elastic fibres produced by dermal fibroblasts. The dermis also contains various structures such as sebaceous gland, blood vessels and nerves. The hypodermis is similar to the dermis but contains larger blood vessels and nerves as well as fat structure, providing thermal insulation.

As stated, the *stratum corneum* provides a formidable barrier to the external environment but where substances penetrate this outer defence, they can interact with more sensitive regions of the skins. For example, compounds that reach the *stratum granulosum* can interact with the viable

keratinocytes, possibly triggering an inflammatory reaction whilst compounds reaching the *stratum spinosum* may interact with Langerhans cells (from the immune system) and initiate an allergic reaction. Compounds that cross the epidermis into the dermis may potentially access the systemic circulatory and lymphatic systems. This can result in translocation to other sites within the body, distal to region of entry, which could potentially lead to a wide range of toxicological effects and disease such as systemic inflammation, organ toxicity and cancer.

1.5 Nano-particles and their use in Cosmetic Applications

Cosmetics are a ubiquitous component of everyday life that has been around for many thousands of years, with early evidence of their use dating back to ancient Egypt to enhance the skins appearance (Li, Wu et al. 2011). This early use has transformed into the multi-billion dollar global cosmetics market we see today, with the European market worth an estimated €69 billion alone in 2014 (Statista 2015). The market can be divided into five main business segments of skincare, haircare, colour (make-up), fragrances and toilettes and of these five segments, 35.3% of the global market consists of skincare products which account for the largest single part of the cosmetics market (Statista 2015). Table 1 provides an overview of the market volume of cosmetic and personal care products by country in Europe (only those countries with a volume of €1 billion or greater are shown). These figures help demonstrate what a significant impact the cosmetics industry has on the European economy with an estimated 170,000 people directly employed in the European cosmetic industry in 2013 (Statista 2015).

Given the scale of the cosmetics market, exemplified by table 1, it is clear to see that the cosmetics are important consumer products and used in their various forms (e.g. make, moisturiser, lotion etc.) on a daily basis by consumers.

TABLE 1: MARKET VOLUME OF COSMETICS AND PERSONAL CARE IN EUROPE IN 2014 BY COUNTRY. SOURCE STATISTA[1]

Country	Market volume in billion Euros
Germany	13.01
France	10.58
United Kingdom	10.4
Italy	9.39
Spain	6.35
Poland	2.95
Netherlands	2.82
Switzerland	2.04
Belgium/ Lux	2.01
Sweden	1.8
Austria	1.35
Portugal	1.28
Norway	1.26
Romania	1.09
Denmark	1.01

The focus of this review is in on the consumer use of nanomaterials in cosmetic applications and to better understand the specific application of these materials, it is useful to first consider what a cosmetic is. The EU cosmetic regulation 1223/2009 (replacing directive 76/768/EC) defines a cosmetic product as:

[1] http://www.statista.com/statistics/382100/european-cosmetics-market-volume-by-country/. Accessed November 2015

> "(a) 'cosmetic product' means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours;"

This definition is similar to that of the US Food and Drug Administration (FDA) which specifies the purposes of cosmetics as "cleansing, beautifying, promoting attractiveness or altering the appearance" .

The use of nanomaterials in cosmetic applications is increasing steadily (further reviewed in section 5) and a 2010 review detailed more than 400 products containing metal nanomaterials and destined to topical applications for consumers (Robertson, Sanchez et al. 2010). One of the most common uses of nanomaterials in cosmetics is the use of nano-sized TiO_2 and ZnO in sunscreens as alternatives to either chemical or micron-sized particulates. This is due to fact that such nanoparticles provide an effective filter against UV radiation but also appear transparent when applied to the skin in contrast to larger, micron-sized TiO_2 which can appear milky and opaque.

Within the European Union cosmetic regulations, nanomaterials are defined as "an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm" (Regulation (EC) No 1223/2009). This regulation also provides a mechanism for notification, labelling, and safety evaluation of cosmetic products containing nanomaterials within this definition. However, whilst this definition encompasses many of the nano-scaled particles used in cosmetics (such as TiO_2 and ZnO used as exemplars above), it does not cover all.

As described in section 3.2, soluble nanomaterials such as liposomes or solid lipid nanoparticles (SLN) used are as carrier systems and whilst these meet some of the criteria of this definition (i.e. size and intentional manufacture) they do not meet the full definition due to their inherent solubility and lack of biopersistence. Therefore, nano-sized carrier systems such as liposomes and solid lipid nanoparticles are not classified as 'nanomaterials' within current EU cosmetics regulation.

Despite this lack of presence of in the regulatory definition of nanomaterials, the use of carrier systems of nano-sized particles, sometimes referred to as 'nano-transporters' or 'nano-carriers' are on the increase in cosmetic applications as a strategy to improve absorption of compounds by the skin as well as improving agent stability (Li, Wu et al. 2011). A great deal of interest has been generated around the use of nano-transporters in medical applications such as drug delivery (Dixit, Kohli et al. 2008, Carneiro, Aguiar et al. 2012, Carbone, Cupri et al. 2013) due to the preferential properties encapsulation offers. Whilst advances in medical applications of nano-transporters are of great scientific interest, as stated in section 3.2 the focus of this review is on the **cosmetic** applications of these particles. However the line between the cosmetic and medical uses of nano-transporters is somewhat blurred and this is not just because both applications often share the same technological innovation, i.e. the same Nanostructured Lipid Carrier construct could equally be loaded with a pharmacological agent for use as a therapeutic or another substance for use as a cosmetic agent. This is reinforced by the statement by Puglia and Bonina who note that there are few difference between the application of lipid nanoparticles in pharmaceutical products for dermal delivery and in the cosmetic field (Puglia and Bonina 2012). As discussed by Li *et al.,* there have been advancements in cosmetics surpassing purely covering or camouflaging imperfections through the incorporation of 'active ingredients' which can have a more therapeutic role in the repair and protection of skin tissue (Li, Wu et al. 2011). As such products are more than simply a cosmetic; they are sometimes referred to as 'cosmeceuticals' to describe this hybrid between cosmetics and

topical medication (Kligman 2000, Millikan 2001, Kaur and Agrawal 2007). Examples of such cosmeceuticals include nano-transporters incorporating active substances such as caffeine for its role in slimming, vitamin A for its role in anti-aging and skin tightening, and coenzyme Q10 for its role as an anti-oxidant. As the line between cosmetic and therapeutic has become blurred, this review encompasses cosmeceuticals although does not address purely therapeutic applications of nano-transporters such as the application of anti-inflammatories such as corticosteroids (e.g. (Baboota, Shakeel et al. 2007, Shakeel, Baboota et al. 2009, Baboota, Alam et al. 2011, Silva, Rijo et al. 2015)), anti-acne treatments (e.g. (Stecova, Mehnert et al. 2007, de Almeida Borges, Simon et al. 2013, Lin, Fang et al. 2013, Raza, Singh et al. 2013)) or vaccines (e.g. (Li, Peng et al. 2011, Li, Peng et al. 2011, Rattanapak, Young et al. 2012)).

When considering this blurred line between cosmetic and medical applications of nano-transporters, it is important to consider that notwithstanding the similarity in technology between these two applications, the time between development and application to the market is much shorter for cosmetic products than for dermal pharmaceuticals due to the relatively minor regulatory hurdles (Puglia and Bonina 2012). This can be seen in two ways; the first is that it is imperative to have a good understanding of the interactions of these materials in cosmetics with the skin and the ultimate toxicological effects especially after chronic administration. This is emphasised by the fact that a cosmetic product such as a sunscreen or moisturiser is more likely to be applied over a larger area and for a longer duration of repeated applications that a pharmaceutical which would typically be limited to a period of treatment (although this may be extensive depending on the disease). The second is more positive as it can be seen that where carrier technologies have been validated and approved for medical applications, this provides a high degree of assurance in safety – much higher than that provided for other cosmetic products not subject to clinical trials. Taking this latter point further, the specific application criteria (dose, area and duration of application etc.) should be considered when considering effects (or lack of) noted in clinical testing in relation to cosmetic uses of a carrier.

1.6 Nano-transporters in Cosmetics

The use of nano-transporters in cosmetics has received increased attention over the last 15 years (Muller, Shegokar et al. 2011) and the specific result they have on the skin is, to a large extent dictated by the specific payload (i.e. active ingredient) they carry. However simply adding active ingredients to cosmetics preparations is often ineffective in achieving desired results due to the barrier function of the skin. Instead, the use of carrier technologies are aimed at improving the penetration profile of active compounds, enabling them to have the desired effect within the skin, rather than simply being placed upon the skin.

The specific uses of carrier technologies such as solid lipid nanoparticles (SLN) or Nanostructured Lipid Carriers (NLC) are shown in figure 1.

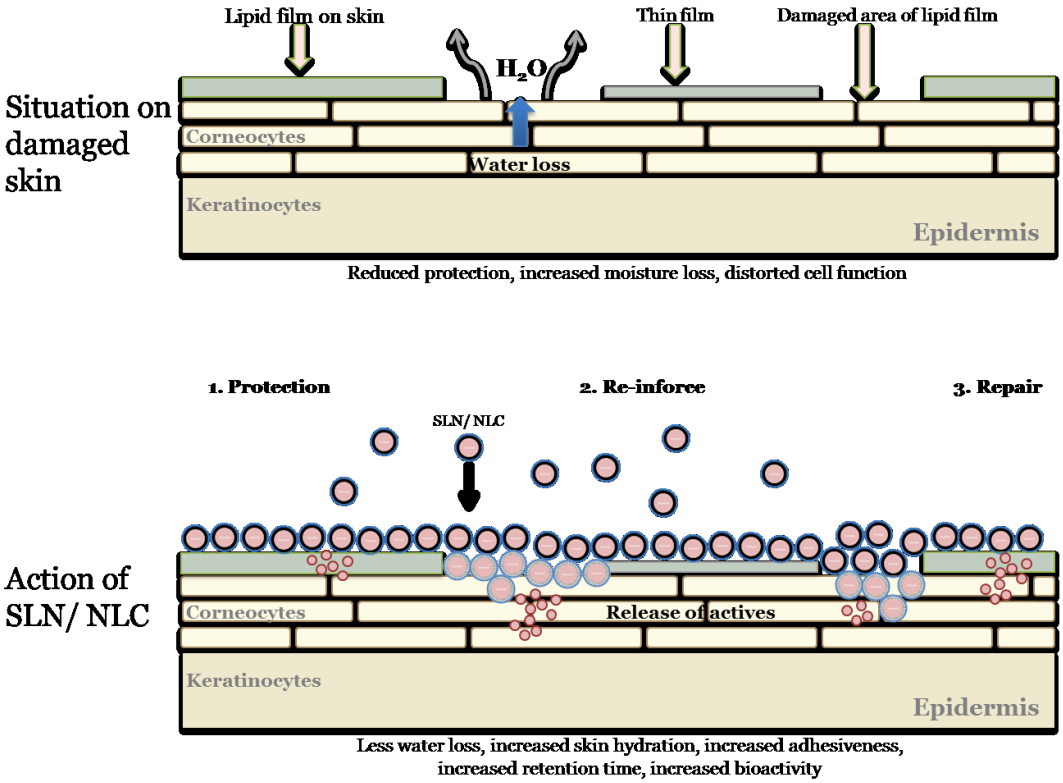


FIGURE 1: SCHEMATIC REPRESENTATION OF THE PROPERTIES OF LIPID NANOPARTICLES ON THE SKIN.

Upper: situation on damaged skin; Lower: action of SLN/NLC). Adapted from Muller *et al.* 2011 (Muller, Shegokar et al. 2011).

The upper diagram shows the situation on damaged skin whereby the protective lipid film on the skin has been eroded (for example due to excess cleansing) resulting in increased water loss from the skin, reduced protection and unfavourable condition for optimum skin performance. The use of nano-carriers such as the SLN and NLC shown act in a multitude of ways. The first is to restore the protective lipid layer as, due to their small size, the lipid nanoparticles adhere to the lipid film through hydrophobic interactions and the fact that small particles possess an adhesive effect and with decreasing particle size, adhesion increases (Muller, Radtke et al. 2002). The result of this restoration is a reduction in moisture loss, improving skin hydration and skin condition with a positive effect on dry skin and wrinkles (Muller, Shegokar et al. 2011). This occlusive effect of lipid nanoparticles is driven by a number of factors including particle size. Figure 2 shows effect of particle size on occlusivity by comparing 200nm lipid particles and 4µm and the results show a profound improvement with the 200nm particles over microparticles.

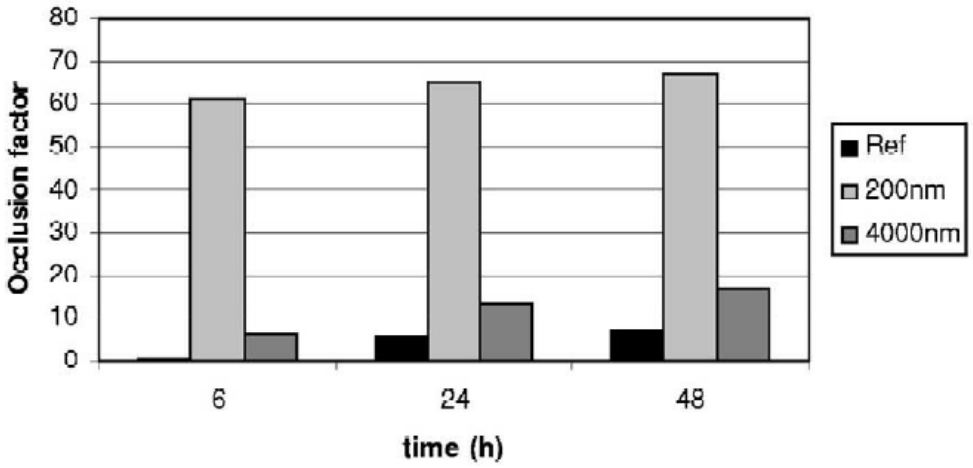


FIGURE 2: OCCLUSIVITY OF 200NM SLN VS. 4 MICRON LIPID PARTICLES AS A FUNCTION OF TIME.

Reproduced from Müller *et al.* (2002) (Muller, Radtke et al. 2002) which is adapted from (Wissing 2002).

Given their lipophilic nature, such lipid nanoparticles can show improved absorption profiles and therefore allow actives with sub-optimal absorption profiles (e.g. hydrophilic compounds) to penetrate through the upper *stratum corneum* and be released. There are many different examples of active compounds used in conjunction with nano-transporters (discussed further later) and examples include coenzyme Q10 which is currently the third most consumed nutritional supplement after fish oil and multivitamins (Cheuk, Shih et al. 2015) and used for its anti-oxidant properties therefore providing a protective effect in the skin (Farboud, Nasrollahi et al. 2011, Schwarz, Baisaeng et al. 2013), particularly useful in photo-protection (Yue, Zhou et al. 2010).

Thus far, this review has used the term 'nano-transporters' as a collective term for soluble nanoparticles which are used for the purposes of administering a substance (active of not) to the skin, through encapsulation for example through. However there are numerous different types of nano-transporters, each with different properties and associated benefits and draw backs. The following section provides a summary of these different particles.

1.6.1 Types of Nano-transporters

Table 2 summarises the various forms of nano-transporter which are available and includes information on the size range. As described, the technology of nano-transporters is ever evolving with first generation particles such as Solid Lipid Nanoparticles (SLN) being surpassed in functional attributes by second generation technologies such as Nanostructured Lipid Carriers (NLC). Below, more detail is given on the properties of each of these nano-transporters and table 3 provides a non-exhaustive list of typical active substances used in conjunction with nano-transporters.

TABLE 2: SUMMARY OF NANO-TRANSPORTER TYPES AND SIZE RANGES

Nano-Transporter	Size (nm)
Liposome	25- few hundred microns
Niosome	100 - 2000
Solid Lipid Nanoparticle	150-300
Nanostructured Lipid Carrier	80 - 300
Nanoemulsions	10-100
Nanoparticulates (nanospheres, nanocapsules)	3-1000

1.6.1.1 Liposomes

Liposomes are spherical, artificial vesicles composed of cholesterol and other natural phospholipids (Akbarzadeh, Rezaei-Sadabady et al. 2013). Of the different forms of nano-transporters, liposomes are perhaps one of the best known and earliest identified transporters, being first described by Bangham and Papahajopoulos in the 1966 (Bangham and Papahadjopoulos 1966, Li, Wu et al. 2011) and applied to dermatological applications in 1980 (Mezei and Gulasekharam 1980). The first liposomal cosmetic was called "Capture" and was an antiaging cream released by Dior in 1986. (Lohani, Verma et al. 2014)

Figure 2 shows a schematic diagram of a liposome and shows that the liposome forms a bilayer structure around an aqueous media (i.e. a payload) with the polar/ hydrophilic head regions of the lipid molecules orientating towards the aqueous phase (either internal – payload facing, or external –vehicle facing). The nonpolar/ lipophilic tail part of the molecule orientates towards the internal aspect of the bi-layer. This amphiphilic property of the liposome means that domains are available for the transport of lipophilic and hydrophilic substances making the liposome constructs a flexible carrier option for active ingredients of differing solubilities (Li, Wu et al. 2011). This coupled with the fact that liposomes encapsulate their active ingredient (as opposed to surface transportation) makes them suitable for the transportation of a wide variety of compounds such as vitamins for the regeneration of skin (Lohani, Verma et al. 2014). Other payloads (not including drugs) include Coenzyme Q10, vitamins such as vitamin A and E (Lohani, Verma et al. 2014), but also substances such as sodium ascorbyl phosphate (SAP) which is a photoreactive agent used in UV protection (Semalty, Semalty et al. 2010). Liposomes improve delivery of active compounds through a variety of modes including intact liposome skin penetration, vesicle adsorption and fusion onto the skin surface and interaction of liposome lipid and the *stratum corneum* (Li, Wu et al. 2011).

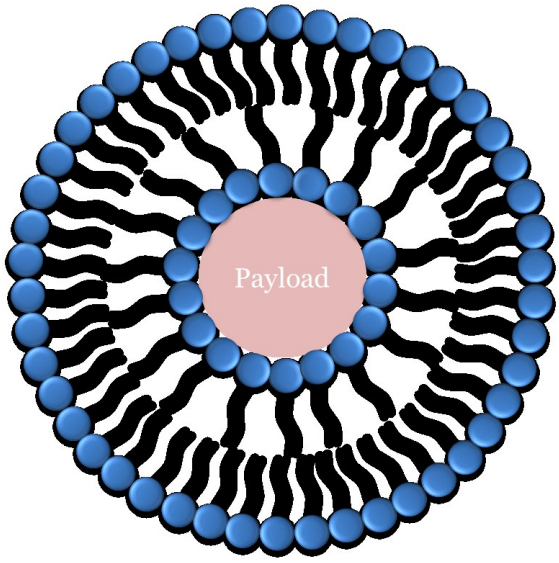


FIGURE 2: SCHEMATIC REPRESENTATION OF LIPOSOME.

Liposomes are extensively used as carriers, not only within the cosmetics and pharmaceutical industries but also within food and farming industries where they have been investigated for their use in encapsulating and protecting unstable compounds (e.g. antioxidants, antimicrobials etc.) (Akbarzadeh, Rezaei-Sadabady et al. 2013).

There are various methods of preparing liposomes but all methods consist of the following four basic stages (Akbarzadeh, Rezaei-Sadabady et al. 2013):

1. Drying down lipids from organic solvents
2. Dispersing the lipid in aqueous media
3. Purifying the resultant liposome
4. Analysing the final product

The loading of liposomes can be performed using passive or active loading techniques with a payload of choice. The specific benefits and drawbacks of liposomes were summarised by Akbarzadeh *et al.* (2013) and are reproduced below:

Advantages of Liposomes	Disadvantages of Liposomes
• Liposomes increased efficacy and therapeutic index of drugs	• Low Solubility
• Liposomes increased stability via encapsulation	• Short half-life
• Liposomes are said to be non-toxic, flexible, biocompatible, biodegradable, and non-immunogenic for systemic and non-systemic administrations	• Phospholipid can undergo oxidation and hydrolysis-like reaction
• Liposomes reduce the toxicity of the encapsulated agent	• Leakage and fusion of encapsulated molecules
• Flexibility to cope with site-specific ligands to achieve active targeting	• High production cost

1.6.1.2 Niosome

Niosomes are similar in structure to liposomes (evolving from them) with a closed bilayer of non-ionic surfactant in lamellar phase surrounding and aqueous cavity (Li, Wu et al. 2011). Compared to liposomes, niosomes have a lower production cost, a high entrapment efficiency and improved stability (Lohani, Verma et al. 2014) as well as avoiding issues such as hydration, oxidation, aggregation and fusion associated with liposomes (Li, Wu et al. 2011). As such, niosomes have been evaluated as carriers for a variety of drugs and cosmetics where they have been found to enhance residence time in the *stratum corneum* and dermis and thereby reduce systemic absorption (Sankhyan, Anchal et al. 2012, Lohani, Verma et al. 2014).

Pro-niosomes are nonionic-based surfactant vesicles which unlike conventional niosome preparations, are hydrated immediately before application or after application by the hydration of the skin to form niosomes. The result is a preparation that is even more stable as the niosomes are not generated until the point of use.

1.6.1.3 Solid Lipid Nano-Particles & Nanostructured Lipid Carriers

Lipid nanoparticles are derived from an oil-in-water (o/w) emulsion where the oil phase has been substituted by a solid lipid (Li, Wu et al. 2011). The term 'solid' refers to the fact that the lipid is solid at body temperature and so remains solid after administration into the body and therefore provides a matrix for controlled release over time (Muller, Shegokar et al. 2011). As shown in Figure 3, the lipid nanoparticles consist of a solid lipophilic matrix into which a payload of active molecules can be loaded and range in size from 50nm to 1000nm (Lohani, Verma et al. 2014) although most common applications are between 150-300nm (Muller, Shegokar et al. 2011).

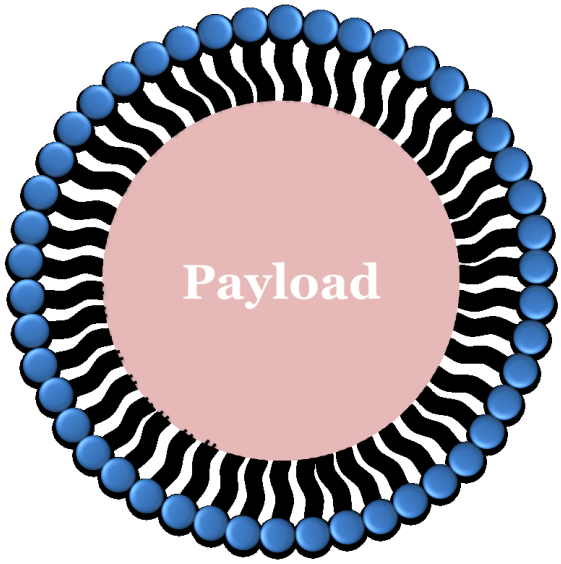


FIGURE 3: SCHEMATIC REPRESENTATION OF SOLID LIPID NANOPARTICLES.

Solid Lipid Nano-Particles (SLN) were developed in the early 1990's as an alternative to emulsions, liposomes etc. and are considered to be the first generation of whilst Nanostructured Lipid Carriers (NLC) where developed later to overcome issues with low loading capacity in SLN and are considered second generation technology (Muller, Shegokar et al. 2011). The major difference between SLN and NLC are the types of lipids they incorporate. SLN are made purely from solid lipids such as tristearine whilst NLC are made from a blend of solid and liquid lipids (oils) although the overall effect of both carriers are that they are both solid at body temperature. The addition of oils means that the formation of lipid crystals is distorted resulting in more imperfections and therefore allowing a greater uptake capacity for a payload. For example Jenning and Gohla (2001) found loading of retinol (vitamin A) was enhanced from 1% to 5% by formulating SLN from

mixtures of liquid and solid lipids (Jenning and Gohla 2001, Muller, Shegokar et al. 2011). As with all nano-transporters, there is considerable variety in composition of the different forms of SLN and NLC. Table 3 shows a list of the main ingredients used in the production of SLN and NLC, showing typical lips, oils and surfactants. As with any material, particle or otherwise, the biological interactions (e.g. toxicity, penetration profile etc.) are very much determined by the physicochemical properties and therefore it can be expected that an SLN (or indeed any other nano-transporter) with one composition may differ from another SLN of a completely different composition; irrespective of them both being classed as SLN.

There are two basic production methods for SLN which are high pressure homogenisation as developed by Muller and Lucks or the micro emulsion technique developed by Gasco (Muller, Radtke et al. 2002). With the high pressure homogenisation technique, the active compound to be loaded is dissolved or dispersed in a melted lipid. Using the hot production method, the lipid/ active compound mix is mixed with a hot surfactant solution at the same temperature and this 'pre-emulsion' mix is then passed through a high pressure homogeniser. The cold method, the lipid/ active compound mix

Is cooled and the solidified lipidic mass is ground to produce microparticles which are then dispersed in a cold surfactant solution by stirring. This suspension is then passed through a high pressure homogeniser to break down the microparticles into SLN (Muller, Radtke et al. 2002). Of the two methods, hot homogenisation is the most frequently used from laboratory scale batches producing 40-500ml of SLN up to large scale batches producing tonnage quantities.

TABLE 3: MAIN INGREDIENTS USED FOR THE PREPARATION OF LIPID NANOPARTICLES FOR TOPICAL APPLICATIONS.

Reproduced from Puglia & Bonina (2012) (Puglia and Bonina 2012)

Solid Lipids	Ref.
Bees wax	(Nikolic, Keck et al. 2011, Zhang and Smith 2011)
Carnuba wax	(Xia, Saupe et al. 2007, Mitri, Shegokar et al. 2011, Nikolic, Keck et al. 2011)
Cetyl alcohol (Lorol® C16)	(Teeranachaideekul, Muller et al. 2007)
Cetyl Palmitate (Predifac® ATO 5, Cutina® CP)	(Dingler, Blum et al. 1999, Jenning and Gohla 2001, Wissing, Lippacher et al. 2001, Wissing and Muller 2002, Kristl, Volk et al. 2003, Ricci, Puglia et al. 2005, Souto, Muller et al. 2005, Grabnar, Zajc et al. 2006, Shah, Date et al. 2007, Junyaprasert, Teeranachaideekul et al. 2009, Jensen, Magnussson et al. 2010, Jensen, Petersson et al. 2011, Mitri, Shegokar et al. 2011)
Glyceryl behenate (Compritol® 888 ATO)	(Jenning, Gysler et al. 2000, Maia, Mehnert et al. 2000, Jenning and Gohla 2001, Maia, Mehnert et al. 2002,

Solid Lipids	Ref.
	Sivaramakrishnan, Nakamura et al. 2004, Castelli, Puglia et al. 2005, Souto, Muller et al. 2005, Pople and Singh 2006, Puglia, Filosa et al. 2006, Souto and Muller 2006, Castro, Orefice et al. 2007, Xia, Saupe et al. 2007, Puglia, Blasi et al. 2008, Bhalekar, Pokharkar et al. 2009, Durand, Habran et al. 2010, Jensen, Magnussson et al. 2010, Castro, Oliveira et al. 2011, Gonzalez-Mira, Nikolic et al. 2011, Nikolic, Keck et al. 2011, Puglia, Sarpietro et al. 2011, Puglia, Bonina et al. 2012)
Glyceryl Cocoate (and) Hydrogenated Coconut oils (and) Ceteareth-25 (Softisan® 601)	(Sivaramakrishnan, Nakamura et al. 2004, Souto, Muller et al. 2005)
Glyceryl monostearate (Imwitor® 900, Geleol®)	(Sivaramakrishnan, Nakamura et al. 2004, Souto, Muller et al. 2005, Joshi and Patravale 2006, Shah, Date et al. 2007, Teeranachaideekul, Muller et al. 2007, Bhalekar, Pokharkar et al. 2009, Jensen, Magnussson et al. 2010, Zhang and Smith 2011)
Glyceryl palmitostearate (Precirol® ATO 5)	(Maia, Mehnert et al. 2000, Maia, Mehnert et al. 2002, Sivaramakrishnan, Nakamura et al. 2004, Munster, Nakamura et al. 2005, Liu, Hu et al. 2007, Stecova, Mehnert et al. 2007, Fang, Fang et al. 2008, Bhalekar, Pokharkar et al. 2009, Passerini, Gavini et al. 2009, Agrawal, Petkar et al. 2010, Jensen, Magnussson et al. 2010, Lin, Huang et al. 2010, Jensen, Petersson et al. 2011)
Glyceryl Trimyristate (Dynasan® 114)	(Maia, Mehnert et al. 2000, Wissing, Lippacher et al. 2001, Maia, Mehnert et al. 2002, Souto, Muller et al. 2005, Bhaskar, Anbu et al. 2009, Jensen, Magnussson et al. 2010, Pople and Singh 2012)

Solid Lipids	Ref.
Glyceryl Tripalmitate (Dynasan® 116)	(Jenning and Gohla 2001, Wissing, Lippacher et al. 2001, Wissing and Muller 2002, Souto, Wissing et al. 2004, Souto, Muller et al. 2005, Souto and Muller 2006, Shah, Date et al. 2007, Souto and Muller 2007, Ruktanonchai, Bejrapha et al. 2009, Jensen, Petersson et al. 2011)
Glyceryl Tristearate (Dynasan® 118)	(Souto, Muller et al. 2005, Jain, Jain et al. 2010, Jensen, Magnussson et al. 2010, Nikolic, Keck et al. 2011)
Hard fat (Witepsol® E 85, Suppocire® NA 150)	(Uner, Wissing et al. 2005, Padois, Cantieni et al. 2011)
Hydrogenated Coco-Glycerides (Softisan® 142)	(Jensen, Magnussson et al. 2010)
Hydrogenated Palm Oil (Softisan® 154)	(Nikolic, Keck et al. 2011)
PEG-8 Beeswax (Apifil®)	(Teeranachaideekul, Muller et al. 2007, Ruktanonchai, Bejrapha et al. 2009, Nikolic, Keck et al. 2011)
Stearic acid	(Iscan, Wissing et al. 2005, Jain, Chourasia et al. 2005, Puglia, Bonina et al. 2009, Silva, Santos et al. 2009, Trombino, Cassano et al. 2009, Gonzalez-Mira, Nikolic et al. 2011)
Stearyl alcohol	(Souto, Muller et al. 2005)
Oil	**Ref**
Caprylic/Capric Triglyceride (Miglyol® 812)	(Jenning, Gysler et al. 2000, Wissing and Muller 2002, Sivaramakrishnan, Nakamura et al. 2004, Souto, Wissing et al. 2004, Castelli, Puglia et al. 2005, Ricci, Puglia et al. 2005, Uner, Wissing et al. 2005, Puglia, Filosa et al. 2006, Souto and Muller 2006, Stecova, Mehnert et al. 2007, Xia, Saupe et al. 2007, Puglia, Blasi et al. 2008, Junyaprasert, Teeranachaideekul et al. 2009, Ruktanonchai, Bejrapha et al. 2009, Gonzalez-Mira, Nikolic et al. 2011, Mitri, Shegokar et al. 2011, Nikolic, Keck et al. 2011, Puglia, Sarpietro et al.

Solid Lipids	Ref.
	2011, Puglia, Bonina et al. 2012)
Castor oil	(Joshi and Patravale 2006, Gonzalez-Mira, Nikolic et al. 2011)
Squalene	(Fang, Fang et al. 2008, Lin, Huang et al. 2010)
Oleic acid	(Stecova, Mehnert et al. 2007, Silva, Santos et al. 2009, Agrawal, Petkar et al. 2010)

Surfactants	Ref
Poloxamer 188 (Lutrol® F68, Pluronic® F68)	(Maia, Mehnert et al. 2000, Maia, Mehnert et al. 2002, Kristl, Volk et al. 2003, Sivaramakrishnan, Nakamura et al. 2004, Castelli, Puglia et al. 2005, Jain, Chourasia et al. 2005, Ricci, Puglia et al. 2005, Pople and Singh 2006, Puglia, Filosa et al. 2006, Souto and Muller 2006, Stecova, Mehnert et al. 2007, Fang, Fang et al. 2008, Puglia, Blasi et al. 2008, Ruktanonchai, Bejrapha et al. 2009, Silva, Santos et al. 2009, Lin, Huang et al. 2010, Puglia, Sarpietro et al. 2011, Puglia, Bonina et al. 2012)
Polysorbate 80 (Tween® 80)	(Wissing and Muller 2002, Sivaramakrishnan, Nakamura et al. 2004, Jee, Lim et al. 2006, Joshi and Patravale 2006, Liu, Hu et al. 2007, Sanna, Gavini et al. 2007, Shah, Date et al. 2007, Teeranachaideekul, Muller et al. 2007, Fang, Fang et al. 2008, Bhalekar, Pokharkar et al. 2009, Bhaskar, Anbu et al. 2009, Passerini, Gavini et al. 2009, Puglia, Bonina et al. 2009, Agrawal, Petkar et al. 2010, Jain, Jain et al. 2010, Jensen, Magnussson et al. 2010, Pople and Singh 2010, Jensen, Petersson et al. 2011, Mitri, Shegokar et al. 2011)
Polysorbate 20 (Tween® 20)	(Iscan, Wissing et al. 2005, Joshi and Patravale 2006,

Surfactants	Ref
	Trombino, Cassano et al. 2009)
Tyloxapol	(Wissing, Lippacher et al. 2001, Wissing and Muller 2002, Souto, Wissing et al. 2004, Grabnar, Zajc et al. 2006, Souto and Muller 2006, Souto and Muller 2007)
Polyglyceryl-3 Methylglucose Distearate (TEGO® CARE 450)	(Dingler, Blum et al. 1999, Wissing, Lippacher et al. 2001, Wissing and Muller 2002, Uner, Wissing et al. 2005, Teeranachaideekul, Muller et al. 2007, Junyaprasert, Teeranachaideekul et al. 2009)
Sodium cholate	(Maia, Mehnert et al. 2002, Souto and Muller 2006)
Phosphatidylcholine (Epikuron® 200, Phospholipon® 80/H)	(Kristl, Volk et al. 2003, Grabnar, Zajc et al. 2006, Shah, Date et al. 2007, Fang, Fang et al. 2008, Padois, Cantieni et al. 2011)
Soybean lecithin (Lipoid® S75)	(Maia, Mehnert et al. 2000, Jain, Chourasia et al. 2005, Liu, Hu et al. 2007)

The beneficial properties of SLN and NLC for dermal applications such as use as carriers in cosmetics and cosmeceutical products has been summarised as follows by Li and colleagues (2011)(Li, Wu et al. 2011):

a. They are composed of biocompatible and biodegradable lipids which exhibit low toxicity (Pardeike, Hommoss et al. 2009);
b. They are reported to enhance dermal penetration (Wissing and Muller 2003);
c. They provide controlled release profiles for many substances (Jenning, Schafer-Korting et al. 2000);
d. They are able to enhance the chemical stability of compounds sensitive to light, oxidation and hydrolysis (Teeranachaideekul, Muller et al. 2007), thereby improving shelf-life.

Due to these beneficial properties, numerous active ingredients have been incorporated into SLN and NLC and examples of these relevant to cosmetic applications can be found in table 3.

The stabilisation effect of SLN is a very useful property, especially when using sensitive molecules such as retinol. When exposed to light and oxygen, retinol decomposes to a variety of structures and this can be overcome by stabilisation of the molecule by incorporation into an SLN. As summarised by Müller and colleagues (Muller, Radtke et al. 2002), studies have shown that the stabilisation effect can be influenced by the choice of lipids as well as surfactant meaning that particularly for susceptible active compounds; the choice of materials can have an important bearing on product performance.

1.6.1.4 Nanoemulsions

Nanoemulsions consist of colloidal oil droplets dispersed in an immiscible liquid (Morales, Valdes et al. 2015). The lipid phase of nanoemulsions can be composed of natural or synthetic oils such as Witepsol® (Cremer Oleo Divisions), Myritol® (BASF), isopropyl myristate and examples of the surfactants include polysorbate and Polyethylene glycol (PEG). The aqueous phase of nanoemulsions can be composed simply of water of co-surfactants such as glycerine and if required, organic solvents may be added and evaporated as an additional stage in production (Morales, Valdes et al. 2015).

The production of the liquid-in-liquid dispersion requires an energy input and this is either classified as a high energy or low-energy. These differ quite markedly as the high energy method works by breaking up the course macroscopic phase into smaller, nano-scale droplets using high energy devices such as high-shear stirrers, ultrasonic homogenisers and colloid mills. The Low energy methods however do not serve to size reduce larger droplets but instead rely on the spontaneous formation of nanoemulsions as a result of changes in interfacial properties (Morales, Valdes et al. 2015).

Nanoemulsions are valued because of their sensorial and biophysical properties and are said to be stable with studies indicating that then can be stable for 15 years which is an advantageous property for carriers in cosmetic products (Li, Wu et al. 2011).

The specific mechanism of penetration enhancement of nanoemulsions is not yet fully understood and theories include the small droplet size providing close interaction between the active payload and skin cells, the improved hydration of skin due to the occlusive effects enhancing penetration and as well as the surfactant and co-surfactants in the preparation acting as penetration enhancers.

1.6.1.5 Nanoparticulates (nanospheres, nanocapsules)

Nanocapsules are hollow vesicles in which a polymeric membrane surrounds and encapsulates a liquid core (usually an oil) whilst nanospheres (nanoparticles) are solid polymeric particles. Various polymers can be used in the production of nanoparticulates such as cellulose derivatives, poly (alkylcyanoacrylates), poly (methylidene malonate), polyorthoesters, polyanhydrides and polyesters such as poly (lacticacid), poly (glycolic acid) and poly (ε-caprolactone) (Hommoss 2008). See table 4 for a more comprehensive list of polymers used in the construction of polymeric nanoparticles for transdermal delivery. The oily core of nanocapsules can be comprised of saturated or nonsaturated natural or synthetic oils such as Miglyol, tricaprulin, ethyl laureate, ethyl oleate, corn oil, sunflower oil, sesame oil as well as others (Morales, Valdes et al. 2015). They can be formed using a variety of processes including interfacial polymerisation, solvent diffusion methods, solvent evaporation, as well as degredative methods such as high pressure homogenisation or micro fluidation.

For the nanocapsules, active agents can either be dissolved in the oily centre (lipophilic agents) or attached onto the polymer surface whilst for polymeric nanoparticles, the agents can be absorbed to the surface or incorporated into the polymer matrix. A range of active cosmetic ingredients such as vitamin A (Jeong, Son et al. 2015, Morales, Valdes et al. 2015), coenzyme Q10, collagen, nano-gold (Li, Wu et al. 2011), sunscreen agents (Alvarez-Roman, Barre et al. 2001), vitamin K1 (da Silva, Contri et al. 2013) as well as others. One of the benefits of nanocapsules and polymeric nanoparticles is that they can provide greater protection for sensitive active payloads than liposomes and emulsions (Hommoss 2008) and the first nanocapsules-based cosmetic was produced by L'Oreal in 1995 (Poletto, Beck et al. 2011, Lohani, Verma et al. 2014).

TABLE 4: EXAMPLE OF POLYMER MATERIALS USED IN POLYMERIC NANOPARTICLES FOR TOPICAL DELIVERY.

Adapted from Fang, Aljuffali et al. (2014)

Polymer Materials	Diameter (nm)	Ref.
Polystyrene	20, 200	(Alvarez-Roman, Naik et al. 2004)
Polystyrene + HEMA	37-74	(Wu, Griffin et al. 2009, Wu, Price et al. 2009, Kimura, Kawano et al. 2012)
Polystyrene (Fluoresbrite®)	50	(Kimura, Kawano et al. 2012)
Polyvinylalcohol + PLA + PGA	320	(Lademann, Richter et al. 2007)
PLA	228, 365	(Rancan, Papakostas et al. 2009)
PLGA	122, 230, 300, 470, 643, 860	(Patzelt, Richter et al. 2011)
Ethyl cellulose	96	(Morgen, Lu et al. 2011)
Ethyl cellulose + chitosan	113 – 705	(Arayachukeat, Wanichwecharungruang et al. 2011)
Ethyl cellulose	50, 100, 500, 1000	(Abdel-Mottaleb, Moulari et al. 2012)
Porous nylon	5000	(Sumian, Pitre et al. 1999)
Polystyrene	750 – 6000	(Toll, Jacobi et al. 2004)
PLGA + chitosan	170, 180	(Mittal, Raber et al. 2013)
PLA	150	(Mattheolabakis, Lagoumintzis et al. 2010)
Polystyrene	40, 750, 1500	(Vogt, Combadiere et al. 2006)
Polystyrene	40, 200	(Mahe, Vogt et al. 2009)
Chitosan	150 - 270	(Huang, Li et al. 2009)

HEMA: Poly-2-hydroxyl methacrylate; PGA: Polyglycolic acid; PLA: Polylactic acid; PLGA: Pol(lactic-co-glycolic) acid.

1.6.2 Summary

Nano-transporters are a diverse array of vehicles, which serve the dual purpose of both protecting active ingredient payloads and improving their delivery to the skin. Compositionally they can differ markedly and can include polymers and lipids as well as a diverse range of surfactants. The different forms of nano-transporters such as *liposomes, niosomes, SLN (Solid Lipid Nanoparticles), NLC (Nanostructured Lipid Carriers), nanoemulsions, nanospheres/nanocapsules* etc. are classified to an extent on structure and within these different groupings, may differ in terms of their specific compositions meaning that, for example, not all nano-emulsions have exactly the same mix of lipids and surfactants.

This can have implications on toxicity because, for example, liposomes are composed primarily of phospholipids generally regarded as safe and indeed many of which are found in the skin itself, niosomes contain surfactants which could act to irritate the skin (Li, Wu et al. 2011). A similar point was raised in relation to nanoemulsions as a large amount of surfactant is required during their production and as such, the choice of surfactants should be made judiciously (Li, Wu et al. 2011). This may also have implications in terms of drawing broad conclusions addressing specific types of

nano-transporter as there may in fact be considerable variability within a type. The same issue is seen within the wider nanotoxicology literature whereby variations in composition, shape, size, surface area, surface coating and many other physicochemical properties can effect toxicity (Donaldson, Schinwald et al. 2012, Donaldson and Poland 2013, Braakhuis, Park et al. 2014) and therefore hinder the drawing of general statements of toxicity for a type of nanomaterial such as carbon nanotubes or metal oxides. Instead, a more nuanced and specific approach is often needed whereby specific groups of characteristic or physicochemical properties (such as size) may better describe observed effects rather than classes of material per se.

Another interesting and challenging component of nano-transporters is the intrinsic link between carrier and payload. Whilst conventional solid, insoluble nanoparticles such as TiO_2 can be modified (e.g. surface coating) and thereby display modified toxicity profiles, a nano-transporter can be radically altered by the nature of its active payload. For example, a nano-transporter carrying a cytotoxic chemotherapeutic may show differing toxicity than the same transporter carrying a more benign payload such as an anti-oxidant. Therefore, in evaluating nano-transporters it is imperative to try and distinguish between the intrinsic toxicity of the nano-transporter itself and that of the payload (which can be subject to change and reformulation). Table 5 provides a non-exhaustive list of active ingredient payloads used with nano-transporters of different types in cosmetics.

TABLE 5: EXAMPLE OF ACTIVE INGREDIENT PAYLOADS USED WITH NANO-TRANSPORTERS IN COSMETICS.

Active Substance	Carrier Type	Reference
Coenzyme Q10	Liposomes, SLN, NLC, nanocapsules	(Teeranachaideekul, Souto et al. 2007, Terroso, Kulkamp et al. 2009, Obeidat, Schwabe et al. 2010, Farboud, Nasrollahi et al. 2011, Gokce, Korkmaz et al. 2012, Korkmaz, Gokce et al. 2013, Schwarz, Baisaeng et al. 2013)
Retinol (vitamin A)	Liposomes, nanocapsules	(Li, Wu et al. 2011, Lohani, Verma et al. 2014, Jeong, Son et al. 2015)
Ascorbic Acid (vitamin C)	Liposomes, SLN, NLC, nanoemulsions	(Uner, Wissing et al. 2005, Palma, Manzo et al. 2007, Teeranachaideekul, Muller et al. 2007, Teeranachaideekul, Souto et al. 2008, Fathi-Azarbayjani, Qun et al. 2010, Saino, Monti et al. 2010, Li, Wu et al. 2011, Lautenschläger 2012, Janesirisakule, Sinthusake et al. 2013)
Vitamin E	Liposomes	(Lohani, Verma et al. 2014)
Vitamin K	Liposomes, nanocapsules	(da Silva, Contri et al. 2013, Lohani, Verma et al. 2014)
Lycopene	Liposomes	(Lohani, Verma et al. 2014)
Carotenoids	Liposomes	(Lohani, Verma et al. 2014)
Caffeine	Nanoemulsions	(Li, Wu et al. 2011)
Ceramide	Liposomes	(Byoung Kee Jo, Gi Woong Ahn et al. 2005)
Sunscreen agents	Nanocapsules, liposomes	(Alvarez-Roman, Barre et al. 2001, Golmohammadzadeh, Imani et al. 2011)

2 Methodology

2.1 Approach

The primary objective of Phase I was to carry out an extensive literature search and assessment of the reliability and relevance of studies regarding the uses and effects of nano-enabled technologies in cosmetics. This initial phase of the project was divided into three component tasks in order to achieve these objectives:

- **Task 1.1** involved the development and approval of the literature search strategy. The development of the search strategy was carried out by IOM/SAFENANO with input from both DHI and the Danish EPA, with overall approval sought from the Danish EPA;

- **Task 1.2** aimed to carry out the approved literature search in a number of databases and identify studies of relevance for consideration in the project;

- **Task 1.3** aimed to assess the relevance and reliability of the studies identified within task 1.2 using a standardised approach for critical review of each information source.

This report marks the culmination of phase II, a report in to the dermal penetration and toxicity of nano-transporters and role physicochemical properties such as (but not restricted to) size, surface charge, composition, shape, etc. play in dermal penetration/ absorption and toxicity.

2.2 Literature Search Strategy

The peer-reviewed literature provides the most substantial resource of information for this review. In order to collate relevant references from the literature, a systematic literature search strategy was developed in Task 1.1 based on the main concepts being examined in the project.

As a first stage in the development of the literature search strategy, key search terms and phrases of relevance to the project scope were derived from recognised standard terminology and nomenclature (e.g. current documents from the BSI and ISO/CEN). Following consultation with and further input from members of the Danish EPA and DHI, a strategy for the systematic identification of potentially relevant studies to assessing the effects and uses of nano-transporters in cosmetics was agreed. A systematic matrix-based search strategy was developed by using Boolean logic operators (AND, OR and NOT) to combine the key search terms into defined search strings. The search strategy is detailed below and consists of the following search string:

> ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter" OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND "Search Term"

The key search terms were split into those assessing *effects* and those relating to *uses* of nano-transporters. The search terms relating to *effects* applied sequentially with the search string above as the “Search term” were:

"absorption"
"ADME"
"allergen"
"allergenic"
"allergic"
"allergy"
"assay"
"bioavailability"
"biodistribution"
"carcinogen"
"carcinogenicity"
"clearance"
"corrosion"
"corrosivity"
"cutaneous"
"cytotoxicity"
"derma-abrasion"
"dermal"
"disease"
"distribution"
"dose-response"
"dosimetry"
"excretion"
"exposure"
"genotoxicity"
"hazard"
"human health"
"in silico"
"*in vitro*"
"*in vivo*"
"irritation"
"metabolism"
("modeling" OR "modelling")
"mutagen"
"mutagenicity"
"PBPK"
"penetration"
"permeation"
"persistence"
"phototoxicity"
"PK/TK"
"QSAR"
"reprotoxicity"
"risk"
"safety assessment"
"safety evaluation"
"skin"
"susceptibility"
"systemic"
"test guideline"
"test method"
"testing strategy"
"toxic effect"
"toxicity"
"toxicokinetics"
"toxicological"
"toxicology"
"translocation"
"uptake"
("characterisation" OR "characterization")
("physicochemical" OR "physico-chemical")
("sensitisation" OR "sensitization")

The key search terms relating to *uses* were:

"anti-age"
"anti-aging"
"anti-wrinkle"
"consumer product"
"cosmetic application"
"cosmetics regulation"
"cream"
"cutaneous delivery"
"dermal delivery"
"dermatological"
"dermatology"
"firming"
"formula"
"health care product"
"inventory"
"lotion"
"nanodermatology"
"patent"
"personal care"
"personal hygiene"
"product"
"serum"
"skin care"
"skin cream"
"sun block"
"sun cream"
"sun lotion"
"sunscreen"
"survey"
"topical application"
"wrinkle"
("cosmetic" OR "cosmetics")
("moisturiser" OR "moisturizer")
("use" OR "uses")
("utilisation" OR "utilization")

In certain instances, the combination of search string and search term provided an unacceptably high number of hits (owing to a high number of irrelevant references) and, in such cases, the search string was further refined to reduce the number of hits to those which are relevant.

For the search on *effects,* the following refined search string, which has the addition of the words "dermal" and "skin" was employed to further reduce the number of hits to those which are relevant:

> **REFINED 'EFFECTS' SEARCH 1:** ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter"

OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND ("dermal" OR "skin") AND "Search Term".

Where the number of hits was still considered too high, additional refinement to the *effects* search string was made by including the words "cosmetic" and "cosmetics" and "personal care" as follows:

REFINED 'EFFECTS' SEARCH 2: ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter" OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND ("dermal" OR "skin") AND ("cosmetic" OR "cosmetics" OR "personal care") AND "Search Term".

For the search on *uses,* the following refined search string which has the addition of the words "application", "use", "uses", "utilisation", "utilization", and "product" was employed to further reduce the number of hits to those which are relevant:

REFINED 'USES' SEARCH 1: ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter" OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND ("application" OR "use" OR "uses" OR "utilisation" OR "utilization" OR "product") AND "Search Term"

Where the number of hits was still considered too high, additional refinement to the *uses* search string was made by including the words "dermal" and "skin" as follows:

REFINED 'USES' SEARCH 2: ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter" OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND ("application" OR "use" OR "uses" OR "utilisation" OR "utilization" OR "product") AND ("dermal" OR "skin") AND "Search Term".

2.3 Identification of Studies

In order to gain a comprehensive summary of the available evidence, in Task 1.2 the final search strategy was undertaken in the following databases:

- The United States National Library of Medicine 'PubMed';
- Thomson Reuters 'Web of Science'.

The references obtained from each individual search were then collated into in-house reference manager software (Endnote). An inherent consequence of performing multiple searches across several databases is the inclusion of duplicate references in the collated dataset. Endnote was

therefore used to remove duplicates in the collated set of references to form a final "computational dataset". The numbers of references obtained from each search in the two databases are presented in Tables 1 and 2 overleaf. Overall, following the removal of duplicates, the computational dataset consisted of 1260 unique references for the "effects" search, 957 unique references for the "uses" search and 1940 unique references when "effects" and "uses" searches were combined.

Results of a systematic matrix-based search strategy will inherently contain references which are not relevant to the scope of the project. The titles and, where required due to non-specific titles, abstracts of the references in the computational dataset were therefore manually screened by an expert reviewer to identify any which were considered not to be of relevance to the aims of the project. For example, the systematic searches identified a 2010 publication by Jafari *et al.*[2] investigating the use of nanocapsules containing inhibitors for the prevention of corrosion of copper surfaces, considered to be outside of the scope of the project.

Those determined to be of potential relevance for the project in terms of scope were selected by the reviewer and included in a "screened dataset" for taking forward into Task 1.3. In total, 286 references from the peer-reviewed literature were included in the screened dataset for further appraisal and amongst them, 181 were patents. These references and patents involved both cosmetic and clinical applications of nano-transporters and a preliminary review of these showed around 30% of the journal publications (89 in total) to relate to cosmetic applications. To maintain the focus of the project, only the publications referring to cosmetic applications were brought forward for appraisal and incorporation into the project database (a list of the 89 appraised publications and a link to the Access database from the literature search can be found in Appendix 1).

In addition to searching the peer-reviewed literature in the above databases, the project team also searched for published information and reports from relevant organisations such as the Scientific Committee on Consumer Safety (SCCS), Commonwealth Scientific and Industrial Research Organisation (CSIRO), Organisation for Economic Co-operation and Development Working Party on Manufactured Nanomaterial (OECD WPMN), Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), as well as standards organisations such as ISO, BSI and CEN, FP6/7 projects, and other international organisations such as the National Institute for Occupational Safety and Health (NIOSH). In addition to searching specific websites for publications, a general search was carried out using Google to ensure coverage of additional relevant literature. Based on the results of this searching, a further 5 references were included in the screened dataset. Thus, a total of 94 information sources were taken forward for further appraisal in Task 1.3.

[2] Jafari, A.H., S.M. Hosseini, and E. Jamalizadeh, Investigation of Smart Nanocapsules Containing Inhibitors for Corrosion Protection of Copper. Electrochimica Acta, 2010. 55(28): p. 9004-9009.

TABLE 6. NUMBER OF REFERENCES OBTAINED FOR THE FINAL SEARCH STRATEGY IN PUB MED AND WEB OF KNOWLEDGE IN TERMS OF "EFFECTS"

Search term	No. of references obtained for the final search strategy: ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter" OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND "*Search Term*"	
	PubMed	**Web of Science**
"ADME"	1	1
"allergen"	2	8
"allergenic"	0	4
"allergic"	8	49
"allergy"	9	62*
"carcinogen"	1	11
"carcinogenicity"	0	0
"corrosion"	4	40
"corrosivity"	0	0
"derma-abrasion"	0	0
"dermal"	59	136
"dose-response"	91	89
"dosimetry"	5	12
"excretion"	12	18
"genotoxicity"	8	25
"hazard"	8	8
"human health"	18	20
"in silico"	4	6
"irritation"	37	84
("modeling" OR "modelling")	50	6
"mutagen"	0	1
"mutagenicity"	0	0
"PBPK"	0	0
"persistence"	9	19
"phototoxicity"	18	27
"PK/TK"	0	0
"QSAR"	0	0
"reprotoxicity"	0	0
"risk"	43	15*
"safety assessment"	2	6
"safety evaluation"	10	9

Search term	No. of references obtained for the final search strategy: ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter" OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND "*Search Term*"	
	PubMed	**Web of Science**
"susceptibility"	26	69
"test guideline"	0	0
"test method"	0	0
"testing strategy"	0	0
"toxic effect"	6	16
"toxicokinetics"	1	1
"toxicological"	22	48
"toxicology"	41	26*
"translocation"	22	55
("sensitisation" OR "sensitization")	5	10
"absorption"	13†	38†
"assay"	22*	47*
"bioavailability"	26*	88*
"biodistribution"	2*	9*
"clearance"	7*	13*
"cutaneous"	11†	10†
"cytotoxicity"	17*	41*
"disease"	28*	13†
"distribution"	41*	15†
"exposure"	20*	33*
"*in vitro*"	14†	31†
"*in vivo*"	87*	13†
"metabolism"	10†	15†
"penetration"	77*	45†
"permeation"	6†	24†
"sensitivity"	13*	17*
"skin"	45*	11†
"systemic"	32*	4†
"toxicity"	47*	75*
"uptake"	21*	50*
("characterisation" OR "characterization")	52*	9†
("physicochemical" OR "physico-chemical")	36*	97*
TOTAL (inc. duplicates)	2728	
TOTAL (exc. duplicates)	1260	

* Refined Effects Search 1 employed, incorporating 'AND ("dermal" OR "skin")' into the search string.

† Refined Effects Search 2 employed, incorporating 'AND ("dermal" OR "skin") AND ("cosmetic" OR "cosmetics" OR "personal care")' into the search string.

TABLE 7. NUMBER OF REFERENCES OBTAINED FOR THE FINAL SEARCH STRATEGY IN PUB MED AND WEB OF KNOWLEDGE IN TERMS OF "USES"

Search term	No. of references obtained for the final search strategy: ("nanocarrier" OR "nano-carrier" OR "nano carrier" OR "nanostructured carrier" OR "nanocapsule" OR "nano-capsule" OR "nano capsule" OR "nanostructured carrier" OR "nanoemulsion" OR "nano-emulsion" OR "nano emulsion" OR "nanostructured emulsion" OR "nanosome" OR "nanoliposome" OR "nanostructured liposome" OR "nanosphere" OR "nano-sphere" OR "nano sphere" OR "nanostructured sphere" OR "nanotransporter" OR "nano-transporter" OR "nano transporter" OR "nanostructured transporter" OR "lipid nanoparticle") AND "*Search Term*"	
	PubMed	**Web of Science**
"anti-age"	0	0
"anti-aging"	2	16
"anti-wrinkle"	2	11
"consumer product"	1	3
("cosmetic" OR "cosmetics")	95	102†
"cosmetics regulation"	0	0
"cutaneous delivery"	1	4
"cream"	21	62*
"dermal delivery"	10	30
"dermatology"	18	71
"dermatological"	14	73*
"firming"	0	3
"formula"	25	83*
"health care product"	0	0
"inventory"	0	1
"lotion"	2	88
("moisturiser" OR "moisturizer")	1	8
"nanodermatology"	0	0
"patent"	10	13
"personal care"	8	25
"personal hygiene"	0	1
"product"	97	109†
"skin care"	3	32
"skin cream"	0	0
"sun block"	0	0
"sun cream"	0	0
"sun lotion"	0	0
"sunscreen"	10	46

"survey"	5	17
"topical application"	41	72
("use" OR "uses")	43	68†
("utilisation" OR "utilization")	64	70*
"wrinkle"	2	47
"cosmetic application"	1	6
"serum"	38*	12†
TOTAL (inc. duplicates)	1587	
TOTAL (exc. duplicates)	957	

* Refined Use Search 1 employed, incorporating 'AND ("application" OR "use" OR "uses" OR "utilisation" OR "utilization" OR "product")' into the search string.

† Refined Use Search 2 employed, incorporating 'AND ("application" OR "use" OR "uses" OR "utilisation" OR "utilization" OR "product") AND ("dermal" OR "skin")' into the search string.

2.4 Assessment of the Relevance & Reliability of the Identified Studies

A standardised process was developed and used to appraise each information source in the screened dataset in terms of its relevance and reliability for use in the project. This process was facilitated in an Access Database, a screenshot of which is provided in Figure 5.

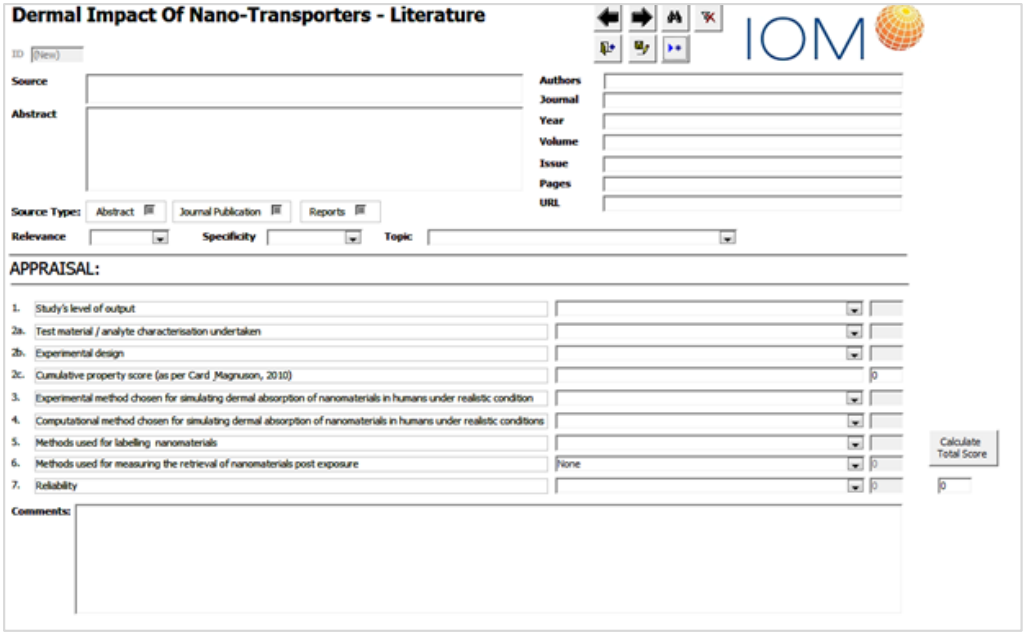


FIGURE 5. SCREENSHOT OF THE ACCESS DATABASE TEMPLATE USED TO APPRAISE THE SCREENED DATASET

For each information source, an entry in the database was created providing a full reference and an indication of the source type (i.e. abstract, journal publication or report). Based on a more comprehensive assessment of the information available in the abstract, an assessment of the relevance (high, low) and specificity (high, low) of the information source was made by the reviewer. The criteria for relevance were based on whether or not the publication addressed the topic of nano-transporters for cosmetic uses. In terms of specificity, this was based on the question of whether the article specifically dealt with dermal penetration/ toxicity or if this was only part of the considerations of the article.

The standardised appraisal framework, detailed in Table 8, was employed to help rank the studies when considering their impact and contribution to the objectives of the project and provide a basis for the consistent assessment of the evidence base identified. The framework draws principally on two published methodologies for literature assessment:

- Klimisch *et al.* (1997), which describes a standard base set of criteria and corresponding codes (Klimisch codes) for assessing the quality and potentially the validity of toxicological and eco-toxicological scientific studies and data sets;

- Card and Magnuson (2010), which describes a two-step process where application of Klimisch codes to assess study reliability comprises the first step. The second step focuses on assessing the level of nanomaterial characterisation which was done by comparing the information presented within a study against ten parameters of nanomaterial characterisation. In this approach, the greater the number of nanomaterial characteristics described within a study, the greater the score.

The developed framework consists of 7 criteria against which the information source is assessed. Each criterion comprised of a set of scored categories. These individual scores are then summed to provide an overall score for the study in terms of its relevance and reliability allowing the identification of studies considered to be of highest quality and impact. Whilst those studies obtaining a high score would be considered to be of the highest quality and impact, those obtaining a low score will not be discounted but instead will be considered in light of any caveats which has impacted on its score.

A summary diagram indicating the tasks and the sequential approach to refining the dataset with results is shown below in Figure 6.

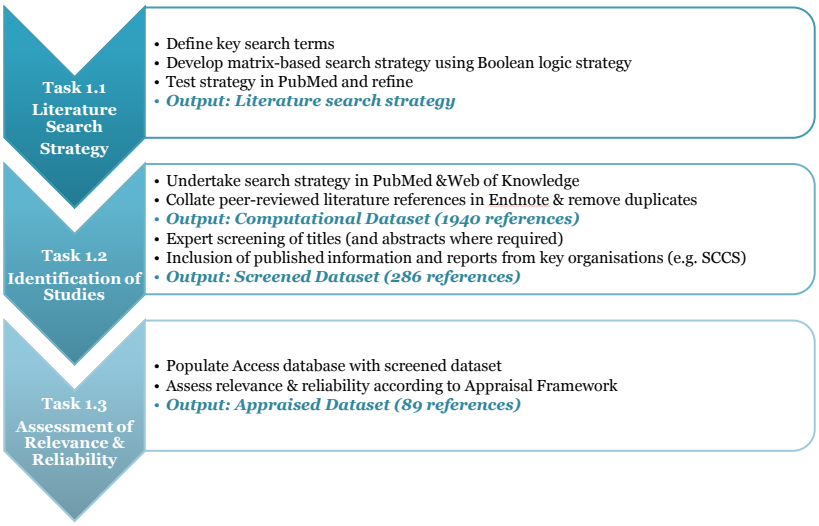


FIGURE 6. OVERVIEW OF PHASE I TASKS AND APPROACH

In addition to the 7 criteria for appraisal, the database also included a topic descriptor to identify studies relating to the uses of these materials, dermal absorption/ penetration, dosimetry, persistence and toxicity (e.g. acute, chronic, localised, systemic etc.).

TABLE 8. APPRAISAL FRAMEWORK USED TO ASSESS THE IDENTIFIED STUDIES

1. Study's level of output	6	Systematic review
	5	Review
	4	Journal publication
	3	Final report
	2	Interim report
	1	Abstract
2a. Test material / analyte characterisation undertaken	4	Determined & reported, with *in situ* characterization
	3	Determined & reported - characterized as supplied
	2	Reported - as per label
	1	Inferred from data presented
	0	None
2b. Experimental design	3	Internationally accepted standard method e.g. OECD TG
	2	Non-standard, documented method
	1	Undocumented method
	0	No experimental detail provided
2c. Cumulative property score (as per Card & Magnuson, 2010)	A score of 1 is accrued per key physico-chemical property reported	
3. *Experimental* method chosen for simulating dermal interaction (e.g. absorption/ toxicity) of nanomaterials in humans under realistic conditions	5	Human
	4	*In vivo* - Direct application
	3	*Ex vivo* – Skin absorption models
	2	*In vitro* – Skin absorption models (multi-cellular e.g. EPiSkin)
	1	*In vitro* – Skin absorption models (single cell e.g. keratinocytes)
4. *Computational* method chosen for simulating dermal absorption of nanomaterials in humans under realistic conditions	3	PBPK model (multi-compartment)
	2	PK/TK model (multi-compartment)
	1	PK/TK model (single-compartment)
5. Methods used for labelling nanomaterials	4	Validated with post exposure confirmation
	3	Validated without post exposure confirmation, or non-validated with post exposure confirmation
	1	Non-validated without post exposure confirmation
	0	None
6. Methods used for measuring the retrieval of nanomaterials post exposure (e.g. in skin, blood, outlying organs etc.)	5	Validated method for *in situ* detection
	4	Non-validated method for *in situ* detection
	3	Validated method for *ex situ* detection e.g. mass balance study
	2	Semi-quantitative detection e.g. local accumulation only
	1	Qualitative detection e.g. histopathology with microscopy
	0	None

7. Specificity & relevance to assessing the dermal interaction of soluble nano-transporters	3	High specificity & High relevance
	2	Low specificity but High relevance
	1	High specificity but Low relevance
	0	Low specificity & Low relevance
8. Reliability	4	Reliable, without restriction
	3	Reliable, but with restriction
	2	Not reliable
	1	Not assignable

3 Use of Nano-Transporters in Cosmetics

The literature search regarding the use and effects of nano-enabled technologies in cosmetics has been performed as described and the overall results in terms of number of hits presented. This section will intend to give a preliminary overview of the uses of nano-enabled technologies in cosmetics. The definition from the EC indicates that: 'Cosmetic product' means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours (*EU Cosmetic Regulation 1223/2009/EC, Art 2. 1)*.

Given their modified and enhanced properties, nanotechnologies have been of interest to the cosmetic industry for many years. Examples of such uses of nanomaterials include:

- TiO_2 and ZnO nanoparticles are typically used as UV filters in sunscreens.
- Nanogold and nanosilver particles are used as antibacterial and antifungal agents but also sometimes as carrier agent.
- Fullerenes are used for their anti-oxidants properties to protect from aging.
- Finally nanotechnologies are used as carrier systems for delivery of agents.

In relation to nano-transporters, these have been seen as particularly attractive for the cosmetic industry since they present many advantages including:

- The protection of sensitive agents
- Enhanced dermal penetration of active ingredients
- The controlled release
- A reduction in the amount of agents and additives
- Longer shelf life and hence greater product effectiveness

Currently, nano-enabled technologies are found in a wide range of cosmetic products and Table 9 provides a list of example cosmetic products currently on the market containing lipid nanoparticles and tables 13 and 14 within Appendix 2 provide a further, more comprehensive list of nano-enabled cosmetic products employing carrier technologies. As can be seen in tables 9, 13 & 14, there is considerable diversity of active ingredients carried by nano-enabled technologies for different purposes. For example, Retinol A typically is used as an anti-wrinkle agent whilst biomolecules such as vitamins A and E as well as antioxidants such as CoQ10 and carotenoids are commonly used in skin care cosmetics and the nano-enabled technologies provide overall better effectiveness of the active ingredient.

The major classes of what have been termed 'nanocosmeceuticals' (Lohani, Verma et al. 2014) are discussed in the next section.

TABLE 9. EXAMPLES OF COSMETIC PRODUCTS CURRENTLY ON THE MARKET CONTAINING LIPID NANOPARTICLES. Reproduced from Pardeike, Hommoss et al. (2009)

Product name	Producer/ distributor	Release	Main active ingredients
Cutanova Cream Nano Repair Q10	Dr. Rimpler	10/2005	CoQ10, polypeptide, hibiscus extract, ginger extract, ketosugar
Intensive Serum NanoRepair Q10		10/2005	CoQ10, polypeptide, mafane extract
Cutanova Cream NanoVital Q10		06/2006	CoQ10, TiO_2, polypeptide, ursolic acid, oleanolic acid, sunflower seed extract
SURMER Crème Legère Nano-Protection	Isabelle Lancray	11/2006	Kukuinut oil, Monoi Tiare Tahiti®, pseudopeptide, milk extract from coconut, wild indigo, noni extract
SURMER Crème Riche Nano-Restructurante			
SURMER Elixir du Beauté Nano-Vitalisant			
SURMER Masque Crème Nano-Hydratant			
NanoLipid Restore CLR	Chemisches Laboratorium	04/2006	Black currant seed oil containing -3 and -6 unsaturated fatty acids
Nanolipid Q10 CLR	Dr. Kurt Richter, (CLR)	07/2006	Coenzyme Q10 and black currant seed oil
Nanolipid Basic CLR		07/2006	Caprylic/capric triglycerides
NanoLipid Repair CLR		02/2007	Black currant seed oil and manuka oil
IOPE SuperVital Cream Serum Eye cream Extra moist softener Extra moist emulsion	Amore Pacific	09/2006	Coenzyme Q10, -3 und -6 unsaturated fatty acids
NLC Deep Effect Eye Serum	Beate Johnen	12/2006	Coenzyme Q10, highly active oligo saccharides
NLC Deep Effect Repair Cream			Coenzyme Q10, highly active oligo saccharides
NLC Deep Effect Reconstruction Cream			Coenzyme Q10, acetyl hexapeptide-3, micronized plant collagen, high active oligosaccharides in polysaccharide matrix
NLC Deep Effect Reconstruction Serum			

Regenerationscreme Intensiv	Scholl	6/2007	Macadamia ternifolia seed oil, avocado oil, urea, black currant seed oil
Swiss Cellular White Illuminating Eye Essence	La prairie	1/2007	Glycoprotiens, panax ginseng root extract, equisetum arvense extract, Camellia sinensis leaf extract, viola tricolor extract
Swiss Cellular White Intensive Ampoules			
SURMER Creme Contour Des Yeux Nano-Remodelante	Isabelle Lancray	03/2008	Kukuinut oil, Monoi Tiare Tahiti® , pseudopeptide, hydrolized wheat protein
Olivenöl Anti Falten Pflegekonzentrat	Dr. Theiss	02/2008	Olea europaea oil, panthenol, acacia senegal, tocopheryl acetate
Olivenöl Augenpflegebalsam			Olea Europaea oil, prunus amygdalus dulcis oil, hydrolized milk protein, tocopheryl acetate, rhodiola rosea root extract, caffeine

3.1 Major Classes of Nanocosmeceuticals

Cosmeceuticals represent one of the fastest growing segments of the personal care industry. Recent forecasts indicate that, with a compound annual growth rate of 7.7%, the global cosmeceutical market could reach over $31 billion by 2016 (RNCOS E-Services Pvt. Ltd. 2013). The use of nanoparticles in cosmeceuticals is considered to afford a number of advantages to existing personal care products, including improving the stability of cosmetic ingredients, protecting the skin from the sun, increasing product aesthetics, targeting active ingredients to the desired sites, and controlling active ingredient release to achieve prolonged effects (Padamwar and Pokharkar 2006, Mu and Sprando 2010).

A comprehensive review of nanotechnology-based cosmeceuticals present in the market has recently been published by Lohani, Verma et al. (2014). Incorporation of nanotechnology in cosmeceuticals is principally aimed at anti-aging products, moisturisers to maintain skin hydration, sunscreens to protect the skin, as well as hair, skin, lip and nail care products to maintain personal hygiene and appearance but also other novel uses such as fragrance release (Hosseinkhani, Callewaert et al. 2015). Each of these application classes is briefly described below. Further information on specific nanocosmeceutical products currently on the market across all of these application classes, as well as a summary of patents for products under development, is provided in Appendix 2.

3.1.1 Anti-aging Products

Aging of the skin can occur as a result of abrasion and/or exposure to chemical products as well as a range of environmental factors including pollution, stress, irradiation from infrared (IR) and ultra-violet (UV) rays. Skin aging can manifest itself in a variety of ways, including dryness, loss of elasticity and texture, thinning, damaged barrier function, blemishes, and wrinkles. Collagen, the main structural protein in the extracellular space of mammalian connective tissues, plays a vital role in skin rejuvenation and wrinkle reversal. Anti-aging products are the main type of cosmeceutical currently on the market involving the application of nanotechnology, with numerous products incorporating various nanosomes, nanospheres and nanocapsules, and claiming to have anti-wrinkle, firming, moisturising, lifting, and skin toning properties. For example, L'Oreal markets a number of products in its 'Revitalift' range which contain Pro-Retinol A encapsulated in nanosomes and claim to instantly retighten the skin and reduces the appearance of wrinkles. It is understood that the retinol is intended to increase collagen synthesis, epidermal water content, epidermal hyperplasia, and cell renewal, as well as inhibit collagen breakdown.

3.1.2 Moisturisers

Water from the primary barrier of the skin (the *stratum corneum*) can evaporate rapidly and potentially lead to dehydration. When moisturisers are applied to the skin, a thin film of humectant is formed which retains moisture and improves appearance. A range of nano-transporters (including liposomes, nanoemulsions, and solid lipid nanoparticles) are being applied in moisturising formulations affording prolonged effects. As well as maintaining general skin hydration, these products are considered to be useful for the management of various skin conditions (e.g. atopic dermatitis, psoriasis, and pruritus). As an example of a product in this application class, Lancôme manufacturers a moisturiser called 'Hydra Zen Cream' which contains nanoencapsulated triceramides and claims to keep skin fully hydrated and "protected from signs of daily stress".

3.1.3 Sunscreens

Sunscreens are widely used to protect the skin from harmful ultra-violet (UV) rays, and typically contain zinc oxide (ZnO) or titanium dioxide (TiO_2) mineral-based ingredients to protect the skin from sun damage. These minerals act to form a barrier on the skin, reflecting UVA and UVB rays from penetrating down to the deeper layers of skin. The major disadvantage of conventional sunscreen products is that they leave a visually unappealing chalky white layer on the skin. In contrast, sunscreen products incorporating ZnO or TiO_2 nanoparticles are transparent, less greasy, and have increased aesthetic appeal, thus forming one of the major application classes of nanomaterials in cosmetic products. Dior market a product called 'Dior Snow Pure UV Perfect Brightening Protective Base' which contains nano-UV filters "for ultraprotection against the damaging effects of UVA and UVB rays". In addition to particulate barriers to UVR, the use of chemical barriers (both conventional and novel such as Safranal (Golmohammadzadeh, Imani et al. 2011, Khameneh, Halimi et al. 2015)) in conjunction with nano-transporter technology have been explored(Alvarez-Roman, Barre et al. 2001, Krishnan, Elmets et al. 2006, Krishnan, Pradhan et al. 2006, Xia, Saupe et al. 2007, Vettor, Bourgeois et al. 2010, Montenegro, Sarpietro et al. 2011, Morabito, Shapley et al. 2011, Nesseem 2011, Siqueira, Contri et al. 2011).

3.1.4 Hair Care Products

Widespread research is currently on-going into the use of nano-transporters in hair care products with the aim of improving the shine, silkiness, and overall health of hair, as well as for the prevention of hair loss. In contrast to traditional hair products, it is envisaged that nanoemulsions in hair cosmetics will not destroy hair cuticles to penetrate into the hair strands. Sericin (composed of cationic sericin nanoparticles) is an active area of hair cosmeceutical research, with studies indicating that sericin nanoparticles can easily adhere to the surface of hair, seal and treat the damaged cuticles.

3.1.5 Skin Cleansers

Skin cleansers are another promising class of nanocosmeceuticals. The skin is covered with a hydrolipid film (composed of secretions from sebaceous glands, apocrine and eccrine sweat glands, as well as decomposition products from corneocytes) which provides natural defence against pathogenic organisms, but also attracts dirt and pollutants from the environment. Microorganisms on the surface of the skin surface may also act on components of the film and create undesirable by-products resulting in body odour. Regular cleansing to remove debris, dirt, and odour is a vital part of maintaining the overall health and appearance of the skin. Silver nanoparticles are considered to be effective for as skin decontamination and disinfection owing to their anti-bacterial properties. For example, Nano Cyclic currently markets a skin cleansing product called 'Nano Cyclic Cleaner Silver' which blends silver nanoparticles with natural ingredients and claims to kill harmful bacteria and fungi, treat acne, exfoliate dead skin cells, diminish age spots, deodorise the body, and fight the appearance of wrinkles.

3.1.6 Lip Care Products

Several cosmetic companies are displaying interest in the incorporation of nano-transporters into lipsticks, lip gloss and other lip care products with the aim of softening or soothing the lips by preventing transepidermal water loss. For example, Lancôme currently manufactures a lip treatment product called 'Primordiale Optimum Lip' which contains vitamin E in nanocapsules and claims to reduce lip bleeding and feathering due to fine lines and wrinkles. Kara Vita also market a product called 'Lip Tender' which claims to contain 'ten bioactive ingredients that are precisely calculated to work within lyphazomes, delivering a 4-in-1 formula and brining long-lasting hydration

for fast and dramatic lip repair". It has also been suggested that gold, silver and silica nanoparticles may help improve the appearance and distribution of pigments in lipsticks, and prevent pigments from migrating or bleeding into the fine line of lips.

3.1.7 Nail Care Products

Nanocosmeceutical nail products are considered to offer numerous advantages over conventional products. A US patent has been registered for a nail polish containing nanoparticles which claims to improve the toughness and impact resistance of mammalian nails (Amato, Farer et al. 2007). In a press release, Nano Labs Corp. (2012) announced that they have been awarded a provisional patent for a nano nail polish and lacquer which dries to a very hard state, resists shock, cracking, scratching, and chipping. There is also interest in the incorporation of metal and metal oxide nanoparticles with anti-fungal activity into nail polish for the treatment of fungal toenail infections.

3.2 Patent Search Results

When considering the spread of nano-transporter technology, the patent records also provide a useful resource to understand where carrier technology is employed and what types of nano-transporters are predominantly used.

During the literature search, a total of 181 patents were found involving the use of nano-enabled technologies and around 165 patents using these nano-enabled technologies had direct applications in cosmetics. As summarized in figure 7, of the patents looked at 8% were found to have only medical applications whilst 91% had both cosmetics and medical applications.

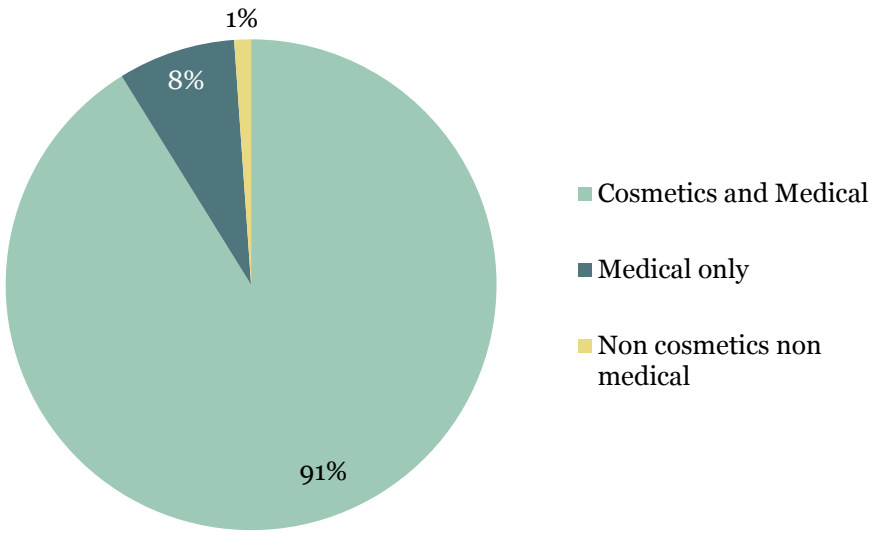


FIGURE 7. RESULTS REGARDING THE USE OF NANO-ENABLED TECHNOLOGIES AMONGST PATENTS.

As can be expected, the proposed uses of nano-transporters within cosmetics mentioned within the patents varied substantially showing the wide potential of this nanotechnology. Specific examples include:

- UVR protection
- moisturisers for skin, hair and nails,
- antioxidants,

- skin whitening,
- prevention of aging and wrinkles,
- promotion of collagen synthesis,
- anti-cellulite,
- treatment of skin allergy and
- repairing skin injury

Other, more medical, applications were anti-microbial, prevention and treatment of alopecia, acne, psoriasis, vasculitis, cancer, diabetes as well as skin protection against chemicals. As well as understanding the specific uses of nano-transporters, it is also useful to understand the types of nano-transporters being employed. Figure 8 shows the results of an assessment of the patents in which the types of nano-transporter were recorded. The results show that within the patents, nano-emulsions and nano-liposomes are the most commonly proposed nano-transporter whilst somewhat surprisingly newer technologies such as NLC were less well represented within only 3 patents mentioning NLC or SLN.

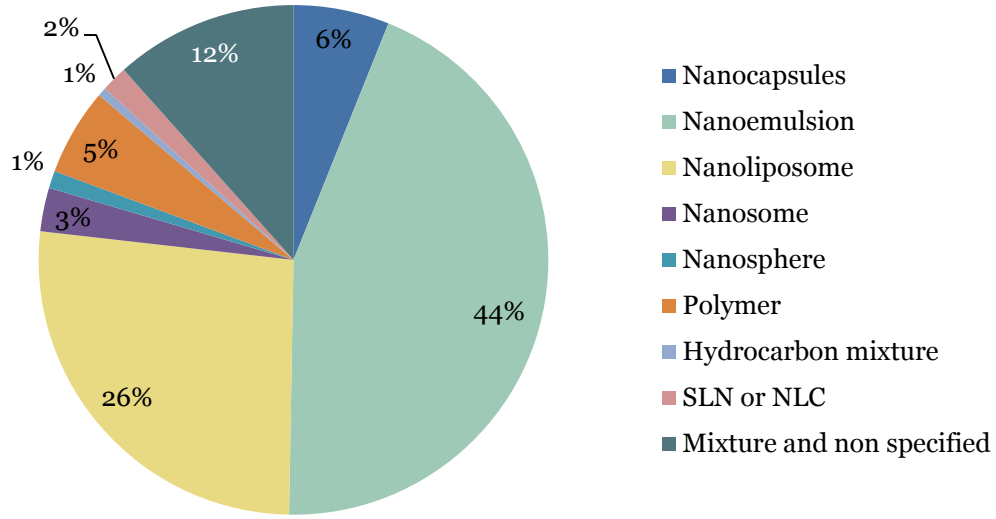


FIGURE 8. RESULTS REGARDING THE TYPES OF NANO-ENABLED TECHNOLOGIES USED AMONGST PATENTS.

3.3 Nano-transporter Characterisation

An aim of this report is to identify potential links between physicochemical properties of nano-transporters and their absorption/ toxicity profiles; so called 'structure-activity relationships'. This is because it is well established that the properties, behaviour and biological effects of engineered nanomaterials can be influenced by a range of physicochemical parameters, including size, shape and surface area as they form the interface with the biological environment. In relation to nanomaterials, size is seen as one of the crucial modifiers of a particles ability to cross biological barriers and nanoparticles in the lower nanometre range may penetrate biological membrane barriers that normally prevent the entry of (larger) particulate materials (Jani, Halbert et al. 1990, Geiser and Kreyling 2010).

Due to the intrinsic link between particle properties and activity (absorption, toxicity etc.) the adequate characterisation of a nanomaterial is necessary to accompany any toxicity study and forms an integral part of the risk assessment (Zuin 2007). This is supported by that good characterisation

data using a minimal characterisation dataset should accompany and be required for all studies on nanomaterials (Boverhof and David 2010). In acknowledgment of this and the effect it has one study quality (and utility) the Card and Magnussen methodology for assessing study quality has been an integral part of the literature appraisal in this project.

Owing to the complexity but also the importance of good physicochemical characterisation, the International Committee on Standardisation (ISO) has published a guidance document outlining a list of physicochemical properties for detailed description of manufactured nano-objects subject to toxicological testing (ISO 2012). These properties, as well as proposed methods for their characterisation, are outlined in Table 10.

TABLE 10: OVERVIEW OF PHYSICOCHEMICAL PROPERTIES REQUIRED FOR DETAILED DESCRIPTION OF MANUFACTURED NANO-OBJECTS SUBJECTED TO TOXICOLOGICAL TESTING, USING SELECTED STANDARD TERMINOLOGY FROM THE ISO CONCEPT DATABASE.(ISO 2013)

Property	Description	Example measurement methods
Equivalent diameter	Diameter of a sphere that produces a response by a given particle-sizing instrument, that is equivalent to the response produced by the particle being measured	Dynamic light scattering; nanoparticle tracking analysis; small angle X-ray Scattering; size exclusion chromatography; analysis of SEM, TEM or SPM images; differential mobility analysis.
Aggregation/ agglomeration state	Aggregate: particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components; Agglomerate: collection of weakly or loosely bound particles or aggregates or mixtures of the two in which the resulting external specific surface area is similar to the sum of the specific surface areas of the individual components.	Analysis of SEM or TEM images; small angle X-ray scattering; X-ray diffraction; small angle neutron scattering; rheology methods; centrifugal liquid sedimentation; nanoparticle tracking analysis.
Shape	External geometric form of a powder particle.	Analysis of SEM, TEM or SPM images.
Surface area	Extent of available surface area as determined by given method under stated conditions	Methods based on gas or liquid adsorption isotherms; liquid porosimetry; analysis of SEM, TEM or SPM images.
Surface chemical composition	Material composition within a few atomic layers of the surface	Auger electron spectroscopy (AES); X-Ray photoelectron spectroscopy (XPS); Secondary ion mass spectrometry (SIMS); Energy Dispersive X-Ray Analysis; Electron Energy Loss Spectroscopy (EELS); Raman and other molecular spectroscopies.

Property	Description	Example measurement methods
Surface charge and surface charge density	Type of charge (positive or negative) and the amount that can be bound to the surface of a material.	Isoelectric point; electrophoretic light scattering; electrophoresis; electrosmosis; electric sonic amplitude; colloidal vibration current
Solubility	Maximum mass [of a substance] that is soluble in a given volume of a particular solvent under specified conditions.	There are no specific methods for the assessment of the solubility of nano-objects, however, consider reporting equilibrium dialysis, inductively coupled plasma mass spectrometry (ICP-MS) or inductively coupled plasma optical emission spectroscopy (ICP-OES) as possible measurement methods.
Dispersibility	Degree to which particles can be broken down to some minimum size.	Methods to assess the dispersibility are based on equivalent diameter measurements stated above.

The properties listed in Table 10 represent an ideal characterisation of a particle but as well as understanding what to measure, it is also important to determine when and where the properties of nanomaterials should be characterised. Typically, particles are characterised as-produced or as-supplied and the literature surrounding nano-transporters is no exception to this. This is because such characterisation tends to be much more straight forward than trying to characterise a material in a complex environment such as the body or within a product. For example, in its guidance on the safety assessment on nanomaterials in cosmetics (SCCS 2012), the SCCS highlights that characterisation of a nanomaterial in a cosmetic formulation is more difficult compared to characterisation in a raw material. However, whilst it may be easier to characterise a particle as produced the actual properties may be modified during incorporation into a product, during storage, during application, during residence in the skin or other environment and indeed may change throughout the particle life cycle. From a toxicological perspective, it is the physicochemical characteristics at the point of application and during residence in or on the skin that is most relevant. This is because this is point of biological interaction and it is the properties at this time which may drive a biological effect.

It has been suggested that for adequate particle characterisation in a toxicological system, there should be three distinct phases of characterisation of primary, secondary, and tertiary (Sayes and Warheit 2009) and are summarised as:

- primary characterisation is performed on particles as-synthesised or as-received in its dry native state;
- secondary characterisation is performed on particles in the wet phase as a solution or suspension in aqueous media (which could be ultrapure water, vehicle solution or cell culture media); and
- tertiary characterisation are performed on particles following interactions with cells under *in vivo* or *in vitro* condition.

This last phase could include characterisation in biological fluids such as blood, lung lining fluid, as well as interactions with proteins, fats, and specific cell types. This final stage is most certainly the most challenging and the difficulties in establishing accurate *in situ* characterisation should not be underestimated. However, as stated, this is the point of biological interaction and therefore the most relevant for establishing true structure-activity relationships.

Whilst the characterisation of biodurable and solid nanoparticles in the biological environment is highly challenging, the situation is likely to be even more difficult for biosoluble nano-transporters. This is because such transporters are somewhat labile, for example due to lipids fusing with the skin or size potentially changing through fusion (as opposed to aggregation). Whilst these labile properties' can be beneficial to the action of the nano-transporters (e.g. causing release of the active payload) they also provide additional challenges to the identification and characterisation of the nano-transporters after application. Indeed, this becomes apparent when considering that in the assessment of dermal penetration, *in vitro* studies tend to measure the penetration of the active payload into the receptor fluid rather than measure the nano-transporters directly. This provides ambiguity as to i) if the nano-transporters themselves can penetrate the skin and ii) how long they can persist in the skin/ body.

It is clear to see that there are significant challenges for proper characterisation of nano-transporters but as with any toxicological evaluation, proper characterisation of the test material is essential for correct interpretation. Given the specific nature of nano-transporters (the labile and transient nature), further tools to detect and characterise these particles in the biological environment are likely to be needed.

4 Dermal absorption/ Penetration by Nano-transporters

4.1 Introduction

As summarised by Bocca *et al.*, up until the latter half of the 20th century it was generally considered that cosmetics remain on the surface of the skin and as such, local effects were the primary concern and available test method reflected this. However laterally it has been recognized that topically applied substances can potentially penetrate into or through the skin and result in systemic exposure (Bocca, Pino et al. 2014). This issue of dermal penetration has raised considerable concern in relation to nanomaterials and these have been reiterated in numerous reports and studies (Monteiro-Riviere , Santamaria and Rachman , Ryman-Rasmussen, Riviere et al. 2006, Nohynek, Lademann et al. 2007, Warheit, Borm et al. 2007, Nohynek, Dufour et al. 2008) from as early as 1996 (Tan, Commens et al. 1996) and despite the concerns, there is still a great deal of confusion. In relation to the dermal absorption/ penetration of nanomaterials meeting the current definition of a 'nanomaterial' under cosmetics regulations (i.e. non-soluble) a previous project investigated the evidence. It was found that:

> *"Whilst there are many conflicting results, on balance the literature seems to suggest that absorption of particles in the nano-range through the skin is possible although occurs to a very low degree and that the level of penetration, depending on chemistry and experimental conditions, may be greater than for larger particles" (Poland, Read et al. 2013)*

Whilst this evaluation showed that penetration, where it does occur, does so to only a small degree, nano-transporters are specifically intended to improve the penetration efficiency of active compounds and so the absorption/ penetration profiles of these materials may differ markedly from other forms of nanomaterials.

4.1.1 Dermal Absorption

When considering the passage of nano-transporters through the skin and into the body, it is useful first to define what is meant by dermal absorption. Dermal absorption can be described simply as the transport of a compound from the outer surface of the skin (the *stratum corneum*) into the skin and from there, into the body where it may become systemically available. The relationship between permeation, penetration and absorption is summarised in figure 9 and these terms differ considerably in the actual movement of materials in the skin. Permeation occurs where a compound diffuses into a specific skin layer such as the *stratum corneum* whilst penetration occurs when the compound moves through a layer into another dermal layer. For example a compound may penetrate through the *stratum corneum* to localise in the *stratum granulosum*. Absorption occurs when the compound is able to move through the dermal layers and to the site of action or systemic circulation.

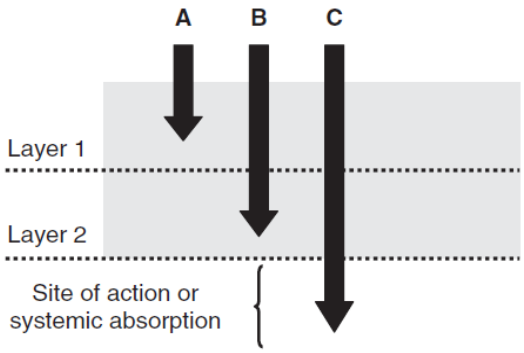


FIGURE 9: REPRESENTATION OF SKIN PERMEATION (A), SKIN PENETRATION (B) AND SKIN ABSORPTION (C).

Permeation is diffusion of a penetrant into a certain skin layer. Subsequent diffusion through that layer represents penetration (in this example, the substance has "penetrated" layer 1). Penetration through layers of skin to either the site of action or systemic circulation represents absorption.(Chilcott, Price et al. 2008)

The passage of a substance into and/or through the skin is governed by many parameters with the specific structure and health of the skin being of great importance. As mentioned previously, the relative thickness of the *stratum corneum* differs markedly depending on anatomical location and the barrier quality also changes with age (Chilcott, Price et al. 2008). In addition, the health status of the skin is of importance in terms of barrier integrity as the presence of lesions such as cuts or ulcers offer potential routes into the skin. Dry, flaky or inflamed skin (e.g. sun burnt) may also present a less efficient barrier to dermal penetration and absorption and this may be enhanced by day-to-day treatments such as clipping, shaving, epilation etc.

As well as the nature of the dermal layer, the physicochemical properties also play a significant role in the ability of a particle to move into and through the skin. Key attributes include surface charge of the particle as mammalian skin also has a charge meaning there can be some interaction. Specifically, mammalian skin is negatively charged and therefore there may be a repulsive effect should a particle also possess a strong negative charge (and vice versa). Indeed, one observation from a critical evaluation of the literature relating to dermal penetration of nanoparticles was that whilst there were conflicting results, there did appear a tendency towards greater uptake of positively charged particles. Other key properties thought to influence absorption include size, hydrophilicity/ hydrophobicity, and surface chemistry. Nano-transporters, as described in section 3.3.1 can be found in a range of sizes and whilst the lipid nature of many of these particles tends to provide a negative surface charge, there are positively charged examples of these materials.

In previously evaluating the dermal absorption of nanoparticles, it was found that particle composition (e.g. TiO_2 or ZnO) played relatively little role in dermal penetration/ absorption. However, in comparison the composition of nano-transporters can be much more complex as they consist not only of lipids and oils (which themselves can interact with lipids within the skin) but also a range of surfactants and co-surfactants which may act as penetration enhancers.

Whilst dermal absorption is of great importance for considering the potential systemic effects of nano-transporters, dermal penetration is also of importance as it indicates breach of the surface barrier and potential interaction between the nano-transporters and more sensitive dermal layers. Therefore, this section aims to address not only dermal absorption, but dermal penetration as well.

4.2 Evidence of Dermal Absorption/ Penetration of Nano-Transporters

4.2.1 Liposomes

A 2012 study by Golmohammadzadeh and colleagues (2012) looked at the ability of nanoliposomes containing Safranal to moisturise and protect the skin from ultraviolet radiation (Golmohammadzadeh, Imani et al. 2011). Safranal is the main aroma factor isolated from saffron, the dried stigmas of *Crocus Sativus* and account for 60% of the volatile components of Saffron. Studies have shown Safranal has a variety of beneficial properties including anti-inflammatory and radical scavenging activities and this has been also utilised with SLN particles (Khameneh, Halimi et al. 2015) (although the toxicity and dermal absorption was assessed and so not discussed here). The aim of this study was to build on previous research by the same authors looking at the sun protection factor (SPF) properties of this compound when packaged within a liposome. The authors made liposomes containing safranal (Lip-Safranal) with soya phosphatidylcholine as the main phospholipid (15% wt.), cholesterol (2% wt.), vitamin E (0.3%), PEG (7% wt.) as well as the parabens, propylparaben (0.02% wt.) and methylparaben (0.1% wt.). Once loaded with Safranal, where the Safranal locates within the bilayer of the liposomes, the mixture was homogenised to produce liposomes ranging from 90 to 135nm in diameter. The zeta-potential of the various preparations (empty liposomes or liposomes containing 0.25-8% Safranal) were all highly negative (-49 to – 35 mV) and became less negative with increasing Safranal loading. The study evaluated the SPF of the liposome preparations as well as the moisture content of the *stratum corneum* following application of the liposomes. The results showed that all preparations (empty and loaded liposomes) increased the water content of the skin in human volunteers and that the SPF of the 8% Li-Safranal was significantly better than the reference material. Penetration studies using a Franz diffusion cell with mouse skin as the dermal barrier showed that 8.06 ± 0.48% of the Li-Safranal penetrated the skin and 0.47±0.42% of Safranal in the liposomes were retained in the skin (Golmohammadzadeh, Imani et al. 2011) over a 24hr period. This study showed negatively charged, small (<150nm) liposomes were able to successfully penetrate mouse skin.

In 2014, a comparative study was published looking at the penetration efficiency of retinyl palmitate (RP; vitamin A) loaded into liposomes (172nm), SLN (271nm) and nanoemulsions (14.42nm), all of which were negatively charged. The study used *ex vivo* human skin obtained from elective, abdominal plastic surgery (Clares, Calpena et al. 2014) held within a Franz diffusion cell with ethanol/ transcutol ® as a receptor medium. Two hundred micrograms of the test samples (equivalent RP) applied to the upper surface over a period of 38hrs and periodically, samples were taken to evaluate penetration. The results showed that all formulations displayed continuous permeation of RP beginning 1hr after application and the cumulative permeation of the drug was 6.67μg (3.3%) for the nano-emulsion, 4.36 μg (2.18%) for the liposomes and 3.64 μg (1.82%) for the SLN. It was noted by the authors that the nano-emulsion had the smallest particle size providing a larger interfacial surface area for drug absorption but furthermore, that the surfactant serves to enhance drug penetration. In relation to skin retention, the results showed that liposomes caused the highest levels of drug retention followed by nano-emulsions and SLN. The reasoning offered here was that higher retention was caused by the presence of biocompatible phospholipids in the liposome constructs, thereby favouring incorporation. It is worth noting that in histological evaluation of the skin structure post exposure (reported in section 7.3.1) treatment with the nano-emulsion appeared to impair barrier function of the skin.

Overall, these results show that dermal penetration of nano-transporter payloads does occur although at a relatively low to moderate level (<10%).

The possession of a negative surface charge may hinder passage of particles into and through the skin due to the negative charge of the skin resulting in repulsive forces. However, relatively few studies utilise positively charged liposomes. Within the study by Carboni, the authors evaluated the permeation of positively charged liposomes (+36 mV) which were 202nm in diameter. A Franz

Diffusion cell utilizing new-born pig skin as the model dermal layer was used and the drug diclofenac was used as the model active ingredient. The authors compared the permeation of the drug encapsulated in the liposomes against the same drug in a gel formulation. Interestingly they found that whilst the gel formulation led to a sizeable permeation of the drug, only 1% of the drug was released through the skin when applied in a liposome formulation. Instead, after application in the liposomes, higher levels of the drug were found in the skin than for the gel application and the predominant zone of accumulation was the viable epidermis, to a lesser extent in the *stratum corneum* and only 0.02% in the dermis. This is an interesting finding as it suggests that the liposomal formulation could cause good dermal delivery with penetration into the dermal layers but relatively poor absorption through the skin for transdermal delivery (Carboni, Falchi et al. 2013).

4.2.1.1 Role of Physicochemical Properties

When looking at the evidence for dermal penetration and absorption, the literature lacks a systematic modification and comparison of physicochemical properties meaning that identifying structure activity relationships is somewhat difficult as one must look between studies to draw conclusions. One area this does appear possible is in the role of surface charge. Studies employing negatively charged liposomes tend to lead to higher levels of transdermal passage (as assessed using Franz Diffusion cells) than that noted with positively charged liposomes. From a biological perspective, this could be anticipated as positively charged particles are more likely to interact with and bind to the dermal layer due to attractive forces between the liposomes and the negatively charged skin. This in turn may favour localisation within the skin (as demonstrated by Carboni *et al.*) rather than transfer through the skin leading to systemic absorption. On the contrary, negatively charged particles would be subject to repulsive forces which may in turn enhance transdermal passage.

However given the relatively low numbers of studies and different methodologies, the relevance of this observation is questionable. For example, the highest level of dermal absorption (8%) was noted in the study of Golmohammadzadeh which employed mouse skin as the model dermal layer. Lower levels (2.18%) were noted in the study of Clares, also using negatively charged liposomes, which was anticipated as this study used human skin as a model which is thicker and therefore offers a more substantial barrier than mouse skin. In the study using positively charged liposomes, the lowest level of absorption was detected (>1%) and this study used new-born pig skin as a model barrier which would provide a more substantial barrier than mouse skin.

4.2.2 Solid Lipid Nanoparticle & Nanostructured Lipid Carrier

In considering the absorption capacity of SLN and NLC, Gokce *et al.* compared the diffusion profiles of NLC and SLN loaded with resveratrol (RSV) (Gokce, Korkmaz et al. 2012) through rat abdominal skin held within a Franz diffusion cell. Both formulations had similar particle size and zeta-potential values with the NLC being slightly smaller (110nm compared to 161nm) and with a less negative charge (-13.8 mV compared to 15.3 mV) although these values were not radically different. HPLC analysis of the receptor fluid showed no trace of RSV suggesting transdermal absorption did not occur whilst analysis of the skin showed similar levels of RSV accumulation after application of the NLC (1.99µg/cm^2) and SLN (1.55µg/cm^2). Through separation of the epidermis and dermis, the authors were able to assess the penetration profiles that interestingly differed between the two carriers although not a significant extent. The SLN appear to accumulate to a greater extent in the epidermis whilst the NLC accumulated to a greater extent in the dermis suggesting enhanced penetration. The authors hypothesised that the phenomenon of RSV accumulation in the skin could be explained by the lipid characteristics of the SLN and NLC preparations (Gokce, Korkmaz et al. 2012).

Whilst a large proportion of published studies addressing dermal penetration/ absorption of nano-transporters use the measurement of a loaded active ingredient as a measure of dermal absorption (e.g. in the receptor fluid of Franz diffusion cell), a 2013 study by Iannuccelli *et al.* took a different

approach (Iannuccelli, Coppi et al. 2013). Here the aim was the *in vivo* detection of SLN and SLM (solid lipid microparticles) by means of tape stripping of the *stratum corneum* after application in human volunteers.

The SLN and SLM were made by obtained by emulsifying stearic acid (4 g), containing the maximum dispersible amounts of titanium dioxide (40 mg) with 2% (w/v) hydrogenated soybean phosphatidylcholine water solution (50 ml) by ultrasound to form the SLN or high-performance dispersing technique for the SLM. The resultant SLN were quite large with an unloaded size of 409nm and a loaded size of 762nm (TiO_2 particle size was 361nm) and these had a negative surface charge of -30.54 mV in the case of the loaded SLN. SLM on the other hand were much larger with a mean diameter of 28.5 μm with no real difference between the loaded and unloaded forms.

To assess the ability of the nano-transporters to be translocated and modified across the *stratum corneum*, the SLN and SLM were embedded into a standard oil/ water (O/W) emulsion. This was then applied to the forearms of female volunteers with a dose of 20mg across an area of 2 x 5 corresponding to a formulation dose of 2mg/cm² and particulate (TiO_2) dose of 0.2mg/cm². The applied dose was left in place for a period of 30 minutes before being wiped clean and the area tape stripped 12 times. The tape strips were then analysed using Energy Dispersive X-Ray Spectroscopy (EDX) for elemental composition and it was considered that the first tape included non-penetrated materials with subsequent strips reflecting penetrated materials. Specifically it is though that from the 2nd to the 5th tape mostly the *stratum disjunctum* is removed and the *stratum compactum* from the 6th to the 12th tape (Jacobi, Weigmann et al. 2005, Iannuccelli, Coppi et al. 2013). The results showed that all tapes removed after application of the SLN had nanosized structures located in rather broad intracellular spaces and in the 6th tape, the characteristics of this resembled the spectral properties of the original SLN preparation. This was seen as evidence of movement of intact SLN reaching the *corneum compactum* along broad channels between the corneocytes. In the 11th and 12th tape strips, intact SLN were also found concurrent with non-encapsulated TiO2 nanoparticles suggesting that biodegradation of the lipid was occurring as a consequence of interactions with dermal lipids. In stark contrast to the results observed with the SLN, application of the much larger SLM did not result in appreciable penetration and the results showed an inability to penetrate even the outermost and loose *corneum disjunctum* layer (as well as an ability to be degraded by the surface environment). Overall, these results indicate a role of size in the dermal penetration of the solid lipid particles (albeit across a wide range) and that with increasing penetration into the dermal layers, the particles are degraded by the biological environment leading to release of the active payload (TiO_2 in this case). These findings support the observation by Gokce (Gokce, Korkmaz et al. 2012) that RSV loaded into lipid nano-carriers did not absorb through the skin but instead was retained within the dermal layers.

Such findings of the ability of SLN to improve drug localisation in the skin and reduce systemic losses (resulting in reduced drug to the specific target) were also by found within other studies (Pople and Singh 2006, Puglia, Blasi et al. 2008, Mitri, Shegokar et al. 2011, Bose and Michniak-Kohn 2013, Hung, Chen et al. 2015). Pople and Singh noted the addition of vitamin A loaded 360nm SLN to polymeric gels led to 10 fold reduction in transdermal permeation of vitamin A into the receptor compartment of a Keshary Chien cell (with human cadaver skin) as compared to the polymeric gel with free vitamin A added (Pople and Singh 2006). In a later study, Mitri *et al.* showed no transfer of Lutin through pig ear skin after loading into SLN or NLC (nano-emulsions showed 0.37% penetration through to the receptor fluid) (Mitri, Shegokar et al. 2011). Further to this, Schwarz and colleagues (Schwarz, Weixelbaum et al. 2012) noted the dermal permeation of SLN and NLC loaded with flufenamic acid, used as a model drug. Here SLN, NLC and nanoemulsions were applied to the upper portion of porcine skin held within a Franz diffusion cell at a dose of 50mg/cm². The particles all displayed a negative surface charge (-35 to -59 mV) and ranged in size from 149nm in the case of the nanoemulsions to 111 and 106nm for the NLC and SLN respectively. All preparations showed good permeation of flufenamic acid with slightly higher levels

after application in the nanoemulsions and NLC preparations. Tape stripping experiments after application showed the deepest penetration to be about 63% of the *stratum corneum* in the case of the NLC (Schwarz, Weixelbaum et al. 2012).

The role of size was further supported by the study of Abdel-Mottaleb, Neumann and Lamprecht (Abdel-Mottaleb, Neumann et al. 2011) who compared the permeation of a range of liposomes, NLC, SLN and polymeric nanoparticles (Abdel-Mottaleb, Neumann et al. 2011). The SLN were composed of Witepsol with varying ratios of the surfactants Cremophor A6 and A25 to produce particles which were 87.8nm or 305nm in diameter. The NLC were also composed of Witepsol with the addition of medium chain triglyceride and the surfactant Cremophor A25 and were 90.nm and 331.7nm in diameter. Application of these particles to the upper surface of pig ear skin mounted in a Franz diffusion cell led to increased permeation of ibuprofen, loaded into the nano-transporters as a model drug and increased permeation was noted with decreasing size. The use of SLN and NLC led to improved skin retention of the active payloads over and above liposomes, which were smaller in size. One reason for the improved permeation of the drug is thought to be from the occlusive effect of the lipid nano-transporters forming a film over skin surface. This results in improved dermal hydration resulting in SC swelling and opening with higher drug permeation (Abdel-Mottaleb, Neumann et al. 2011) as shown in Figure 1.

Whilst many studies noted improved dermal retention with SLN/ NLC and lesser dermal transfer, this was not universally the case. The recent study by Schwarz et al (2013) noted that CoQ10 loaded into ultra-small NLC (~80nm in diameter and -34 mV zeta-potential) resulted in permeation through pig skin mounted in Franz diffusion cells and this was enhanced when compared to larger NLC 226nm in diameter (-54 mV zeta-potential). However whilst CoQ10 was detected in the receptor fluid, due to large standard deviation in the experimental results none of the results were significant (Schwarz, Baisaeng et al. 2013). Similar results showing transdermal delivery have been noted in a study by Goindi *et al.* (Goindi, Guleria et al. 2015) who observed a 3 fold increase in drug permeation though mouse skin using 150nm SLN with a weakly negative charge of -0.17 mV.

4.2.2.1 Role of Physicochemical Properties

The results of dermal penetration and absorption of SLN/ NLC show that dermal penetration can occur resulting in localisation within layers as deep as the dermis. Such penetration appears to be influenced by particle size with smaller particles able to penetrate more efficiently and effectively into the skin than much larger particles. However, whilst dermal penetration into and through the SC was certainly apparent, transdermal penetration resulting in absorption through the skin was rarely seen with the majority of studies evaluated showing effective skin retention. This may be influenced by composition whereby degradation of the SLN/NLC occurs as they move through the skin (possibly due to lipid extraction) meaning that they are unable to penetrate the full skin thickness intact. Studies of cosmetic applications employing different surface charges were not noted but the effect would likely be the same as noted elsewhere.

4.2.3 Nanoemulsions

From evaluating the literature, it is evident that there are numerous publications addressing nanoemulsions for cosmetic applications such as whitening creams (Al-Edresi and Baie 2009) and moisturising agents (Ribeiro, Barreto et al. 2015) however many of these only address the specific characteristics of the nano-emulsions and not their interactions with the skin in relation to dermal absorption, penetration or toxicity (Guglielmini 2008, Pawar and Babu 2014, Tsai, Fu et al. 2014, Cheuk, Shih et al. 2015, Yukuyama, Ghisleni et al. 2015).

Corresponding to the observations on *Carboni et al.* (Carboni, Falchi et al. 2013) in relation to liposomes, Baspinar and Borchert evaluated the dermal penetration of positively and negatively charged nanoemulsions (Baspinar and Borchert 2012). This publication did not specifically address cosmetic uses of nanoemulsions and instead utilised the corticosteroid Prednicarbate as a model

drug but as the production process and compositional attributes of nanoemulsions (and other nano-transporters) are relatively common across cosmetic and medical applications, the study is deemed of relevance owing to its novel nature. The positively charged nano-emulsion was composed of 0.25% Prednicarbate, 0.6% phytosphingosine (which provide the positive charge), 20% Eutanol, 2% Tween 80, 2% Lipoid E80, 0.03% α-tocopherol and 0.1% potassium sorbate. For the negatively charged formulation the fatty acids myristic acid, stearic acid, lauric acid and palmitic acid were tested (Baspinar and Borchert 2012). The mean particle size of the positively charged nano-emulsion was 157nm and this changed little over storage of up to 12 months. Similarly the negatively charged nano-emulsion was relatively small at 136nm and showed similar stability during storage. The stability was driven by the high surface charge which was approximately +50 mV in the case of the positively charged nano-emulsion and -34mV for the negatively charged samples which complies with the basic rule stated by the authors that:

> *"A basic rule signifies, that a formulation with a zeta-potential value over 30 mV, independent from the positive or negative prefix, is generally regarded as physically stable" (Baspinar and Borchert 2012)*

Dermal penetration was assessed using a Franz diffusion cell with excised female human abdominal skin as the dermal barrier with 500µl of the nano-emulsion preparations applied to the upper surface over a period of 24hrs with samples taken at 1,2,3,6 and 24hrs. As is most often the case with nano-transporters, the levels of the active substance (in this case Prednicarbate) were used to evaluate penetration rather than a direct measure of the lipid nanoparticles themselves. The results showed that 6.5% of the negatively charged and 15.5% of the positively charged nano-emulsions penetrated into the skin. However, analysis of the receptor compartment fluid showed no detectable concentrations of Prednicarbate meaning that whilst there was clear penetration of the skin, there was no evidence of absorption through the dermal barrier. The authors explained that the increased penetration pf the positively charged particles was due to an increased interaction and absorption of the particles with the negatively charged coenocytes of the *stratum corneum* (Piemi, Korner et al. 1999, Song and Kim 2006, Baspinar and Borchert 2012).

This lack of absorption through to the receptor fluid of a Franz diffusion cell, this time employing pig skin, was also observed in the study of Gianeti *et al.* after 6 hours incubation with a Retinyl Palmitate (RP) loaded nanoemulsion with a particle size between 100-200nm. However, RP was detected in the *stratum corneum* and epidermis (with the *stratum corneum* and dermis removed) (Gianeti, Wagemaker et al. 2012) suggesting again that penetration can occur but that significant absorption through the skin may be hindered, potentially through lipid interactions leading nanoparticle degradation.

The 2006 paper by Calderilla-Fajardo (Calderilla-Fajardo, Cazares-Delgadillo et al. 2006) compared the influence of composition in the dermal penetration of Octyl Methoxycinnamate (OMC) which had been formulated in to nanoemulsions, emulsions and also nanocapsules. OMC is a chemical UV filter and used in some sunscreens and the aim of this study was to investigate the influence of sucrose laureate and sucrose oleate on the enhancement of dermal penetration. This is due to the positive effect sucrose esters (non-ionic surfactants) can have on permeability due to the ability of these molecules to extract and fluidize lipid domains (Calderilla-Fajardo, Cazares-Delgadillo et al. 2006). The nanoemulsions and emulsions were prepared in the same as the nanocapsules but without the addition of a polymer and this resulted in an emulsion with a particle size of 2644 – 2889nm, a nano-emulsion of 124-127nm and a nano-capsule preparation of 362 - 458nm. The preparations were applied to the forearms of 3 healthy volunteers at a dose of 400µl/cm^2 of each preparation containing 1% OMC. After one hour, the area was cleaned and sequential tape stripping performed and the amount of OMC in each tape determined. The results showed that application of the nano-transporters without sucrose esters led to better penetration of the nano-emulsion and emulsion when compared to the nano-capsules but when the sucrose esters

were include within the nano-emulsion and nano-capsule preparations OMC penetration improved appreciably (2 fold over control). As the penetration depth did not differ amongst the control formulations, the authors concluded that size had no effect on OMC penetration whereas composition (i.e. the use of a sucrose ester) seemed to have a greater impact.

4.2.3.1 Role of Physicochemical Properties

The results suggest that of the physicochemical parameters evaluated, composition and surface charge have a predominant effect on dermal penetration of nano-emulsions with positively charged particles leading to enhanced dermal accumulation of actives than negatively charged particles. However, whilst dermal penetration is apparent the results seem to suggest that dermal absorption, that is crossing the full dermal barrier with the potential for systemic availability, is much less efficient and active compounds often not detected in receptor fluids (Kong, Chen et al. 2011, Baspinar and Borchert 2012, Gianeti, Wagemaker et al. 2012).

4.2.4 Nanoparticulates (nanospheres, nanocapsules)

Polymeric nanoparticles offer a useful alterative to other forms of soluble nano-transporter which due to their structures and composition may be more labile and prone to fusion and offer less payload separation and protection than encapsulation. Such nanoparticles vary markedly in composition and can be used for a variety of purposes from the delivery of vitamins to the skin to anti-oxidants such as CoQ10. However, not all of the studies appraised use a payload intended for cosmetic applications and instead often use surrogate payloads such as proteins or labels. This is so that the transmission of payloads through the skin and/or localisation in dermal layers can be more easily tracked than can the case with certain cosmetic payloads, which do not, for example, fluoresce.

A range of studies have been published evaluating the dermal permeation, penetration and absorption of nanocapsules. Of these studies, the most common experimental approach has been the use of *ex vivo* skin samples mounted within a Franz diffusion cell. An example of such an approach is the 2011 study Abdel-Mottaleb, Neumann and Lamprecht (Abdel-Mottaleb, Neumann et al. 2011) which compared the permeation of polymer-based versus lipid based nano-carriers. Specifically, the authors compared a range of liposomes, NLC, SLN and polymeric nanoparticles, the latter of which were composed of ethyl cellulose or PLGA which differ in their hydrophobicity and biodegradability (Abdel-Mottaleb, Neumann et al. 2011).

The particles were loaded with ibuprofen as a model drug and Nile Red (NR) as a fluorescent marker. In the nano-transporter constructs, the authors included nano and non-nano comparators of the liposomes, SLN, NLC and polymer nanocapsules. For example, within the liposomal test samples, three were ~50nm in diameter and one was 289nm in diameter. Similar ranges were seen for the SLN (87 vs. 305nm), NLC (90 vs. 331nm) and polymer nanoparticles (~50 vs. 529nm). The results showed that after application to pig ear skin mounted in a Franz diffusion cell (300µl), significantly higher permeation occurred for the smaller, lipid based particles ($p < 0.05$). In contrast however, the ethyl cellulose based particles showed increasing drug permeation with increasing size whilst for the PLGA constructs; there was no difference between the 50 and 500nm particles. In support of this, analysis of skin retention showed good retention which was also increased with larger particles and the greatest level of retention was seen for the 500nm PLGA nanoparticles (Abdel-Mottaleb, Neumann et al. 2011).

The reduced efficiency in polymer nanocapsules permeation was also noted in a recent study by Alnasif *et al.* (Alnasif, Zoschke et al. 2014) who noted that over a period of 6hrs, flexible dendritic core-multi-shell (CMS) nano-transporters remained within the SC and only penetrated further after 24hrs exposure. The CMS were dye-tagged with NR or indocarbocyanine (ICC) to facilitate detection using fluorescence lifetime imaging microscopy (FLIM) and measurements showed that

the ICC loaded nanocapsules formed aggregates of up to 140-160nm whilst the NR loaded particles formed aggregates up to 200-240nm.

Penetration was measured into human abdominal skin obtained during elective surgery by application of the test samples (20µl/cm^2) to the upper surface of the skin in a culture insert. Whilst penetration through the SC took up to 24hrs in the case of healthy skin, the authors also used a 'disease model' induced by repeated tape stripping to reduce the barrier quality of the SC. Here the SC was 17.2µm thick in the case of healthy skin and 3.3µm thick after tape stripping. This reduction in barrier thickness meant that at 6hrs, penetration into the viable skin layers was already evident and even more prominent by 24hrs (Alnasif, Zoschke et al. 2014). In addition to addressing penetration of barrier impaired (tape stripped) model of human skin, the authors also attempted to investigate penetration using *in vitro* models mimicking skin diseases. The models were one of autosomal recessive generalized peeling skin disease (PSD) and non-melanoma skin cancer (NMSC). PSD is a keratinization disorder linked to corneodesmosin deficiency and so was induced in an RHS model through the use of siRNA targeted to knock-down the corneodesmosin gene in keratinocytes used to generate a RHS model. Histopathologically, PSD is characterised by orthokeratotic hyperkeratosis and increased detachment of coenocytes from the granular layer(Alnasif, Zoschke et al. 2014) . Despite this barrier impairment, penetration of the ICC labelled CMS into the viable layers were not seen after 3hr incubation although permeation of standard compounds such as caffeine did show improved permeation. In the case of NMSC, the human squamous cell carcinoma line SCC-12 were seeded onto the RHS that induced barrier impairment. The results showed that penetration of the loaded CMS into the viable cell layers was significantly enhanced. Overall, the results showed that within human skin, the CMS constructs were able to penetrate into the viable cell layers after prolonged exposure although rapid penetration did not occur (Alnasif, Zoschke et al. 2014). Barrier impairment induced by tape stripping or through the addition of cancer cells to an *in vitro* multi-cell model also led to enhanced penetration and, in the case of the human skin model, penetration rate. However due to the design of the experiment, it is unknown if dermal absorption passed the viable cell layers occurred and therefore, whether systemic absorption is likely.

Two studies which did assess full transdermal penetration were that of Kim *et al.* (2010) (Kim, Shim et al. 2010) and da Silva *et al.* (2012)(da Silva, Contri et al. 2013) .The study of da Silva used a Franz diffusion cell covered with porcine skin to investigate the permeation of nanocapsules formed from the polymer, poly(ε-caprolactone) loaded with vitamin K. The zeta-potential of the particles was -14.9mV and the average diameter of the particles were 211nm by laser diffraction and 162nm by dynamic light scatter (DLS) which shows that different techniques can give significantly different size readings of the same samples. Figure 10 shows the results of dermal penetration of the porcine skin 8hrs after 300µl of the nanocapsules or control formulation (vitamin K dispersion) was added. The results clearly show that whilst high levels of vitamin K were observed in the receptor fluid of the control formulation (vitamin K alone) indicating full transdermal penetration, this was not seen for the nanocapsules. Indeed, in the case of the nanocapsules, improved penetration of the skin layers was observed for the SC, epidermis and dermis whilst at the same time, limiting systemic absorption. Similar results have been noted in other studies for different compounds (Alves, Scarrone et al. 2007, Vettor, Bourgeois et al. 2010, Siqueira, Contri et al. 2011) and such a targeted effect holds promise for improved and selective dermal delivery.

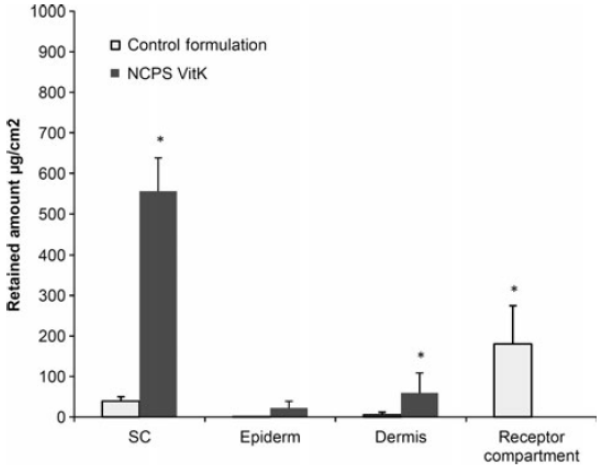


FIGURE 10: SKIN PENETRATION AFTER 8 H FOR VITAMIN K1–LOADED LIPID CORE NANOCAPSULE SUSPENSION (NCPS VITK) AND CONTROL FORMULATION.

Reproduced from Da Silva *et al.* (2013) (da Silva, Contri et al. 2013).

Similar results were noted by Kim *et al.* who assessed the transdermal delivery of CoQ10 loaded polymers formed using an emulsifier-free emulsion process. The authors synthesised negatively charged polymers using the monomers butyl methacrylate or methyl methacrylate as well as positively charged particles using trimethyl ammonium ethyl methacrylate chloride. The polymers were loaded with either CoQ10 or NR, the latter of which was used as a fluorescent label and this resulted in ~100nm and 40nm diameters for the positively (+60mV) and negatively (-60mV) charged nanocapsules respectively.

Skin permeation was analysed using guinea pig skin mounted in a Franz diffusion cell as well as the direct application of the nanocapsules (1% wt.) to the skin of live guinea pigs. This latter approach adds an important dimension as whilst skin held within a Franz diffusion cell is stationary and immobile, skin on a living creature is subject to frequent flexion and movement which may affect penetration. The results showed that whilst the payload (CoQ10) was released through the skin and could be detected within the receptor fluid of the diffusion cell, the nanocapsules were retained within the skin. To visualise the location of the positively charged polymer within the skin, the authors conjugated the yellow dye, Lucifer Yellow to the polymer. As shown in figure 11, confocal laser scanning microscopy (CLSM) was used to visualise the location of the polymers and NR payload in the skin, which had been removed from the animals by punch biopsy. The location of the yellow signal shows that the polymer remained in the SC and in contrast, the NR diffuses into the lower skin layers. Overall the reaction seems to be that the polymers interact with the upper layers of the skin resulting in the release of the active payload which is free to diffuse through to the lower layers into the deep skin (Kim, Shim et al. 2010).

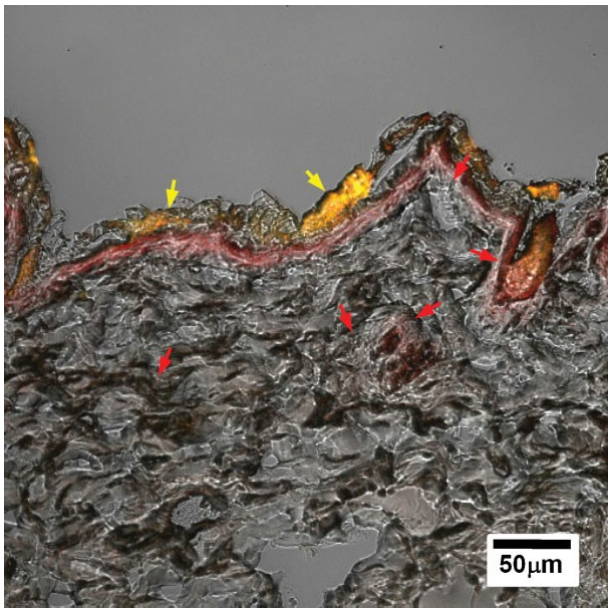


FIGURE 11: DISTRIBUTION OF A PROBE MOLECULE, NILE RED, IN THE SKIN OF ALBINO HARTLEY GUINEA PIG.

The nanocapsules encapsulating Nile Red in PBMA-C5 are tagged with Lucifer Yellow. The concentration of Nile Red was 0.05 wt.-%. Arrows indicate the locations of Nile Red and PBMA-C5 with Lucifer Yellow. Reproduced from Kim *et al.* (2010) (Kim, Shim et al. 2010).

Whilst studies that of da Silva (da Silva, Contri et al. 2013) and Kim (Kim, Shim et al. 2010) have shown that polymeric nanoparticles offer good localisation within the skin yet poor transdermal penetration making them useful for dermal delivery, there are studies showing good dermal absorption. The study of Choi *et al.* (2012) used human cadaver skin placed within a Franz diffusion cell to evaluate the skin permeation of soluble proteins encapsulated within pluronic based nano-carriers (Choi, Lee et al. 2012). These nano-carriers were either evaluated in their bare form or chitosan-conjugated form and were loaded with either bovine serum albumin (BSA) or insulin labelled with the fluorophore fluorescin isothiocyanate (FITC). The nanocapsules were 60nm in diameter and a surface charge of -4.5mV in the case of the bare particles and 70nm and +11mV in the case of the chitosan conjugated particles. After application of the nanocapsules (1.5mg), receptor fluid was removed from the Franz Diffusion cells at a range of time points up to 24hrs and analysed using a spectro-fluorophotometer to detect the labelled protein. As well as analysing the receptor fluid (to detect full thickness penetration (absorption)), the skin samples were also sequentially tape stripped to separate the skin into SC, epidermis and dermis (Choi, Lee et al. 2012). As a method, this is somewhat crude to isolate the various layers as there is most certainly the potential for mixed separation.

The results showed that the pluronic based nanocapsules and especially the chitosan conjugated versions were effective in transporting the loaded proteins across the skin and into the receptor fluid. When comparing the various dermal layers, greater levels of the fluorophores were detected in the epidermis and dermis than in the SC but most had transferred through to the receptor fluid(Choi, Lee et al. 2012) . The observation of increased efficiency in transdermal delivery with the chitosan conjugated nanocapsules is interesting. The two forms of nanocapsules do not differ markedly in size (~10nm) but these two samples do differ in surface charge with the chitosan particles being positively charged. As mentioned in several instances in this report, this positive surface charge reduces repulsive effects and may aid movement through the dermal layers. Given these results, this would suggest that transdermal delivery of agents is possible with pluronic based

nanocapsules which could result in systemic absorption. However, as is most often the case it was only the simulated active payload (in this case a FITC-labelled protein) that was detected in the receptor fluid and deep skin layers and therefore the suggestion of transfer of the complete nanocapsules would be speculative.

4.2.4.1 Role of Physicochemical Properties

The literature suggests that in relation to polymer based nanocapsules, these tend to preferentially locate within the skin with limited transdermal passage through the skin. This effect does not seem to be specifically influenced by particles size although the evidence in this respect is somewhat limited. Instead, the major contributor seems to be the role of surface charge due to the enhanced interaction of positively charged particles with the negatively charged skin cells.

4.2.5 Summary

The literature provides a convincing picture of effective dermal penetration (~5-10%) achieved through the use of nano-transporters with both lipid and polymer based transporters allowing the passage of active substances such as drugs, anti-oxidants or labels through the SC. The ability of these materials to penetrate efficiently through the SC marks a considerable difference between these soluble nano-transporters and more conventional solid nanoparticles such as TiO_2, and ZnO which typically show minute penetration. For example, it was shown that after that after 22 days of administration of submicron (300-500 nm) or nano-sized (30-50 nm) TiO_2 to mini-pigs there was no detectable levels of TiO_2 within the sentinel organs (liver, spleen) for either size range. Very low levels of TiO_2 were noted in cell layers below the SC, including the dermis by TEM, but these were very small which led the authors to conclude that minimal penetration occurs through the viable epidermis, with TiO_2 particles primarily found in the SC and upper follicular lumens, and highly aggregated between the layers of keratin in the SC (Sadrieh, Wokovich et al. 2010).

The result of improved penetration over that of solid, insoluble nanoparticles into the dermal layers could mean that the relative dose of soluble nano-transporters received by these lower layers is greater than that of conventional solid nanoparticles. In terms of full transdermal penetration resulting in systemic absorption, the evidence suggests to the most part nano-transporters are retained within the skin. Indeed, as noted by Puglia et al., "lipidic or polymeric nanoparticles have shown the peculiarity to reduce and/or suppress the permeation (transdermal delivery) through the skin while enhancing the penetration (dermal delivery) into the upper skin layers (Jenning, Gysler et al. 2000, de Jalon, Blanco-Prieto et al. 2001, Alvarez-Roman, Naik et al. 2004, Lombardi Borgia, Regehly et al. 2005, Chen, Chang et al. 2006, Liu, Hu et al. 2007)" (Puglia, Blasi et al. 2008). This observation is of crucial importance for considering the fate of nano-transporters (applied topically) in the body. Specific localisation and retention within the skin limits the potential unforeseen and unintended impact of nano-transporters themselves (rather than their payload which may dissociate and travel further) and therefore provides a more localised focus on health impact. However whilst the evidence does point towards a relatively high level of dermal retention of nano-transporters, these findings are not unanimous and more evidence would be needed to rule out absorption and systemic availability of nano-transporters. A big part of this is the actual analysis of the fate of the nano-transporters themselves and not purely their payloads.

One issue within the literature is a distinct lack of detection or characterisation of the actual nano-transporters within the skin (or beyond). Studies almost exclusively determine the depth of penetration and/ or absorption through the detection of the active payload rather than detection of the specific nanoparticle as is more commonly the case for insoluble nanoparticles. The reason behind this is the distinct technical challenges associated with detecting very small, labile particles within a complex environment, which shares many of the structural components with the particles (i.e. lipids). There are a few exceptions where specific labelling or analysis of the particles themselves has been conducted and the results of this are of high value as they speak of the actual behaviour of the particle within the skin rather than that of the payload which may not one and the

same. For example, Kim et al. used the relatively common approach of loading the nano-transporters they were studying with Nile Red as a detectable florescent marker but coupled this with a further fluorescent dye (Lucifer Yellow) to the polymer shell enabling the two entities to be tracked separately. Using confocal microscopy to analyse treated skin sections they located the two markers and by overlaying them in an image were able to see that location of the yellow signal showed that the polymer remained in the SC whilst the NR payload was free to diffuse into the lower skin layers (Kim, Shim et al. 2010). Such an approach is of high value as it provides evidence that the detection of the active payload in the skin, in particular the lower layers, does not necessarily mean the nano-transporter is present or intact. Secondly, the approach also provided specific evidence of the locality of the transporters and showed that transdermal penetration did not occur. In another study were analysis of the nano-transporter as well as payload was investigated, the authors used tape stripping to investigate sequential layers coupled with scanning electron microscopy and EDX analysis. This approach enabled them to detect SLN in the skin (and specifically within channels suggesting a route of penetration) but also, with deeper layers they found both intact SLN and non-encapsulated payloads (TiO_2 particles). This then led the authors to suggest that at this level, biodegradation of the lipid was occurring possibly as a consequence of interactions with dermal lipid (Iannuccelli, Coppi et al. 2013).

These examples used either polymeric nanocapsules or SLN which may provide lesser challenges than other forms of nano-transporters such as liposomes whose composition is more closely matched to the dermal lipids. However, the greater knowledge and evidence such efforts provide is clear and greater efforts need to be made in characterising the specific dermal interactions, localisations and fate of nano-transporters – not their payloads, in the skin and beyond.

One aspect of nano-transporter fate, which has great relevance to the comparison to insoluble nanoparticles is their biodegradation. A key aspect of this is in understanding the rate of degradation, which in turn may affect the level of penetration which can be achieved before the nano-transporters loses its integrity, releasing its payload. Differing compositions and differing types of nano-transporters (e.g. polymeric nanocapsules vs. liposomes) will degrade at different rates with some persisting longer and offering greater protection to their payloads whilst others may be more susceptible to the dermal environment. The rate of biodegradability and the effect this has on potential risks is very much subject to debate as some nanoparticles such as ZnO are indeed soluble and do release zinc ions systemically (Gulson, McCall et al. 2010) but would likely be classified with insoluble nanoparticles within the current definition. Similarly, through modification nano-transporters could be produced to display sufficient resilience to degradation but the point at which they may be classified as a nanoparticle currently is unclear.

In relation to the physicochemical determinants of dermal absorption, the literature very much lacks systematic evaluations and comparative analyses of nano-transporters differing in specific physicochemical attributes. However, some studies do occur and similar to what is noted for conventional nanoparticles, size does appear to have an effect on the ability of particles to penetrate with smaller particles able to penetrate. One of the most convincing series of experiments was that of Abdel-Mottaleb et al. who compared the dermal penetration of a range of different nano-transporters, each of which were created in several sizes (large and small). The use of several sizes for each class of material allowed the authors to detect a size difference with smaller particles resulting in increased drug permeation but the use of numerous classes of nano-transporters further supported the applicability of this observation.

Another physicochemical property to receive attention within the literature is surface charge. As stated previously, the net charge of the skin is negative owing to its composition and so particles with a strong negative charge would be subject to repulsive forces whilst particles with a strong positive charge would be subject to attractive forces, possibly increasing interaction with the skin cells. Evidence for nano-transporters such as nanoemulsions suggests that a positive surface charge

results in enhanced dermal accumulation of actives although this does not necessarily translate to dermal absorption of nano-transporters. Instead, it may be the case that a positive charge facilitates improved release of the active payload within the skin (with or without improved penetration into the skin.

It is worth noting that size and surface charge in addition to composition were the physicochemical properties most commonly described within the literature with further characteristics rarely described.

The effect of a compromised dermal barrier such as a result of sunburn or other damage has received a great deal of attention with conventional nanoparticles (Monteiro-Riviere, Wiench et al. 2011) but much less so in relation to nano-transporters. Where barrier impairment has been assessed, this did show that nano-transporters penetrated more rapidly into the skin but due to the experimental design, it is not known if this could have resulted in transdermal penetration.

5 Dermal toxicity of nano-transporters

5.1 Introduction

The ability of a substance to cause an adverse effect in the skin of considerable concern as this can range from the mildly irritating to highly debilitating effects on the skin and examples of localised skin disease ranging from simple dry skin, psoriasis to serious ulceration and necrosis. Such toxicity may be associated with the intrinsic toxicity of a substance itself (e.g. corrosive or inflammogenic) or may occur due to the body's immunological reaction to a substance in the case of allergic skin disease. As a result of dermal penetration/ absorption, systemic toxicity can also occur and this may manifest in many different depending on the target organ (e.g. hepatic, renal, central nervous system etc.) and/or region of accumulation such as within the reticuloendothelial system.

Regulatory testing is in place in order to detect possible adverse effects on the skin and this section summarises the current evidence of toxicity of the various forms of nano-transporters. As nano-transporters differ markedly in physico-chemical properties such as composition and size, an adverse effect caused by one is therefore not necessarily representative of a response to all. Therefore, the following subsection is sub-divided by nano-transporter type and where possible, scientifically justified generalisation made about the role of specific properties.

5.1.1 Dermal Toxicity

The term 'dermal toxicity' in this context is meant to mean any adverse health effect resulting from dermal exposure to nano-transporters applied to the skin. This may mean localised effects occurring either acutely (e.g. after a single exposure) or after repeated exposures (e.g. sub-acute, sub-chronic or chronic) but also includes systemic toxicity such as hepatotoxicity. When considering systemic effects, this report deals exclusively with effects resulting from dermal absorption and/ or penetration of nano-transporters. It does not address systemic toxicity arising from other routes of exposure such secondary to inhalation (e.g. through the use of transporters applied via a spray product) or ingestion caused, for example though hand-to-mouth contamination.

From a regulatory perspective, dermal toxicity is considered in terms of endpoint such as:

- Skin irritation/ Corrosion
- Phototoxicity
- Skin sensitisation

Typical reactions of the skin to an insult is localised inflammation resulting in reddening of the skin (Erythema), swelling (oedema) and may also result in localised cell death given rise to a lesion such as an ulcer. These can be well characterised, providing a quantitative measure of toxicity and table 11 provides an example of the scoring of such dermal effects.

Dermal irritation is defined as the production of reversible damage and localised inflammation such as Erythema and oedema whilst dermal corrosion as defined as irreversible damage of the skin

(OECD 2015). Such irreversible damage could include visible necrosis through the epidermis and into the dermis and would be typified by ulcers, bleeding, bloody scabs etc.

TABLE 11: GRADING OF SKIN REACTIONS IN THE ASSESSMENT OF DERMAL IRRITATION (OECD 2015)

Effect	Score
Erythema and Eschar Formation (maximum possible = 4)	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4
Oedema Formation (maximum possible = 4)	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond area of exposure)	4

Broader evaluations of dermal toxicity include 'Acute' (OECD 1987), 'Repeated dose' (21/28 day) (OECD 1981) and 'sub-chronic' (90 day) (OECD 1981) exposure studies. Such tests are usually carried out on rats, rabbits or guinea pigs and therefore are not suitable for specific evaluation of materials for cosmetic purposes. For the application of a test substance, fur is either carefully clipped or shaved from the dorsal area of the trunk of the test animals (so as to avoid abrasion which may alter permeability) although shaving must be performed ~24hrs prior to the application of the test substance as it can cause irritation. In the case of repeated dose studies, repeat hair removal is often needed at weekly intervals. A minimum of 3 dose intervals are usually selected and then area of not less than 10% of the animal body surface is used for application. The selection of the doses, in line with other OECD test methods is intended to show clear evidence of toxicity although not induce fatalities in the highest dose, minimal toxicity in the intermediate dose and no evidence of toxicity in the lowest dose. Where there are useable estimations of human exposure (e.g. typical application rate) then the lowest dose should exceed this.

These test methods have been described as broader than more end-point focused test methods such as OECD TG404: Acute Dermal Irritation/Corrosion (OECD 2015) as the examinations made address a broad evaluation of health. These include haematological evaluations such as differential leucocyte count and platelet count, clinical biochemical measurements such as liver and kidney function in addition to gross necropsy and histopathological evaluation of normal and treated skin and on organs and tissues as well as all gross lesions.

In relation to acute dermal toxicity (OECD 1987), the endpoints evaluated are far more limited and focus on death with the derivation of an LD_{50} (median lethal dose) being the main endpoint under evaluation and as such, this test is useful primarily for Classification and Labelling purposes and much less useful for identifying modes of toxicity.

As well as *in vivo* test methods described above, there are also numerous *in vitro* alternatives such those employing reconstituted human epidermis (RHE) as a model barrier. The 7th Amendment to the Cosmetics Directive 76/768/EEC enacted on 27th February 2003 contained animal testing bans restricting the use of animals in the safety evaluation of cosmetics. This came into force on 11th of March 2009 with the exception of *in vivo* testing for repeated dose toxicity, toxicokinetics and repro-toxicity testing which were banned 2 years later.

In vitro alternatives do exist for many endpoints and table 12 describes alternative test methods adopted as OECD Testing Guidelines.

TABLE 12: *IN VITRO* ALTERNATIVE TEST METHODS

Endpoint	Assay	Test Guideline
Phototoxicity *in vitro*	3T3 Neutral Red Uptake (NRU) phototoxicity	OECD 432
Skin sensitisation	In Chemico skin sensitization: Direct peptide reactivity test (DPRA)	OECD 442C
	In vitro Skin Sensitisation: ARE-Nrf2 Luciferase test method	OECD 442D
	In vitro Skin Sensitisation: Human cell line Activation test (h-CLAT)	draft OECD TG
Skin corrosion	Skin corrosion using RHE models	OECD 431
	In vitro Membrane Barrier Test Method for Skin Corrosion	OECD 435
	In vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER)	OECD 430
	In vitro Skin Irritation	OECD 439
Eye corrosion, severe eye irritation	Bovine corneal opacity and permeability (BCOP)	OECD 437
	Isolated chicken eye test method for identifying ocular corrosives and severe irritants	OECD 438
Dermal penetration	Skin Absorption: *In vitro* Method	OECD 428
Genotoxicity	Bacterial Reverse Mutation Assay (Ames)	OECD 471
	In vitro Mammalian Cell Micronucleus Test	OECD 487
	In vitro Mammalian Cell Gene Mutation Test	OECD 476
	Chromosomal aberration test	OECD 473
	Unscheduled DNA synthesis *in vitro*	OECD 482

Whilst *in vitro* alternatives do exist, certain endpoints such as reprotoxicity, sensitisation, systemic toxicity, carcinogenicity and toxicokinetics prove particularly challenging as simplified *in vitro* systems struggle to effectively recreate such complex endpoints.

As the preceding description shows, there are numerous regulatory test methods available for the evaluation of toxicity (both dermal and more broadly) however such test methods are not always employed. There are numerous reasons behind this including the fact that regulatory test methods are primarily used during regulatory testing which tends to be performed by industry, with the results being commercially sensitive and therefore not subject to publication in scientific journals. The use of internationally accepted, standardised methods has obvious benefits to demonstrating the robustness of a study but that is not to say that results produced by other testing approaches are of limited or no use. On the contrary, such data can be very useful, in particular for the purpose of investigating mode of action through more detailed investigations than normally required within test guidelines. To reflect the assurance the use of standardised test methods have on study quality, the coding within the literature appraisal assigns the highest score to internationally accepted standard method e.g. OECD TG (score of 3) whilst non-standard documented methods gain a lower score (2) and undocumented methods such novel investigations using new methods gain the lowest score (1). In the following discussion of the evidence of toxicity, high value test results are noted.

5.2 Nano-Transporter Induced Dermal Toxicity

The range of nano-transporters, their different composition and production methods means that to gain a true insight into the dermal toxicity of this technology, it is essential that the different classes of nano-transporters be considered separately. This can then be followed by the assessment of the role of certain properties such as surface charge may have on induced effects to identify specific structure activity relationships.

Below, the evidence base for each of the main classes of nano-transporters is summarised and whilst it is important to identify any evidence of dermal toxicity, it is also important to note where these materials have been assessed and show no signs of toxicity. This is because to make an informed decision as to the potential challenges and risks associated with nano-transporters it is important to provide a balanced view of actual toxicities. The provision of data showing a lack of toxicity may be hampered as the reporting of solely negative results in the peer reviewed literature can be challenging which may provide an unbalanced view of actual toxicity (Poland, Miller et al. 2014). This is may in turn be compounded by the relative lack of openness of industry who, through a need to protect intellectual property and competitiveness maybe reluctant to disseminate test data outside of the specific requirements for regulatory compliance.

5.2.1 Liposomes

Liposomes are widely employed within the cosmetics market and as Figure 8 demonstrated, is one of the most commonly proposed nano-transporter types within patents. The use of biocompatible lipids has led to the suggestion that nano-liposomes are low toxicity and incorporate well into the skin. Within the literature, the evaluation of toxicity tends to not be the main focus of a study which instead, aims to describe the efficacy of the liposomes with limited assessment of biocompatibility. An example this is the study by Jo et al. (2005) (Byoung Kee Jo, Gi Woong Ahn et al. 2005), whereby the authors investigated the role of nano-sized liposomes as an anti-irritant in cosmetics. The hypothesis was that liposomes composed of ceramides would serve to reduce potential irritation caused by cosmetic due to the role ceramides play in the treatment of various skin disorders such as atopic dermatitis and psoriasis (Byoung Kee Jo, Gi Woong Ahn et al. 2005). Indeed studies have shown decreased ceramide levels in the *stratum corneum* of patients with skin barrier abnormalities (Stoughton, Clendenning et al. 1960) and it has been suggested that insufficiency in ceramides may provide an etiological basis for dry and barrier-disrupted skin (Byoung Kee Jo, Gi Woong Ahn et al. 2005).

Jo *et al.* compared the anti-irritation effects of formulas containing nanoliposomes composed of ceramide and those containing dispersed ceramide against a placebo by investigating the recovery rate of transepidermal water loss (TEWL) after tape stripping in human volunteers. The authors also looked at the ability of the preparations to inhibit a generalised cutaneous erythema, or 'flush' resulting from the topical application of methyl nicotate or skin irritation patch test.

The nanoliposomes were composed of a mixture of glycerine (65% wt.), caprylic/ capric triglyceride (12% wt.) phospholipid (5% wt.), ceramide III (8% wt.) and water (10% wt.). High pressure homogenisation was used size reduce macro sized liposomes to the nano-range to provide a particle size of 300-600nm

The nanoliposomes composed of ceramide led to a significantly accelerated repair of skin barrier damage (caused by tape stripping) as well as inhibition of a dermal flush caused by the application of methyl nicotinate (40.4% in the case of the nano-liposomes vs. the placebo effect of 16.8%). Similar results were noted for the human patch test, which caused a 45% reduction in irritation vs. positive control and a 25% reduction in stinging caused by the application of lactic acid. To investigate the cause of this reduction in dermal flushing, irritation and stinging, Jo *et al.* investigated the *in vitro* skin penetration of lactic acid using a Franz diffusion cell with skin from a hairless mouse used as the model dermal barrier. They found that application of the 300-600 nm nano-liposomes composed of ceramide led to a lower penetration of lactic acid, which led them to conclude that inhibition of the dermal effects to the various triggers of irritation was due to improved barrier function resulting from the application of the ceramide nano-liposomes. In all of the experiments, the application of the nano-liposomes either composed of, containing or not containing (i.e. placebo) ceramide did not induce a worsening of skin effects induced by tape-stripping, lactic acid application etc.

Whilst the study by Jo *et al.* utilised human volunteers as a test model, a recent study by Clares *et al.* evaluated the toxicity of liposomes, SLN and nanoemulsions loaded with retinyl palmitate (RP; vitamin A) on *ex vivo* human skin obtained from elective, abdominal plastic surgery (Clares, Calpena et al. 2014). The skin was dermatomed to 400 μm thickness and placed within a Franz diffusion cell and 200μg of test samples applied to the upper surface over a period of 38hrs. The liposomes were formed of a 1:1 ratio of L-α-Phosphatidylcholine and RP dissolved in chloroform, dried and dispersed to form liposomes. The SLN were produced using the ultrasound method with 5% Compritol® 888 ATO used as the lipid phase and the nanoemulsions prepared by mixing RP (1% wt.) with oil (Labrafac lipophile, 15% wt.), surfactant and co-surfactant (Labrasol (53.47%) and Plurol oleique (13.27%)). The nanoemulsions had the lowest particle size at 14.42nm followed by the liposomes at 176nm and the SLN were 271nm in diameter. The lipid nanoparticles were all negatively charged (-42.2 to -55.26 mV).

Evaluation of the dermal toxicity of the three nano-transporters was performed by assessing the structure of the dermal layers by histological evaluation of the skin sections post exposure in the Franz diffusion cell. The results showed that treatment with the free drug (RP) led to clear disruption of the *stratum corneum* caused by cutaneous irritation. The skin treated with the nano-emulsion had a seemingly rigid appearance and appeared to be fragile with a loss of flexibility. The authors hypothesised that the oil and surfactant in the emulsion had interacted physically with *stratum corneum* bilayers, causing lipid extraction resulting in dehydration with significant loss of moisture (Shakeel, Baboota et al. 2008) and causing a disorganize structured (Gonnet, Lethuaut et al. 2010). Based on this and the skin permeation studies, the authors suggested that nano-emulsions with the highest flux could induce skin irritation or sensitization, cause damage and reduce the barrier function (Clares, Calpena et al. 2014).

In contrast to the free drug and nano-emulsion, after treatment with the liposomes and SLN the *stratum corneum* appeared uniform with evidence of interlamellar gaps between the *stratum*

corneum and uppermost layer of the epidermis after treatment with the liposomes. This was suggested to be caused by improved hydration of the skin. These results suggest that whilst all preparations had a beneficial effect on the toxicity of the free drug through encapsulation, the nano-emulsion RP preparation was associated with dermal irritation. However, as blank nano-emulsions were not used as a compactor, it is impossible to state whether the toxicity observed with the nano-emulsion RP preparation was due to intrinsic toxicity of the emulsion or the sub-optimal prevention of RP mediated toxicity. The view of the study author was that the oils and surfactants in the emulsion led to lipid extraction from the *stratum corneum* and therefore this would suggest intrinsic incompatibility between the emulsion and the skin.

A 2012 study by Gokce and colleagues (Gokce, Korkmaz et al. 2012) evaluated coenzyme Q10 loaded liposomes in comparison with SLN for their biocompatibility and cytotoxicity using *in vitro* cell culture. The liposomes were composed of Phospholipid-Lipid S100, cholesterol and coenzyme Q10 (payload) and homogenised to produce liposomes which were 301nm in diameter (367.9 nm in the case of the blank liposome). In comparison, the SLN were much smaller at 152.4 nm in diameter (164.1nm in the case of the blank SLN) and similar to the study of Clares (Clares, Calpena et al. 2014), were composed of Compritol® 888 ATO as a lipid base with Poloxamer and Tween 80 used as surfactants and co-surfactants respectively which were then size reduced using high-shear homogenisation. All particles were negatively charged although the liposomes were much more so at -32-36 mV (-13-18mV in the case of the SLN).

Cytotoxicity was evaluated using normal human dermal fibroblasts using the MTT assay across a dose range of 10-250 μM. Further analysis was performed on proliferation at 25 and 50 μM doses, again using the MTT assay. Both nano-transporters showed significant ($P< 0.05$) cytotoxicity at the highest dose of 250 μM but it was at lower doses that differences in the biocompatibility of the two transporters became apparent. At the dose of 100 μM, 80% cell viability was maintained after administration of the CoQ10 loaded SLN but in the case of the CoQ10 loaded liposomes, viability was less than 20%. Analysis showed the critical concentration of the CoQ10 loaded nano-transporters to be 50 μM and at this concentration and below, treatment with the liposomes indicated stimulation of cellular proliferation. Further testing to demonstrate the protective effect of CoQ10 administration involved culturing the cell under oxidative conditions (presence of H_2O_2) and assessing viability which showed that whilst the liposomes provided a protective effect (greater than that of CoQ10 alone at the same dose), the SLN did not.

The reason behind the differential toxicity and moderate dose was not discussed within this study and the specific cause is difficult to ascertain given the different size, compositions and surface charges of these two nano-transporters. A detraction from the quality of the study is that whilst the authors appear to produce blank versions of the liposomes and SLN (size and charge data were reported), they did not test this for cytotoxicity or efficacy in protecting the cells from oxidative stress. This means it is not directly possible to assess the impact of the particle payload on toxicity although as toxicity was noted with the liposomes at 100 μM yet not the SLN, this suggests that CoQ10 was not the main driver per se. An additional detraction was the fact that the biocompatibility testing was rather limited. Indeed, best practice dictates that multiple assays are used in order to test a specific endpoint rather than relying on a single test method (MTT in this case) (ISO 2014) in case of assay interference caused by the particles (a common issue (Kroll, Pillukat et al. 2012)).

Within many of the studies published, the surface charge of the liposomes and indeed many lipid based nanoparticles are negative (Clares, Calpena et al. 2014). The surface charge of a material can influence several factors such as its propensity to agglomerate/aggregate, its interaction with charged molecules, such as proteins, and its cellular uptake. Given that the particle surface is point of interaction with biomolecules and cells, it plays a significant role in determining *in vivo* behaviour of nanomaterials, whether they result in benign, beneficial consequences, or cause toxicity (Kim, Saha et al. 2013). Indeed, it is hypothesised that particles with a highly charged

surface may also adversely affect cells by strongly binding and disrupting membranes. Evidence for this effect is reported in the literature for solid insoluble nanoparticles (Cho, Duffin et al. 2012, Greish, Thiagarajan et al. 2012, Nagy, Steinbruck et al. 2012) as well as for other conventional particles such as asbestos (Light and Wei 1977, Light and Wei 1977) and airborne particulate matter (PM) (Veronesi, de Haar et al. 2002). For example, Goodman and colleagues examined the effects of gold nanoparticle surface charge on cytotoxicity by studying cationic (amine) and anionic (carboxyl) gold nanoparticles on Cos-1 cells, red blood cells, and *E. coli*. It was concluded that cationic gold particles were moderately toxic and anionic particles were non-toxic, pointing to the initial electrostatic binding of the particles to the negatively charged cell membrane as the probable mechanism of toxicity and suggesting that electrostatic repulsion may limit anionic and neutral particle interaction with the cell surface (Goodman, McCusker et al. 2004). As such, within this experiment it can be seen that cationic gold nanoparticles can be expected to exhibit more toxic effects relative to anionic particles. In another study, Schaeublin *et al.* modulated the surface charge of 1.5 nm gold particles and examined the interactions and effects on a human keratinocyte cell line (e.g. cellular morphology, DNA damage-related gene expression etc.)(Schaeublin, Braydich-Stolle et al. 2011). Whilst the positively charged, neutral, and negatively charged particles all caused alterations in cellular morphology and dose dependent toxicity, the authors did note increased toxicity at lower concentrations as well as mitochondrial stress with the charged particles. Perhaps most interestingly, they also noted that different surface charges induced differential effects on cellular process and mechanisms of cell death, specifically neutral nanoparticles caused necrotic cell death whilst the charged nanoparticles caused apoptosis (Schaeublin, Braydich-Stolle et al. 2011).

Whilst many studies utilise negatively charged liposomes, the 2013 study by Carboni *et al.* developed a range of liposomes which were composed of monoolein (~3% concentration) and increasing amounts of lauroylcholine ranging from 0.3% (termed LPS0.3) to 1.3% (termed LPS1.3). The result was a range of liposomes of roughly equal size (77-87nm in diameter) which varied in surface charge although all were positive charged (+57mV (LPS0.3) to +82 mV (LPS1.3)). As well as developing unloaded liposomes, the authors also loaded a liposome with diclofenac as a model drug. The result of this loading was an increase in particle size from 82nm in the case of the empty comparator to 202nm and a reduction in zeta-potential from +57mV to +36 mV.

Whilst this study does not specifically address the cosmetic applications of these liposomes and indeed the use of a model drug suggests a more medical focus to the study, it is one of the few studies to sequentially modify the surface charge of a nano-transporter in the positive range. Therefore, this study is of considerable interest.

The authors characterised the toxicity of the liposomes using an *in vitro* cell model composed of mouse 3T3 fibroblasts. The 3T3 fibroblast cell line is of embryonic origin and is a standard fibroblast cell line model and used both as feeder cell layer (Carlson, Alt-Holland et al. 2008) for culture with keratinocytes and as a dermal cell model.

The cytotoxicity was assessed using the MTT assay and showed that both the liposomes with the lowest surface charge of 57.3 mV (LPS0.3) and the highest surface charge of 82.8 mV (LPS1.3) showed caused significant cell death (>80%) over a period of 24-48hrs. Interestingly, the cytotoxic effect was more rapid with the more positively charged LPS1.3 particles which caused >50% reduction in cell viability in the first 4hrs whilst the more neutrally charged LPS0.3 particles did not. The cells showed a pro-apoptotic appearance with condensed nuclei and altered intracellular lipid distribution. One aspect of this study that causes it stand out from others is that as well as characterising the cellular toxicity of the liposomes, the authors also looked at the toxicity of the constituent ingredients. They found that the addition of monoolein (~3% concentration) or lauroylcholine (0.3%) did not cause toxicity at either short or long term incubation although at a higher, 1.3% concentration lauroylcholine did cause 50% toxicity in the short term suggesting intrinsic toxicity of the liposomal constituent. The authors concluded that at short term exposures and, most importantly at low lauroylcholine content the liposomes were not toxic and that

lauroylcholine favoured internalisation. As lauroylcholine itself showed significant cytotoxicity, it is unclear if the primary driver of toxicity was composition or surface charge although given that liposomes were the same size, size as a factor in differential toxicity in this case can be ruled out. It is thought that positively charged solid particles can interact with negatively charged cell membranes, potentially causing perturbation and rupture leading to cell death. However, given the flexibility and lipid nature of the liposomes, it is unclear if this could occur and instead, enhance fusion with cells leading to internalisation may occur.

5.2.1.1 Role of Physicochemical Properties

In relation to evaluating the role physicochemical properties contributing to observed toxicity, the literature does not lend itself to the critical assessment of the role modification of key physicochemical properties have on toxicity. To do so, preferably involves the modification of single attribute (such as diameter) followed by a systematic comparison of effects. However, this is challenging as it can be difficult (if not impossible) to modify one attribute and keep all others the same but also whilst such studies from a toxicological perspective are very useful, they are perhaps of lesser importance to the specific aim of technological innovation whereby demonstration of function is more important.

The literature seems to suggest that in relation to toxicity, composition of the liposomes is a major factor in determining toxicity. Based on the study of Carboni, it was shown that whilst the size remained the same, compositional (and surface charge) changes resulted in differential toxicity at shorter time points suggesting that size in that situation were of lesser importance.

5.2.2 Solid Lipid Nanoparticle & Nanostructured Lipid Carrier

SLN and NLC are employed in nanocosmaceuticals and NLC represent the further development of SLN technology with their improved loading capacity. Their toxicity has been evaluated across a range of studies but as discussed in section 3 and shown in Table 3, the composition of SLN and NLC can vary widely as well as their resultant physicochemical properties. Of the studies evaluated addressing the dermal toxicity of SLN/ NLC for cosmetic applications, the majority employ *in vitro* methods to investigate cellular interactions.

The *in vitro* approaches used in the investigations of SLN/NLC toxicity range in complexity starting with analysis of the haemolytic potential as the most basic. The haemolysis assay is employed commonly with particle toxicology (Wilson, Stone et al. 2000, Stone, Jones et al. 2004, Cho, Duffin et al. 2013, Horwell, Baxter et al. 2013) yet whilst it does use red blood cells (RBCs) as a model; it is not necessarily a true model of haemocompatibility as the model lacks other crucial blood constituents such as plasma proteins, platelets etc. Instead, the membrane of the RBCs provide a simple model of a cellular membrane which when disrupted, leads to the release of haemoglobin, which can be quantified spectrophotometrically. As such, it is straightforward to quantify adverse reactions leading to cell membrane perturbation and rupture. The haemolysis assay has been employed in several studies looking at the membrane compatibility with SLN/ NLC. The 2014 study by Pizzol *et al.* (Pizzol, Filippin-Monteiro et al. 2014) analysed the biocompatibility of SLN formed of different ratios of stearic acid, polysorbate 80 and Lecithin. The SLN were all negatively charged (-11 to -15 mV) and should show a repulsive effect to the negatively charged RBC membrane. The particles ranged in size from 116nm to 306nm depending on composition. The results of co-incubation of the SLN with RBCs showed stark variation with 6 of the 9 formulations showing no haemolytic activity whilst 3 showed high levels of haemolysis (~50%). The size of these 3 haemolytic particles was not measured due to incompatibility with the technique (highly reflective) and so correlations between size/ charge and toxicity could not be made although the 6 non-haemolytic SLN varied considerably in size suggesting that size may have not been a driver. The one correlating factor was the presence of stearic acid in the composition of the haemolytic SLN whilst this was not present in the others.

NLC were also analysed for their haemolytic potential in a very recent study in comparison to nanospheres, nanocapsules and liposomes. The particles ranged in size from 136nm to 214nm with NLC being middle in the range at around 195nmand had the most negative charge at -19.48 mV followed by the nanospheres (-19.38 mV), nanocapsules (-15.02 mV) and liposomes (-6.86 mV) respectively (Mendes, Delgado et al. 2015). Whilst none of the other particles showed any sign of haemolysis, the NLC sample at high particle number of 2.1×10^{10} and 2.1×10^{11}. The haemolysis results were reflected in further *in vitro* testing using 3T3 fibroblasts as well as lymphocytes obtained from human volunteers with NLC being the most cytotoxic. The authors used differing assays depending on the cell type (the MTT assay with the lymphocytes and Neutral Red assay with the 3T3 cells) and the rationale for this was not immediately clear. Of the particles tested, the liposomes were the least cytotoxic whilst nanospheres and nanocapsules did induce a reduction in cell viability (16 – 35%) (Mendes, Delgado et al. 2015).

This result is interesting not least through the use of particle number as a dose metric which is rarely used within such dermal nanotoxicity studies. In addition, the NLC do not stand out as being significantly smaller or larger than the other nano-transporters analysed and whilst it does have a large surface area, the nanocapsules showed an even greater surface area. Similarly, the surface charge of these particles did not differ appreciably from the other test materials and one would have hypothesised that the negative charge of the NLC would have acted to impede cellular interactions through electrostatic repulsion. One thing that is of interest is that when monitoring cellular uptake, the NLC sample was taken up to the greatest extent by the lymphocytes reaching 66% by 6hrs; over twice that of the closest comparator (liposomes). As such, the main difference between the various nano-transporters was composition and the NLC were composed of glyceril monostearate, oleic acid, poloxamer 188 and sorbitan monosterate as a surfactant.

The lack of specific correlations between observed toxicity and physicochemical properties was also noted by Pizzol who when analysing the results of varying toxicity of SLN in fibroblasts cells noted that the toxicity did not present a size trend. This led them to suggest that a “combination of components should be considered as the primary parameter for cytotoxicity profiles instead of size” (Pizzol, Filippin-Monteiro et al. 2014).

Recently, a study by Gokce *et al.* compared the release and *in vitro* toxicity profiles of NLC and SLN loaded with resveratrol (RSV) (Gokce, Korkmaz et al. 2012). The toxicity model used normal dermal fibroblasts from human foreskins which were exposed to increasing doses of RSV loaded SLN, NLC or RSV alone at 10, 25, 50, 100 and 250 μM and toxicity evaluated using the WST-1 assay which shows cellular viability and proliferation. The results showed that at the higher doses of 250 and 500 μM cell death at levels greater than 40% was seen and this led the authors to determine that a concentration of 50 μM was optimal for RSV loading. Comparing the two nano-transporters, no significant difference was noted in terms of cytotoxicity with both causing similar levels of toxicity at the higher concentration ranges and no toxicity at the lower ranges. The size of the SLN was 161nm with a zeta-potential of -15.3 mV (RSV entrapment efficiency of 73%) and the NLC were developed by using the SLC as a base and adding in varying qualities of Miglyol oil which led to a reduction in size. The optimally selected NLC had a diameter of 110.5nm, a zeta-potential of -13.8 mV and a loading efficiency of 91%. Thus it can be said that whilst compositionally the two nano-transporters differ, the latter containing 15% Miglyol oil, the particulate properties of size and charge do not differ wildly which may account from similar toxicological response which is likely to be driven by the RSV rather than the particle properties. As with many studies, whilst the authors developed and characterised empty carriers, they failed to evaluate the toxicity of these materials and so it is difficult to say if the observed toxicity at 100 and 250 μM was due purely to the RSV content delivered to the cells or if this reflected to some degree an intrinsic toxicity associated with high dose SLN/ NLC application.

The effect of composition on cytotoxicity was evaluated by Weyenberg, Filev et al. (2007) who varied the type and amount if various surfactants in combination with core materials of stearic acid, suppocire, novate or Witespol. In total there were 11 strearic acid SLN whilst the remaining 9 were termed hard fat SLN. All of the particles generated had a negative surface charge with the majority between -20 and -30mV. The size of the SLN similarly varied but was typically between 300 and 400nm in diameter. The toxicity of the SLN was evaluated using only the MTT assay with 3 cell lines including 3T3 mouse fibroblasts, HaCaT keratinocytes and J774 macrophages. The results showed a stark divide between the hard fat and stearic acid SLN with the hard fat SLN showing very little cytotoxicity in the 3 cell lines tested at a higher dose of 5%. In contrast, the SLN formulated with stearic acid were cytotoxic for all cell lines causing more than 80% cell death at a dose of 1%. The macrophage cell line proved to be the most sensitive, which was ascribed to the macrophages function of taking up particles and thereby leading to a greater intracellular dose than the other, more passive cell lines. What is most profound is the clear link between SLN composition (i.e. the presence or absence of stearic acid) and toxicity with no apparent correlation with size or charge, although neither of these attributes changed markedly across the sample ranges. Similar results were also noted in other studies (Pizzol, Filippin-Monteiro et al. 2014).

The detection of toxicity driven by the presence of stearic acid using the haemolysis assay by Pizzol and colleagues (Pizzol, Filippin-Monteiro et al. 2014) suggests that this assay approach may offer a very rapid, cost effect and straight forward way of screening for biocompatibility although further analysis would need to be performed to confirm its utility across a range of substances.

Whilst many of the studies have focused on the generation of cell death, other endpoints are also available including inflammation and genotoxicity. The latter is of considerable concern if particles penetrate to the viable and dividing cell layers of the *stratum basale* and indeed studies show that these particles can penetrate with efficiency and accumulate in the skin. To address, this Doktorovova and colleagues (Doktorovova, Silva et al. 2014) investigated the genotoxic potential of cationic SLN using a human liver carcinoma cell line (HepG2) and human colorectal cell line (Caco-2). The choice of cell lines obviously indicates a more systemic focus of the study with applications such as drug delivery being the main application aim rather than dermal toxicity but the results are of interest. The SLN were produced using high shear homogenisation and 3 forms of SLN were produced. These used either Imwitor 900P or Compritol 888ATO as a solid lipid in conjunction with Lutrol F68 or Miranol C-32 Ultra as the surfactant. The particles all possessed a positive surface charge although this range from +55 mV for particle A, +61.2 mV for particle B and +72.5 mV for particle C. Similarly the size was altered between the 3 samples with sizes of 141nm, 173nm and 222nm for samples A, B and C respectively. Cell viability reduced in a dose dependent manner with significant cytotoxicity noted at the high dose of 1mg/ml tested. The level of genotoxicity was assessed using the Comet Assay which detects DNA strand breaks and the results showed no major DNA damage at concentrations below 1mg/ml and positive results for genotoxicity were only noted at doses shown to be cytotoxic. Overall, these results show that the SLN detected were not genotoxic although at high doses, could induce cell death(Doktorovova, Silva et al. 2014).

In addition to *in vitro* analysis of biocompatibility, studies also utilised *in vivo* assays. One such example is the study of SLN loaded with tretinoin (a metabolite of vitamin A) published by Shah et al.

The study used the draize patch test in which SLN formulations were applied to the backs of rabbits, 24hrs after they have had their hair clipped free. The dose was 0.5g spread across an area of 4cm^2 and the skin was observed for any visible signs of change such as erythema (redness) up to 72hrs after application and graded using the scale detailed in Table 11. As shown in figure 11, the marketed formulation of Retino-A® cream caused significant erythema which was dramatically reduced in the case of the SLN incorporating tretinoin (Figure 11, D). In addition the unloaded SLN

preparation also showed a lack of dermal response indicating a lack of irritation (Shah, Date et al. 2007).

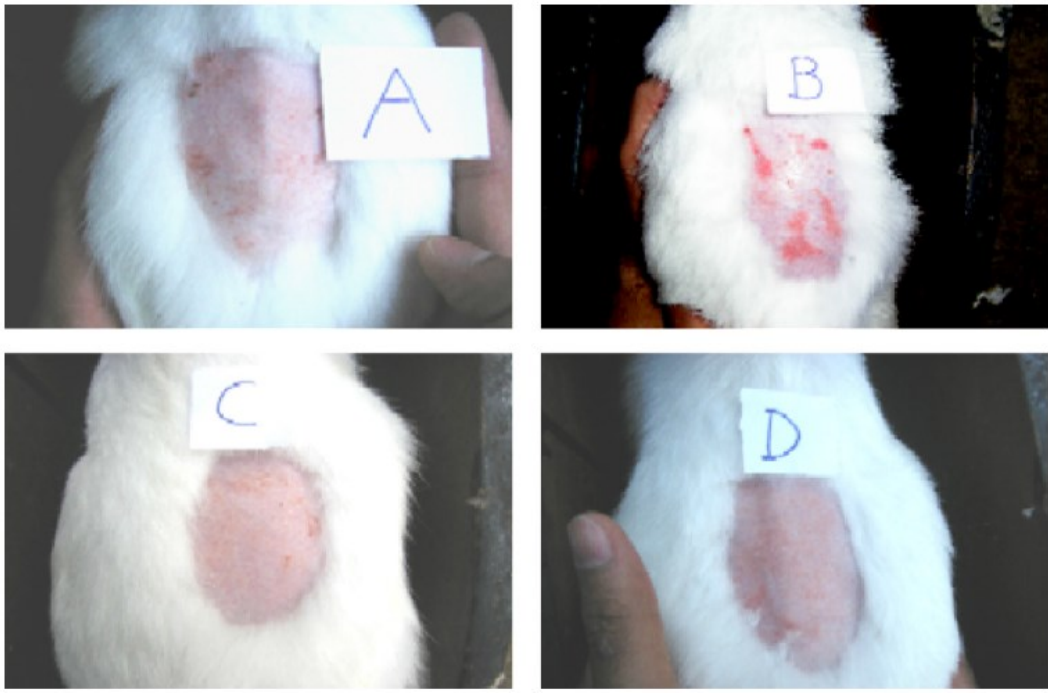


FIGURE 11: PHOTOGRAPHS OF SKIN IRRITATION STUDIES CARRIED OUT ON NEW ZEALAND RABBITS.

(A) control no application); (B) marketed formulation (Retino-A® cream); (C) SLN based gel without TRE; (D) SLN based gel containing TRE (0.05%, w/w). Photographs have been taken after 24 h. Marketed TRE cream clearly shows erythemal lesions, which are not visible in SLN based TRE gel. Reproduced from Shah *et al.* (Shah, Date et al. 2007)

Addressing SLN/ NLC toxicity more broadly (i.e. not focused on cosmetic applications), Doktorovova et al. published an excellent and detailed review in 2014 (Doktorovova, Souto et al. 2014). As noted within our review, Doktorovova stated that they found few studies with the stated purpose of assessing safety and considerable challenges in comparing study outputs due to the plethora of different materials, compositions, species, models, endpoints, assays and data analyses used(Doktorovova, Souto et al. 2014). In order to compare published studies, they systematically collated information on cell lines, methods, exposure duration, dose range, and outcome as well as basic information on the SLN/NLC (solid lipid, surfactant, size and zeta-potential).

In terms of toxicity, they attempted to compare response based on calculations of the IC_{50} (dose corresponding to 50% inhibition/ cell death) but noted that relatively few studies included a sufficient number of doses to calculate an IC_{50}. They found that most IC_{50} values were in the range of 0.1-1mg/ml and some were found to be greater than 1mg/ml. Of the values listed, the majority seem to sit above 0.3 mg/ml. They could not detect any clear tendency of surfactant or lipid core type on toxicity but they did note that the surfactants poly-vinyl alcohol (PVA) and sodium dodecyl sulphate (SDS) led to low IC_{50} values whilst commonly used surfactants such as Tween 80 were associated with higher values. Based on an IC_{50} measure, they were unable to establish if size or surface charge had an effect on toxicity and figure 12 shows the plotting of cell viability data from a range of studies utilizing arrange f models against particle size showing a clear lack of a trend.

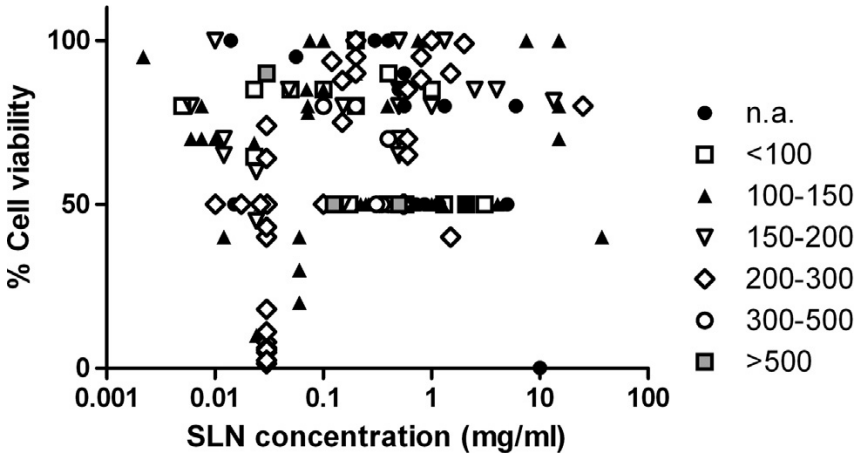


FIGURE 12: THE EFFECT OF SLN/NLC SIZE (Z-AVE, DIAMETERS IN NM) ON CELL VIABILITY *IN VITRO*.

Cell viability (in % viable cells) vs. concentration of SLN/NLC in cell culture media reported in Doktorovova et al. (2014). From reports that list three and more different SLN/NLC concentration, the highest and lowest cell viabilities were used. Citation, in the respective references, as "No decrease in cell viability" was plotted as 100% viability, as "no toxicity" was plotted as 85% viability (as nontoxic could be between 70% and 100% viability) and IC50 data were plotted as 50% viability. Size not available on the reference denoted by n.a. Reproduced from Doktorovova, Souto et al. (2014).

In terms of compatibility, the lowest viabilities were noted from reports on cationic SLN/NLC and also from particles composed of stearic acid (as noted previously) whilst other compositions such as the commonly used Whitpsol SLN were non-toxic. Indeed, when considering the influence of surfactants on toxicity Doktorovova reported that the influence was more pronounced with cationic SLN/NLC with the cationic surfactants/ lipids alone showing signs of toxicity. They also found that single alkyl chain containing surfactants were more toxic than double chained surfactants.

Addressing toxicity more broadly, by looking across the aggregated data it was determined that there is no apparent difference between the toxicity of SLN and NLC but true comparative data was very sparse. In addition, it was also suggested that the biocompatibility of SLN/NLC is better that that of comparable colloidal systems prepared from polymers, possibly owing to improved biodegradability. Overall, the systematic review by Doktorovova point towards the impression of SLN/ NLC as well tolerated carrier systems (Doktorovova, Souto et al. 2014).

5.2.2.1 Role of Physicochemical Properties

Of the physicochemical properties, there is a lack of specific correlations between properties and toxicity and this has been noted within the literature (Doktorovova, Souto et al. 2014, Pizzol, Filippin-Monteiro et al. 2014). Where differences have been associated with specific nano-transporters such as NLC, these often do not differ markedly in size or surface charge (as being the main properties reported) from other, less active comparators. However, one attribute that does seem to show some association with toxicity is composition. A clear example here is the inclusion of stearic acid in the composition of SLN which has been shown to induce haemolysis in RBCs as well as cytotoxicity in a variety of cell models. Other SLN/NLC constituents such as PVA and SDS have also been shown to be sub-optimal in terms of biocompatibility and such issues demonstrate the need for careful consideration of particle composition during development.

5.2.3 Nanoemulsions

The toxicity of nano-emulsions has been evaluated in a range of studies and the approach to toxicity evaluation can be quite varied. One novel example is that of Kong et al. (2011) who as well as utilising histological evaluation of rat skin previously treated in a Franz diffusion cell, also used the haemolysis assay to assess biocompatibility. The haemolysis assay is a simple, accepted and well used assay within then particle toxicology field (Pavan, Rabolli et al. 2014) as a model of the interaction between a particle and a biological membrane. It is not a model of the complex interactions of a particle and a motile cell such as a macrophage nor is it a model for haemocompatibility as crucial blood components such as platelets are removed during processing, leaving only erythrocytes as a simple model biological membrane. Previously published studies on the interaction between haemolytic activity and particle physicochemical properties (Cho, Duffin et al. 2012), specifically charge, size (and surface area), solubility and composition showed that zeta-potential at an acidic pH (5.6) of less than +14mV showed no haemolytic potential whilst the haemolytic potential of nanoparticles with zeta-potential greater than +14 mV, showed a linear relationship with haemolysis. The other parameters that were available for comparison (namely primary size, hydrodynamic size, mass dose, EPR (electron paramagnetic resonance) intensity, solubility, and zeta potential at a basic pH or with the presence of protein) showed poor correlations with haemolytic potential. This was later correlated to the wider toxicological picture whereby in a later study (Cho, Duffin et al. 2013) the same authors investigated a range of *in vitro* toxicological assays in the predictive value for acute respiratory toxicity. The results showed that across a panel of 9 different nanoparticles, the haemolysis assay showed 100% consistency with the lung inflammation if any dose, having statistical significance was considered positivity.

Whilst the utility of the haemolysis assay has been demonstrated in terms of respiratory toxicology, it is not clear how well the relatively brittle red blood cells represent the dermal environment and therefore, its use in evaluating dermal toxicity. As such, the results of Kong *et al.* should be interpreted with caution.

The results showed that incubation with amphiphilic 56-59nm nano-emulsions containing Vitamin E (dose 0.5 – 1mg/ml) led to significant haemolytic activity ranging from 31 to 56% at the highest concentration. Of the nano-emulsions tested, the least haemolytic was taken forward for skin penetration and toxicity experiments. Histological evaluations after the skin had been treated with the nano-emulsion for 10hrs showed that the *stratum corneum* had a rigid appearance, seemingly to have lost its flexibility. This was coupled with extensive inter-lamellar gaps that seemed to suggest lipid extraction from the *stratum corneum* by the nano-emulsion leading to dehydration of the skin. A similar result was seen by Clares *et al.* when evaluating the toxicity of liposomes, SLN and nanoemulsions loaded with retinyl palmitate (RP; vitamin A) on *ex vivo* human skin (Clares, Calpena et al. 2014). Similarly the authors hypothesised that the oil and surfactant in the emulsion had interacted physically with *stratum corneum* bilayers, causing lipid extraction resulting in dehydration with significant loss of moisture (Shakeel, Baboota et al. 2008) and causing a disorganize structured (Gonnet, Lethuaut et al. 2010). Despite this dehydration, Kong *et al.* noted no signs of irritation within the dermis or signs of skin irritation (erythema and oedema) (Kong, Chen et al. 2011). However whilst the authors report no signs of erythema and oedema, it is perhaps unlikely that such dermal effects would be significantly apparent given the skin had been excised and mounted and so was not attached to the vasculature which would limit surface reddening etc.

Considering the role of surface charge, Yilmaz and Borchert investigated positively and negatively charged oil/ water nano-emulsions for their ability to cause erythema (Yilmaz and Borchert 2006). The nano-emulsions contained particles 180-200nm in diameter and did not differ appreciably between the preparations. The surface charge of the positively charged nano-emulsion was +35mV whilst it was -43mV for the negatively charged formulation. To test for skin irritation and erythema, the nano-emulsions were applied to female volunteers twice daily over a period of 28 days with readings taken at day 0, 14, 28 and 29 (1st day after last application) and 31. The results showed that

the positive charge caused a significant improvement in skin humidity and elasticity. In addition, none of the formulations evaluated caused visual signs of irritation or evidence of erythema indicating low toxicity (Yilmaz and Borchert 2006). A similar lack of apparent dermal toxicity as determined using a patch test in human volunteers was also noted after application of negatively charged nanoemulsions prepared from hydrogenated lecithin and silicone oil (Bae, Shin et al. 2009). Thus, within this study it appears that the presence of a high surface charge did not lead to appreciable dermal toxicity.

A number of studies indicate that composition can have a significant effect on biocompatibility and indeed that presence of large levels of surfactants may cause dermal irritation (Li, Wu et al. 2011). An excellent study by Weyenberg and colleagues (Weyenberg, Filev et al. 2007) looked at the cytotoxicity and physical properties of various submicron-emulsions and SLN. The focus of the study was to evaluate the influence of (co)surfactants on the physical properties of carrier systems and subsequently on their cytotoxicity. This stems from concerns as to the potential irritation of surfactantia or co-surfactantia as *in vitro* and *in vivo* data suggest that cationic surfactantia can be the most detrimental followed by anionic surfactantia with non-ionic surfactantia being the least problematic (Harvell, Tsai et al. 1994, Korting, Herzinger et al. 1994, Wilhelm, Bottjer et al. 2001, Weyenberg, Filev et al. 2007). The authors produced a range of 11 microemulsions varying in the amounts of lecithin and utilising different combinations of co-surfactants. The particles ranged in size from 538 – 900nm in diameter and all had a negative surface charge (around – 30mV). The toxicity was evaluated using only the MTT assay but treated 3 cell types including 3T3 mouse fibroblasts, HaCaT keratinocytes and J774 macrophages. Whilst the 3T3 and HaCaT cells are commonly used dermal models, the J774 macrophages are a less obvious choice but were selected due to their ability to phagocytose particles and so were included to address the influence of particle size on cell survival. Across the 11 preparations, only the addition of stearylamine led to cellular toxicity and the concentration was a factor with a concentration of 1% being less toxic than 5%. Given that the size and surface charge of these 11 preparations were relatively similar, this study shows how composition can have a profound effect on toxicity.

5.2.3.1 Role of Physicochemical Properties

The evidence surrounding the toxicity of nano-emulsions suggests in humans (*in vivo*) such preparations well tolerated although *ex vivo* analysis does suggest that the oils and surfactants used in emulsions can interact with *stratum corneum* bilayers, causing lipid extraction resulting in dehydration with significant loss of moisture (Shakeel, Baboota et al. 2008) and causing a disorganize structured. Such effects and indeed, significant cytotoxicity *in vitro* seems to be driven by the choice of lipids and surfactants with some showing considerable cytotoxicity. Overall, physicochemical properties such as surface charge and size do not appear to have a profound effect on dermal toxicity of nano-emulsions although of the studies evaluated, few appear to be truly 'nano' in scale.

5.2.4 Nanoparticulates (nanospheres, nanocapsules)

The cyto- and genotoxic potential of poly(lactide-co-glycolide) nanoparticles was evaluated *in vitro* using the 3T3 fibroblast cell across a range of concentrations (5.4, 54 and 540 μg/ml). The nanosphere were 95nm in diameter with a zeta-potential of -20mV and application of these nanospheres to the fibroblasts is culture did not lead to significant cytotoxicity (as assessed using the MTT assay) despite the relatively high top dose. Analysis of genotoxicity was performed using the cytogenetic assay using isolated human lynphocytes. Co-incubation with the nanospheres caused no significant changes in the mitotic index suggesting a lack of genotoxicity.

Given their potential for drug delivery, the toxicity of polymer-based nanoparticles have been assessed in several studies for their broader toxicity as assessed through more direct routes of exposure to the body. These include intraperitoneal (IP) and intradermal (ID) injection of preparations and the assessment of clinical toxicity as well as localised toxicity, for example at the

site of injection (i.e. intradermal) or target organs such as the liver. These routes of exposure are not directly relevant to cosmetic applications of these products as routes such as intradermal application by-pass the barrier function of the SC and applied the dose directly to the lower layers, where through the injection of a bolus dose may also cause localised damage or changes to permeability. This is important if we consider that these materials may degrade as they pass into and through the skin due to interactions with dermal lipids. Through direct injection into lower layers, this interaction may be limited and as such, potentially increase the chance of systemic availability. One must also consider that in these types of administrations are typically for the purpose of drug delivery and so the dose rate tends to be higher and over a shorter duration (i.e. single administration or repeated dose over a short period of time). On the contrary, through cosmetic applications the internal dose is likely to be much lower as there is very limited evidence of actual dermal absorption and systemic availability of the carriers themselves (likely due to retention and degradation the skin). However the utility of such studies is they provide us with a 'worst-case' scenario of high level systemic availability and the resultant effects.

Bulcão et al. have published a number of such studies, each dealing with the potential toxicity of poly(ε-caprolactone) nanocapsules with an early study published in 2011 (Bulcao, Freitas et al. 2013). Previous studies have shown that poly(ε-caprolactone) nanocapsules loaded with vitamin A can penetrate with efficiency into the skin layers but are retained there and are readily absorbed through the skin (da Silva, Contri et al. 2013). In the 2011 study, the toxicological impact of the nanocapsules (215nm in diameter) were assessed after single and repeated dose administration by IP injection into male Wister rats. Various doses were used based on particle number (18.03×10^{12} – 72.2×10^{12}) in a fluid volume of 12ml/kg in the case of single dose administration or lower doses (6.01×10^{12} – 18.03×10^{12}) in a lower volume of 3ml/kg. The single dose animals received a single injection on day 1 and were monitored for 14 days before being euthanized whilst the repeated dose animals received a daily injection for 28 days, after which they were euthanized. A comprehensive range of measurements were taken including body weights, relative organ weights, histopathological analysis of the liver, spleen, kidney, heart, and brain although oddly the lungs were omitted despite this being a common area of damage. In addition biochemical parameters were measured in the blood and urine as well as blood haematology.

Single or daily injection of polymer nanocapsules did not lead to mortality or permeant changes in body weight and most hepatotoxicity and nephrotoxicity markers were within normal ranges. The authors noted slight haematological alterations (increase in RBC levels and increase in white blood cell count in response to tissue injury). The most significant response was noted in the high dose groups in both the acute and repeated dose exposure scenarios where granulomatous foreign body reactions were noted in the liver and spleen. The location of the granulomas are of importance as these were on the serosal surface of these organs rather than internally which indicates they are a result of the route of application and high dose. Particles administered to the peritoneal cavity come into contact with the mesothelial surface which lines the external surface of the gut and all organs located within the abdominal cavity. Particles which are unable to be cleared from the peritoneal cavity (e.g. via diaphragmatic stomata) can become trapped and elicit a foreign body reaction whereby they are enclosed and effectively 'walled off' by the action of peritoneal macrophages forming a dense granuloma (Poland, Duffin et al. 2008). This is a normal response to an immovable object and does not indicate high toxicity as such a reaction may also occur with low toxicity materials also. Detailed assessment of histology showed no signs of parenchymal inflammatory infiltration, necrosis, apoptosis or vacuolation of these organs indicating that internally, these particles were dealt with no appreciable signs of damage or pathology. Overall, despite the high doses used, the study showed no appreciable toxicity of the biodegradable polymeric nanocapsules leading the authors to suggest they might be a safe candidate for systemic drug delivery (Bulcao, Freitas et al. 2013).

Other studies by the same authors showed similar response after IP injection(Bulcao, de Freitas et al. 2011, Bulcao, de Freitas et al. 2011) although one study did suggest that the nanocapsules may interfere with the levels of renal and haematological markers analysed due to the polysorbate 80 used within their formulation (the polysorbate 80 control showed similar modifications) (Bulcao, de Freitas et al. 2011).

In relation to intradermal administration, in 2014 Bulcão et al. (Bulcao, de Freitas et al. 2014) published a study evaluating the toxicological response after injection of 245nm (-7.5 mV zeta-potential) poly(ε-caprolactone) nanocapsules into the plantar skin on the foot of Wistar rats. The animals received a single dose of 6.01×10^{12} nanocapsules per ml in 0.5ml of fluid and animals receiving repeated doses had daily injections over 28 days of $1.8 \times 10^{12} – 5.4 \times 10^{12}$ polymeric nanocapsules. As with the IP route, intradermal administration led to no mortality or clinical signs or physiological of distress although there was a significant decrease on body weight after administration of the polysorbate 80 control after repeated administration. Similar to previous studies, no significant changes in the blood or urinary markers were observed nor were there any histopathological changes in the analysed organs (Bulcao, de Freitas et al. 2014) although no attempt seems to have been made to analyse the skin tissue which may or may not have shown localised damage.

Most recently, Bulcão et al. undertook a more endpoint specific evaluation of toxicity by focusing on the inflammatory and oxidative damage response to poly(ε-caprolactone) nanocapsules, similarly applied via the IP and ID routes with repeated dosing (Bulcao, Bubols et al. 2015). Similar to previous experiments, the animals received $6.01 \times 10^{12} – 18.03 \times 10^{12}$ nanocapsules by the IP route or the lower dose range of $1.8 \times 10^{12} – 5.4 \times 10^{12}$ by the ID route, daily over 28 days. At the end of the treatment period, the organs were homogenised and the levels of lipid peroxidation and protein oxidation established as markers of oxidative damage was well as quantification of endogenous antioxidants to assess modification in the oxidant balance. Levels of the cytokines IL-6 and IL-10 were also measured in the blood plasma as markers of inflammation.

The results showed that markers of oxidative stress could be observed as enhanced protein damage by carbonylation was found in the intermediate and highest IP doses and whilst lipid peroxidation was not noted in the blood plasma, liver, kidney or cardiac tissue, it was noted in the brain after ID administration of the highest dose nanocapsules and polysorbate 80 control. The exact cause of this given that antioxidant levels and markers of lipid peroxidation were not altered and such a response was not seen with the IP route (applied at higher dose levels) is somewhat puzzling and certainly requires more investigation. The authors did suggest that the intradermal response could have been a consequence of local inflammation but did not specifically address the localised response in terms of markers or histopathology (Bulcao, Bubols et al. 2015).

Overall, inflammatory markers were not increased and the results taken together led the authors to conclude that the nanocapsules appeared safe for use as systemic drug nano-transporters (Bulcao, Bubols et al. 2015). Whilst these IP and ID studies do represent an extreme dose, both in terms of direct access to the body and dose rate used (bolus) they do enable us to see a worst case example of polymeric nanoparticle application to the body and subsequent toxicity. Overall, the response appears very much muted with no appreciable toxicity noted at lower doses and relatively minimal responses noted at higher and repeated doses. The lack of histopathological reaction, especially within the reticuloendothelial system is somewhat surprising and suggest that the particles are dealt with relatively rapidly (potentially though biodegradation). A benefit would have been the inclusion of some form of marker, either in terms of a payload or conjugated to the nanocapsules as this may have allowed better detection in tissues to assess distribution, persistence and excretion.

5.2.5 Nano-transporters and Dermal Sensitisation

An endpoint of considerable concern is the potential for sensitisation and the possible development of allergic disease such as allergic contact dermatitis. Given the relatively commonality of such conditions within the general population and potential for sensitisation/ adverse reaction through repeated application of a product; it would be expected that the allergic potential of nano-transporters would receive considerable attention. However, no specific discussions were found on the potential of these nanomaterials to cause an allergic response although several studies did discuss the use of such technology in their treatment. This issue represents a significant gap in the current knowledge surrounding nano-transporters and dermal health.

In 2007, a case report was published (Clemmensen, Thormann et al. 2007) describing the case of a 54 year old woman developing facial dermatitis after several days using a new anti-wrinkle cream (Lancôme Resolution D-contraxol ®, L'Oréal, Paris). The women had no previous cases of allergy or atopy and identified the cream as the cause of the allergic response as demonstrated by the generation of erythema after application of the cream to her forearm. The cream contained as its active ingredient, Retinyl Palmitate (RP) in conjunction with SLN as a penetration enhancer. By performing a series of patch tests, the case study authors showed that the RP (5%) generated a positive reaction (+), The SLN generated a negative reaction and a stronger reaction to the combined RP and SLN formulation (++).

As noted by the authors, whilst RP is well described as a dermal irritant (indeed, encapsulation within nano-transporters appears to improve the biocompatibility), it is not commonly seen as a dermal sensitizer with only a few cases described. The series of patch tests showed that the specific cause of the allergic reaction was the RP as application of the SLN did not cause a reaction suggesting the nano-transporter itself was not the allergen. However, what is of concern is the role the SLN may have played in the development of the allergic reaction. Specifically, did the SLN act to enhance the development of an allergic response and would this have occurred if the SLN had not been present in the product? From the article, it is not clear if the woman had previously been exposed to RP without incident or if this had been her first application. This is of interest as if she had not previously used RP, it may suggest she was sensitive to RP and would have developed an allergy irrespective of the SLN component but if she had previously used it without incidence, the reformulation with the inclusion of SLN may have triggered a response where previously there was none (Clemmensen, Thormann et al. 2007).

The concern is that whilst the SLN themselves do not act as a sensitizer, the action of improving dermal penetration and retention may lead to an enhanced dose within the skin than would conventionally be seen without the use of nano-transporters. This enhanced dose and/or extended duration in the skin (via controlled release) may have resulted in optimal conditions for sensitisation to occur with a resultant allergic response.

The potential for nano-transporters to increase allergenicity of substances (i.e. known allergens) has been investigated (Madsen, Vogel et al. 2010a+b). Within their 2010 study, Madsen et al. investigated the ability of ethosomes to influence the allergenicity of contact allergens (isoeugenol and dinitrochlorobenzene (DNCB)) using the standard murine local lymph node assay (LLNA). Ethosomes are liposomes with the addition of ethanol and were in the size range of 200-300nm.

Application of the allergens encapsulated within the ethosomes led to significantly increased sensitisation as compared with the allergens dissolved in ethanol/ water. Interestingly, a linear dose response relationship was noted between the concentration of the ethosomes and sensitisation with a significant response being noted at 60 mg/ml. What was also noted was that the ethosomes themselves were not allergenic and instead served to enhance the allergenicity of the contact allergens. To further investigate the role of the ethosomes in generating an allergic response, the authors compared the response to empty ethosomes, DNCB in ethanol/ water, DNCB in ethanol/

water with the main lipid constituent of the ethosomes (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and the encapsulated DNCB. They noted that the addition of the ethosome lipid caused a significant ($P<0.001$) increase in sensitisation which was even further increased by encapsulation ($P<0.05$) (Madsen, Vogel et al. 2010a). This shows that the lipid constituent as well as the form (capsule) have a profound impact on the generation of an allergic response. It is not clear, however, how the lipid alone caused a synergistic enhancement in response and if this was due to improved penetration.

The same authors also undertook a similar study but this time, using human volunteers to test the effect of encapsulation on sensitisation to a known allergen (Madsen, Vogel et al. 2010b). Within the study, several concentrations of the allergens in ethosomes (333 – 463nm), in ethanol/water or blank controls were tested in a randomised and blinded patch test study with volunteers who had previously shown a positive patch test to the allergens. In addition a repeated open application test (ROAT) was performed in a subset of 16 patients, and lag time until a positive response was recorded. As noted within the LLNA, the results showed that the encapsulation of the contact allergen within the ethosomes led to a significant enhancement of the patch test reactions as shown in figure 13. As also demonstrated in figure 13, the ethosomes themselves were not allergenic.

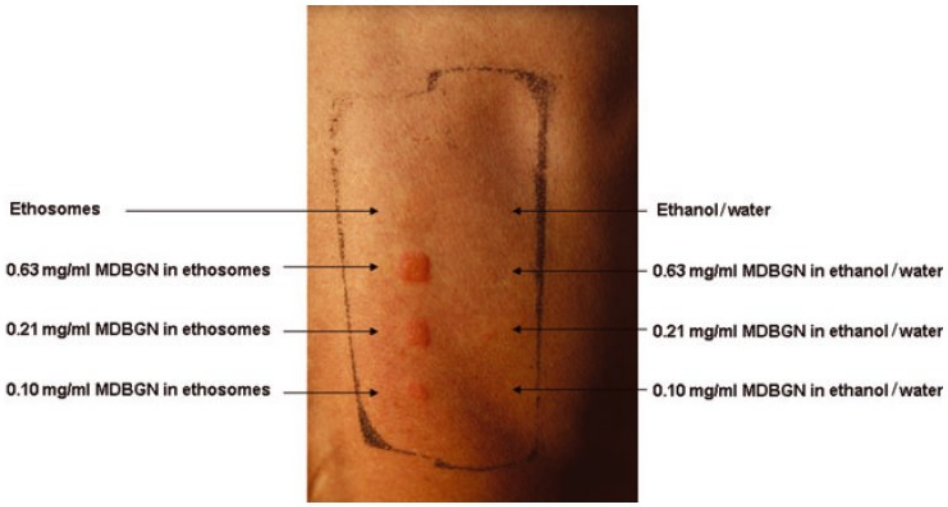


FIGURE 13: CELL RESULT OF A SERIAL DILUTION PATCH TEST IN A SENSITIZED VOLUNTEER WITH METHYLDIBROMO GLUTARONITRILE (MDBGN), USING IQ-CHAMBERS AND 15 ML OF TEST SUBSTANCE FORMULATED IN ETHOSOMES AND ETHANOL/WATER.

Reproduced from Madsen, Vogel et al. (2010b)

In addition to the patch test results shown above, the ROAT assay showed a differential in response between the two contact allergens used. In the case of Isoeugenol (previously used in the LLNA study (Madsen, Vogel et al. 2010a)), the lag time to a positive ROAT was decreased by ~50% (15.3 to 7.7 days) by the encapsulation with ethosomes showing that the use of the nano-transporter led to a more rapid allergic response. However, this was not noted in the case of methyldibromo glutaronitrile which showed little differences between the ethosomes (10.7 days) and ethanol/ water mix (10.1 days). The reason behind this disparity may be to do with the interaction between the ethosomes and the payload. It was described that the isoeugenol is less lipophilic and therefore better retained inside the ethosomes than methyldibromo glutaronitrile as expressed by higher encapsulation efficiency (77% versus 22%) (Madsen, Vogel et al. 2010). This could potentially result in a different penetration/ release profile resulting in better deliver and controlled release of the isoeugenol into the skin, optimal for generating an allergic response.

Similar results have been reported in further publications by the same authors (Madsen, Vogel et al. 2011a+b) but a useful consideration raised by the authors is that the selected allergens are only model allergens and it is unlikely that some of the study allergens (e.g. potassium dichromate encapsulated in liposomes) would find their way into the market. However, some of the allergens may be used and the example was given of isoeugenol as a fragrance ingredient which could very well be encapsulated in liposomes along with the preservative methyldibromoglutaronitrile added an active anti-wrinkle compound like a vitamin A derivative (Madsen 2011).

What these series of studies and case report show is that the nano-transporters themselves do not appear to be allergenic, however, the effect they have on the presentation of allergenic substances to the skin can have an enhancement effect on allergy. This enhancement effect requires the active of interest to possess sensitising ability (i.e. non-sensitizers do not appear to be made sensitizers through encapsulation). The cause of the enhanced response is likely to be due to the improved penetration and potentially sustained release resulting in both an increased and prolonged dose to the responsive dermal layers. What this means for product safety is that the potential for this enhancement effect needs to be taken into account when assessing the potential risks of new and (most importantly) previously authorised substances for use in cosmetics. This is because weakly or rarely sensitising ingredients authorised for use may increase in potency through encapsulation.

5.2.6 Summary of Dermal Toxicity of Nano-transporters

The toxicity of nano-transporters has been assessed through a number of different approaches ranging from *in vitro* assays using unrelated cell types to dermal toxicity (e.g. red blood cells and hepatocytes) to more closely related *in vitro* models including keratinocytes and multi-cell modes such as RHE. In addition *in vivo* models have been used to assess toxicity including both animal models as well as human volunteers. The latter represents the gold standard in terms of relevance but offers limitations in the degree of investigations that can be performed to assess toxicity (i.e. typically non-invasive).

A key challenge in the use and interpretation of *in vitro* data is establishing the relevance of a particular outcome to the *in vivo* situation to determine if a specific adverse effect *in vitro* is predicative of an adverse effect *in vivo*. This is perhaps more difficult for highly simplified single cell models such keratinocytes in culture than more complex models such as RHE which do display some of the barrier proprieties of skin and therefore less sensitive. An example of where this can be a challenge in establishing a true adverse effect of a nano-transporter is in the evaluation of surface charge on toxicity. Within the study of Carboni *et al.,* a range of liposomes were developed which had progressively increasing positive surface charges. *In vitro* analysis across arrange of 3 single cell models showed that whilst both the highest and lowest surface charged particles caused significant cell death, it was much more rapid in the case of the highly positively charged particles. This in turn could suggest that highly charged particles may cause dermal toxicity over a short period of time. However, a study in humans also evaluated the role of surface charge although this time it was with nano-emulsions. Here the application of positively charged particles to human volunteers showed no signs of toxicity (e.g. erythema) and instead showed significant improvement in skin moisture levels and elasticity. Therefore, based on these results it could be concluded that rather than a negative impact on dermal health, a positive charge may have a beneficial effect. It is of course difficult direct comparisons between these studies as the same nano-transporter was not employed, the nano-particles applied to humans were not as positively charged as those used in cell culture etc. However this comparison does serve the purpose of demonstrating that validation of *in vitro* test results is important in drawing solid conclusions.

In terms of endpoints evaluated, the majority of *in vitro* analyses focus on an assessment of cell death as a measure of toxicity which in itself, is a relatively rudimentary assessment of toxicity and says very little about possible sub-lethal effects (e.g. inflammation) or mechanisms. Indeed, detailed assessment of cellular interactions and effects is very much missing from the literature pertaining to

the cosmetic applications of these materials. Assessment of inflammatory potential has been assessed *in vivo* using methods such as the patch or draize test and typically shown no effect and this is also supported by measures of blood cytokines after systemic application of nano-transporters. Genotoxicity is also an area of concern and this has been assessed both *in vitro* and *in vivo* with negative effects in both systems. Given the lack of significant inflammation, the generation of secondary genotoxicity would not necessarily be anticipated but this route of toxicity does not appear to have been investigated.

In relation to allergic responses; nano-transporters themselves do not appear to be allergenic, however, the improved penetration and controlled release of actives does bring with it potential issues. It has been noted in several studies using well established contact allergens that encapsulation, for example into ethosomes can lead to an enhanced and in some cases more rapid allergic response than would normally be seen had the active not been encapsulated. This is possibly due to an increased dose of the allergenic substance being presented to the responsive, epidermis and dermis; possibly over a greater period of time (sustained release).

Taking nano-transporters collectively, the literature provides an impression of relative low toxicity and biocompatibility with these materials although there are exceptions. In relation to toxicity, there does not appear to be a clear relationship between size or surface charge and observed toxicity and this has also been stated within a systematic review of SLN/ NLC including medical uses (Doktorovova, Souto et al. 2014). Indeed, as toxicity with many of these materials lacks a specific trend, it has been suggested that a combination of components should be considered as the primary parameters for cytotoxicity profiles instead of size (Pizzol, Filippin-Monteiro et al. 2014).

One such component that seems to have a profound effect on toxicity is composition. Studies have indicated that components such as lauroylcholine, stearic acid, PLA, and SDS can all have a negative impact on biocompatibility. This may be due to intrinsic toxicity of some of these components or an attribute they may infer. For example, as reported by Weyenberg et al., the potential irritation of surfactantia or co-surfactantia as *in vitro* and *in vivo* data suggest that cationic surfactantia can be the most detrimental followed by anionic surfactantia with non-ionic surfactantia being the least problematic (Harvell, Tsai et al. 1994, Korting, Herzinger et al. 1994, Wilhelm, Bottjer et al. 2001, Weyenberg, Filev et al. 2007). Therefore, it is imperative that adequate consideration be given to the selection of lipids, oils, surfactants and co-surfactants (including their relative concentrations) to ensure a high degree of biocompatibility, particularly as such products are likely to applied repetitively.

6 Conclusions and Knowledge Gaps

6.1 Conclusions

The dermal penetration, absorption and toxicity of a range of nano-transporters have been reported within the literature, consisting of a wide range of compositions and physicochemical properties. One thing that is apparent is the relative flexibility attached to the word 'nano' in defining these transporters. The search strategy employed in the collation of the relevant literature and its evaluation for relevance used to word 'nano' as a defining part of relevance to the current project yet in the critical study of the individual papers, it became apparent that many did not in fact meet the commonly held definition of nano as being within the 1-100nm size range. A key example of this is the study Butnariu and Giuchici who created "nano-emulsions" based on aqueous propolis and lycopene extract as a protective approach against UVA radiation. The study did not provide detailed size analysis data and instead showed images of the nano-emulsion with clear spheres, several microns to tens of microns in diameter. As shown in Table2, the size range across which these supposedly 'nano'-transporters are made is very broad and this is reflected in the literature with many studies not dealing with particles in what may be considered the true nano-size range.

The assessment of dermal absorption and toxicity of nano-transporters has been addressed through a number of studies, which typically involve the uses of Franz diffusion cells with a static dermal barrier and *in vitro* cell cultures. *In vivo* models including both animals and humans have been reported and from these different approaches, several conclusions in relation to dermal absorption and penetration of nano-transporters can be drawn:

- Nano-transporters can penetrate the skin to a greater extent than insoluble solid nanoparticles and locate in and beyond the *stratum corneum*
- Where penetration has occurred, evidence suggests that nano-transporters are to a significant extent retained within the dermal layer
- Nano-transporters interact with the skin cells and degrade, releasing their payload into the skin where it may diffuse further including transdermally
- Size can influence penetration with smaller particles enhancing drug permeation and very large particles (tens of microns) being excluded from penetration into the SC
- Damage to the barrier quality of the SC can lead to faster penetration of the dermal layer
- Surface charge can have an effect on dermal penetration and positively charged particles show enhanced dermal accumulation of actives although this does not necessarily translate to dermal absorption of nano-transporters

A key challenge in assessing the actual dermal absorption of nano-transporters is the lack studies attempting to detect nano-transporters after application. Many studies characterise the nano-transporters before application to a model but thereafter, the focus of analysis is on the active

payload. This is likely to be due to a combination of technical challenges and the fact that many such studies focus on how much dermal penetration of an active ingredient is improved by incorporation into a nano-transporter and therefore the focus is not on penetration of the transporter. However, this leads to a lack of clear evidence as to the fate and behaviour of nano-transporters in the skin. Thus far, the limited evidence seems to suggest effective penetration followed by retention and degradation resulting in release of actives into the skin, which are free to diffuse further including transdermally.

The impact of improved penetration and resultant localisation should be considered both for localised effects and for the systemic effects.

Many studies show that encapsulation within nano-transporters leads to improved penetration of the skin beyond the protective barrier of the SC. This means that unlike insoluble nanoparticles such as TiO_2, interaction with living cell layers found within the epidermis and dermis is increased. This is in many circumstances is an intentional situation with improved penetration of substances such as CoQ10 providing an additional protective effect against damage. However, studies have also shown that this can result in an enhancement of observed negative effects associated with certain actives; owing to intrinsic properties of these substances such as allergenicity. Thus, this increased dose into the sensitive and responsive dermal layers has, in some circumstances, been shown to result in a more rapid and greater allergic response. So, potentiating effect of nano-transporters should be acknowledged in review of product compositions, especially re-formulations.

Considering the systemic effect, studies show both improved transdermal penetration of substances (predominantly assessed using Franz diffusion cells as opposed to *in vivo* studies) as well as reduced transdermal availability (e.g. see Gianeti, Wagemaker et al. (2012)) therefore it is not possible to make generalisations as to the overall effect of nano-transporters in systemic availability of active ingredients. Thus, the possibility for an increased absorption of the active ingredients should be borne in mind when making safety assessment of formulations and products as the nano-transporters in this way may increase the potential for a systemic toxic response.
However, whilst the specific systemic availability of actives is variable, relatively little is known about the systemic fate of nano-transporters applied to the skin as most studies have focused on the penetration and absorption of the actives whereas the fate of the nano-transporters have been over-looked. From the literature, it appears that due to their lipid composition, nano-transporters tend to degrade within the epidermis meaning that systemic availability is likely to be minimal.

An additional issue when assessing dermal absorption is the suitability of the model. The use of Franz diffusions cells to measure dermal absorption is a common and well accepted method for this purpose but the static nature of the skin held within the cell has drawn criticism. Indeed skin on living creatures is subject to contestant movement and flexion and this action of stretching and compression may serve to modify penetration rates. Therefore, it has been suggested models which recreate skin movement may serve as a more realistic model for dermal absorption (Butz 2007, Labouta and Schneider 2013) and modifications of Franz diffusion cell models have been used. However, such flexion models have not been applied to the assessment of nano-transporters.

In relation to the dermal toxicity of nano-transporters, several endpoints have been addressed within numerous models and from these; the following conclusions can be drawn:

- The level of toxicological evaluation of nano-transporters for cosmetic applications is sporadic and not seen as comprehensive, particularly around sub-lethal endpoints in dermal cells such as inflammation, genotoxicity and sensitisation

- Nano-transporters on balance appear to be of relative low toxicity although there is a spectrum of toxicity

- Typical adverse effects noted are cell death within *in vitro* models which are more sensitive than *in vivo* models

- *In vivo*, nano-transporters are well tolerated and even high dose systemic administration causes minimal toxicity

- Nano-transporters do not appear to be genotoxic

- Nano-transporters may enhance the sensitising potency and allergic response of contact allergens

- No clear correlations between size or surface charge and toxicity are apparent

- Within observed toxicity, composition appears to be of primary importance with several substances showing less than optimal biocompatibility

Whilst the literature does provide an optimistic picture of dermal compatibility, the evidence base is not regarded as comprehensive and for many endpoints and nano-transporters, it is seen as incomplete. One endpoint of particularly concern due to its complete omission is that of dermal sensitisation and allergic disease. Nano-transporters have been mooted as a potential therapy for allergic and allergic skin conditions but little or no assessment seems to have been made in relation to topical applications of these materials and their ability to cause allergic disease. Given the key role composition appears to show in toxicity of these materials and given their degradable nature, it may be possible to read-across data for individual components but the effect of altered rate of penetration (and hence presentation of dose) or combinational effects would need to be assessed for impact on risk assessment.

A cross topic issue is one of metrics used within the dosing of models, either for the purpose of toxicity or absorption/ penetration assessment. The use of dose metrics is somewhat sporadic within the literature with various approaches being used including mass (e.g. mg/ml), particle number, active payload loading (specific nano-transporter dose not reported) and molar concentration. Whilst the use of alterative metrics is to be encouraged as it allows the expression of results in different way (potentially showing correlations), it is preferable if a base set such as mass is used consistently to aid cross-comparison. Drawing analogies with more conventional nanoparticles, mass is the most commonly cited dose metric and this may be supplemented with other metrics such as surface area and particle number but mass is often a constant.

6.2 Knowledge Gaps and Research Needs

Based on the review of the literature pertaining to dermal absorption, penetration and toxicity stemming from the application of nano-transporters in cosmetics; the following knowledge gaps and research needs have been identified:

- There is a distinct lack of knowledge on the actual level of penetration/ systemic absorption of the actual carrier (as opposed to the payload). Owing to the difficulties in detecting the rather labile nano-transporters, the vast majority of studies only attempt to detect and quantify the payload either via fluorescence microscopy or analytical techniques such as HPLC. As a result there is a lack of evidence and especially quantitative measure of dermal absorption of nano-transporters and hence, possible systemic dose. The evidence does suggest that as these nanoparticles penetrate into the skin, they degrade through interactions with the dermal lipids resulting in the release of their payloads. The result would be that dermal penetration of these nano-transporters occurs, but not (or to a much lesser extent) dermal absorption. This release, past the barrier of the SC may account for higher levels of dermal absorption of actives but the detection of such actives in the

receptor fluid of Franz diffusion cells may not necessarily mean actual absorption of the nano-transporters.

- The degradation in the skin of the nano-transporters is perhaps more prominent for lipid based nano-transporters such as liposomes or nano-emulsions due to the interaction between the lipids in the skin and in the nano-transporter. It is not clear if polymer based nanocapsules etc. degrade to the same extent and therefore, if these are more likely to become systemically available.

- The Franz-type diffusion cell is the most commonly employed experimental method for the evaluation of dermal permeation, penetration and overall absorption. This is similarly the case for insoluble nanomaterials previously evaluated and in both cases, the experiments usually employ a static system whereby the skin layer is motionless and flat. However, this represents a rather false environment for several reasons. Firstly, upon application of a cosmetic, the formulation would normally be spread and massaged into the skin (for example a face cream or sunscreen) and as previously reported, the action of massage may affect the level of dermal penetration (Poland, Read et al. 2013). The effect of application method has not been addressed in relation to topical cosmetic applications of nano-transporters. Secondly, within a Franz-type diffusion cell, the skin sample from which ever specific is held passively within the apparatus and the test sample applied to the upper layer for a period of time. However this immobility of the skin is not representative of actual application/ wear conditions as the skin of a living creature would be under frequent flexion, compression etc. An example of this would be moisturising cream applied to the face and around the eyes. During the day the skin on the face, especially around the jaw, eyes and brow would be repeatedly be stretch and compressed during talking, laughing eating and facial expressions.

  As the passage into the skin for nanomaterials, including nano-transporters may be driven through a variety of mechanisms, not just diffusion, the flexion of the skin may play prominent role, which is thus far being overlooked. The use of flexion models is apparent in the literature evaluating solid insoluble nanoparticles, albeit to a limited extent(Tinkle, Antonini et al. 2003, Rouse, Yang et al. 2007, Labouta and Schneider 2013) but appears absent in relation to nano-transporters. The use of flexion systems as a more realistic model for dermal penetration has been suggested by many authors and was one of the main conclusions of the NANODERM report for TiO_2 nanoparticles (Butz 2007, Labouta and Schneider 2013) and the arguments for their use with solid, insoluble nanoparticles would be equally relevant to nano-transporters and perhaps more so given their flexible nature.

- When evaluating the literature in relation to more conventional, solid nanoparticles such as TiO_2, nano-gold etc., a significant issue in the ability to draw solid, evidence based conclusions was the variety of experimental and material parameters which confounded cross-comparison. Differences between studies such as experimental models, species, doses, duration, and particles of differing physicochemical characteristics meant that true comparisons could often not be made. This issue is very much compounded in the case of nano-transporters given their complexity in composition. For conventional nanoparticles such as TiO_2, there is indeed a degree of variability such as crystalline state, and the addition of surface coatings but fundamentally, TiO_2 nanoparticles were composed of TiO_2. In the case of nano-transporter, even within single classes there is enormous variation in composition. This is clearly shown in Tables 3 and 4 where there is an extensive list of common oils, lipids and surfactants are noted (Table 3) as well as polymers (table 4). These in turn can be modified in a number of ways though conjugation and functionalization with proteins such as cell penetrating peptides (Desai, Patlolla et al.

2010, Shah, Desai et al. 2012) and obviously can also be produced in a range of sizes with different loadings which may also effect the size and surface properties such as charge.

- The impact of the various compositions on the activity of nano-transporters is not fully apparent and indeed knowledge in this area of active research will be borne out of the desire to improve dermal penetration/ biocompatibility by constructing, characterising and evaluating new formulations. It is well established within the field of nanotoxicology that adequate characterisation of a nanomaterial is paramount in the understanding hazards and forms an integral part of the risk assessment yet this is currently often incomplete. This in itself hinders progressive understanding about the role physicochemical properties (as well as composition) plays in toxicity which in turn, reduces are ability to read-across between and/or group materials based on shared properties relevant to their toxicity (or absence thereof).

- When considering the level of characterisation in relation to nano-transporters as compared with more conventional nanoparticles, on the whole it was better as most studies reported a minimum dataset of:

    - Composition (including sources)

    - Production method

    - Size (often including particle size distribution)

    - Zeta-potential

- In addition, other information such as long term stability was also reported. Information on composition was also aided by the common use of commercially available ingredients such as the lipid Compritol® 888 ATO (Glyceryl behenate). However, whilst charactersaion is better, it is perhaps worth considering the development of a harmonised *minimal characterisation dataset* for nanomaterials (both insoluble and soluble) that should accompany and be required for all studies on dermal penetration of nanomaterials.

- One key issue with the characterisation of nanomaterials, which is an exceptional challenge for nano-transporters is the ability to move away from simple as-produced or as-supplied characterisation of nanoparticles. As discussed in section 5.3 and suggested by Sayes and Warheit (Sayes and Warheit 2009), full characterisation should ideally consist of primary, secondary, and tertiary levels of characterisation. Currently in relation to nano-transporters most if not all characterisation is performed only at the primary level and secondary level (i.e. as-synthesised or as-received in its dry native state; secondary characterisation is performed on particles in the wet phase as a solution or suspension in aqueous media (which could be ultrapure water, vehicle solution or cell culture media)). Tertiary characterisation is very rare but allows us to understand better the modification and ultimate fate of these nano-transporters in the skin as well as the wider body (should absorption occur). As such, efforts are needed to both encourage such tertiary level characterisation and to provide the effective analytical tools to facilitate this overcome the considerable challenges associated with detecting and charactering these materials in situ.

- It needs to be further clarified how the various types of nano-transporters may affect the sensitizing properties of contact allergens.

7 Nano-transporters in a Regulatory Context - Recommendations

A further objective of this project is to discuss if and how nano-transporters can be specifically addressed as nanomaterials in the cosmetic regulation.

When evaluating whether there may be arguments for specifically addressing the nano-transporters in a regulatory context, the main findings of this report have to be evaluated and discussed in relation to the current criteria for defining nanomaterials and in relation to nano-specific hazardous properties of the nano-transporters. Also, it has to be discussed whether the current provisions that apply for nanomaterials also would be relevant for the nano-transporters.

7.1 Main Findings

Although systematic toxicity testing has not been undertaken to identify specific structure activity relationships, and toxicity data on the cosmetic uses of nano-transporters is sparse the available data do not indicate any specific concern regarding adverse effects from the nano-transporters on its own. In general, the substances used for forming the nano-transporters are considered to be of low toxicity.

In some studies dermal irritation was observed in connection with exposure to the nano-transporters. However, this response does not seem correlated to the particle size of the nano-transporter but rather to the inherent properties of the chemical ingredients used for the nano-transporter. Also, it has been found that the use of nano-transporters can enhance the sensitizing properties of known contact allergens as the nano-transporters may result in increased delivery of the contact allergens into the skin.

Thus, as transporters of active ingredients, the nano-transporters ensure delivery of the ingredients into the skin layer after structural degradation of the nano-transporter itself. Due to this delivery, the nano-transporters may enhance the effects of the active ingredients - beneficial effects as well as adverse effects. Thus, in most of the studies the concern was directed towards the substances that are loaded into the nano-transporters rather than to the nano-transporter material itself. Therefore, when utilizing nano-transporters it seems especially important to consider the hazardous effects from the substances transported by the nano-transporters, as the effects from these in some cases are enhanced.

Currently, the nano-transporters as described in this report are not covered by the definition of nanomaterials in the cosmetic regulation, as nano-transporters cannot be considered as insoluble or biopersistent materials. However, as the nanomaterial definition is under discussion, some of the issues and discussions relevant for the nano-transporters and definition as a nanomaterial are shortly described below.

7.2 Nanomaterial Definitions

7.2.1 Overall EU definition of nanomaterials

In 2011, the EU Commission EC (2011) provided an overall definition of nanomaterials as:

> *"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm"*

This definition was a *recommended* definition and it was stated that:

> *"The definition set out in this Recommendation should not prejudge nor reflect the scope of application of any piece of Union legislation or of any provisions potentially establishing additional requirements for those materials, including those relating to risk management. It may in some cases be necessary to exclude certain materials from the scope of application of specific legislation or legislative provisions even if they fall within the definition. It may likewise be necessary to include additional materials, such as some materials with a size smaller than 1 nm or greater than 100 nm in the scope of application of specific legislation or legislative provisions suited for a nanomaterial"*

Thus, it was acknowledged that for specific regulatory implementation of a definition, it could be necessary to make adaptions to this overall definition in order to focus the scope and relevance for the specific area of regulation.

After this overall definition was published, the definition has been intensively discussed and recently the Joint Research Centre (JRC) has published three reports presenting the issues of discussion and also providing a thorough review of the definition (JRC 2014a, JRC 2014b, JRC 2015). These reports now serve as a basis for an update of the definition. The update of the recommended definition is by the EU Commission planned to be available in 2016.

In the review by JRC (2015) it was noted that the current EC definition of nanomaterials refers to particles. A particle is defined as a minute piece of matter with defined physical boundaries, in line with the ISO definition. However, discussion is ongoing about including or excluding, for example, single molecules, micelles and non-solid materials (the latter being important for the discussion of nano-transporters).

In the review, JRC (2014b) further discussed micelles and liposomes as these are considered as nano-objects if they have external dimensions within the nanoscale. Also they are nanostructured materials, or more specifically nanoscale capsules, because their shells have a thickness at the nanoscale and they can enclose, fix, transport or release substances. Being nanostructured materials they can have external dimensions well above the nanoscale. However, as stated by JRC (2014b) the current position of the EU Commission (2012) is that such objects are not covered by their definition.

However, JRC (2014) found that these materials could be relevant from a regulatory perspective, because they are used in applications for cosmetics, food (e.g., carriers, supplements), or for drug delivery. Thus, JRC (2014) considered micelles worthwhile to consider for inclusion in the definition, if their external dimensions were within the nanoscale, even if they were "soft" materials. Also, it was said that if necessary, exclusion of such materials should be possible by sector specific provisions in relevant regulations. To which extent micelles and other "soft" materials will be

covered or specifically excluded in the update of the definition has, however, to be awaited. However, it can be expected that a revised overall definition would highly influence the discussions and update of the definition in the cosmetic regulation.

7.2.2 Nano-Definition in Cosmetics

In the cosmetic regulation, a nanomaterial is not described as containing particles, but other characteristics such as being *insoluble or biopersistent and intentionally manufactured* are specified in the definition (all of which are terms open for interpretation and further specification).

However, various issues have been brought up for revision/clarification of the definition of nanomaterials. Thus, The European Federation for Cosmetic Ingredients has pointed towards the following issues to be clarified (EFfCI 2012, Höfgen-Müller 2012):

> *Applicability:* should the provisions apply to the raw materials / finished cosmetic products?
>
> *Threshold:* inclusion of % of particles (number size distribution) in the nano range
>
> *Criteria and specifications regarding:*
>
> - intentionally manufactured, incidental
> - insoluble, biopersistent
> - external or internal structure
> - constituent particles
> - additional parameter (e.g. volume specific surface area)
> - measurement techniques

Here especially discussion of the criteria for *solubility and biopersistency* can be considered important in relation to the nano-transporters.

Further, the International Cooperation on Cosmetics Regulation (ICCR) (a group of cosmetic regulatory authorities from the United States, Japan, the European Union and Canada) has conducted a review of the nanomaterial definition (ICCR 2010). Based on this review the organization considers a nanomaterial as:

> *"a substance used in a cosmetic is considered a nanomaterial if it is an insoluble ingredient, intentionally manufactured, with one or more dimensions in the realm of 1 to 100 nanometers in the final formulation and is sufficiently stable and persistent in biological media to allow for the potential of interaction with biological systems."*

Further, it is stated that:

> *"nanomaterials should be sufficiently stable and persistent in biological media to allow for the potential of interaction with biological systems. This would include nano-carriers intended to enhance dermal penetration if they remain sufficiently stable upon application. Labile nanomaterials, which disintegrate completely upon application to skin into their molecular components (e.g. microemulsions, nanoemulsions, or labile liposomes), should be excluded. This is consistent with several international bodies including the EU (EU Commission), VCI (Verband der Chemischen Industrie), SCCP (Scientific Committee on Consumer Products) and SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks)".*

Thus, ICCR (2010) distinguish between the most labile nano-transporters such as microemulsions, nanoemulsions and liposomes that are considered outside the definition as a nanomaterial and the more stable nano-transporters that may be considered inside the definition, if they are sufficiently persistent to cause interaction with the biological system. (No specific examples of these more persistent nano-transportes was mentioned by ICCR).

For including the nano-transporters as described in this report it is clear that a change in current definition would be necessary. To include the nano-transporters it would be necessary make changes in the definition regarding e.g. solubility/ persistency, and the upper size limit of 100 nm if the nano-transporters are to be covered more broadly (see size ranges for nano-transporters, table 2).

7.3 Regulatory Aspects

If widening the definition of nanomaterials in the cosmetic regulation in order to include the nano-transporters, the overall aim, purpose and consequences of this should be considered.

When changing a current regulation this should ideally:

- Reflect a regulatory need
- Have a defined purpose/ goal
- Contain clear, specific and relevant criteria and requirements
- Be practical/ feasible in relation both to implementation and enforcement

Therefore, it is important to keep these elements in mind when discussing to which extent the nanomaterial definition for cosmetics should be adapted in order also to cover nano-transporters as described in this report. These elements are discussed point by point as follows:

7.3.1 Regulatory Need

JRC (2014) indicated that it could be relevant to include e.g. micelles and liposomes in the size range of 1-100 nm in the nanomaterial definition, as these materials are used in consumer products such as cosmetics and food. At the same time it was acknowledged that exclusion of these materials should be possible by sector specific provisions in relevant regulations. It is not quite clear to which extent these statements express a need for regulation. The statements should probably be seen as a need for clarification of the issue.

When it comes to the nano-definition, as referred to by the International Cooperation on Cosmetics Regulation (ICCR), this may however indicate a need to include some of the nano-transporters in the regulation (i.e. the nano-transporters that are in the size range of 1nm-100nm and that are sufficiently persistent in a biological media to interact with a biological system).

Apart from the expressions from JRC (2014) and ICCR (2010) no other voices for further regulation of the nano-transporters has been found.

7.3.2 Defined Purpose/ Goal

The overall goal of the cosmetic regulation is to ensure the safe use of cosmetics. Further, within the area of nanomaterials the aim is to generate increased knowledge concerning the use of nanomaterials in the various cosmetic products.

JRC (2014) does not express a specific purpose for a possible inclusion of degradable and labile nanoparticles in the regulations, and as indicated above the suggestion from JRC (2014) may instead be seen as a need for clarification of the issue.

When the International Cooperation on Cosmetics Regulation (ICCR 2010) included some nano-transporters in their nanomaterial definition this most probably reflects concern for human health, as only the transporters that are sufficiently persistent to interact with a biological system should be covered by the definition.

With respect to human health concern, the findings in this report indicate that nano-transporters in general are considered as having low toxicity and also that the size of the transporter particles seems less relevant for a toxic potential compared to the chemical composition. Thus, nano-specific provisions based on human health concern for these materials seems less relevant than for the persistent nanomaterials.

This is in line what has been expressed by SCCP (2007) which found that the conventional risk assessment methodologies would be adequate for assessing these transporter materials as they disintegrate into their molecular components upon application to skin (e.g. liposomes, micro-emulsions, nanoemulsions). Thus, for human health reasons there hardly seems to be a need for specific nano-requirements for these types of labile nanoparticles.

On the other side, the available data is not considered sufficient to conclude whether the same conclusion would apply for the less labile nano-transporters, which have a higher degree of persistency in the biological media. The data from this report indicate that this especially may apply to the nanoparticulates, i.e. nanospheres and nanocapsules.

7.3.3 Specific Criteria and Requirements

In the current cosmetic regulation, information regarding *chemical name, size, and physical and chemical properties* has to be reported for the nanomaterial used.

With regard to *chemical name* this may not be so simple for some nano-transporters e.g. emulsions as they may be generated from a mixture of various chemicals (i.e. they are a mixture rather than a single substance). So, although having the overall chemical composition of the product it may be difficult to describe the proportion of these in the emulsion particles, as this may also vary depending of the emulsification processes.

Regarding *size,* JRC (2015) noted that for emulsions and micelles their external dimensions depend more on chemical and physical (mechanical) forces from their surroundings than those of solid particles. For micelles, it was mentioned that high frequencies of molecules leaving and entering the structure makes their structure highly dynamic.

Such aspects have also been described in a publication by Rocha-Filho, Maruno et al. (2015) that examined how various processing factors and the presence of other constituents in a product may affect emulsion particle size and composition. Thus, not only the particle size but also the particle content may be highly dynamic parameters in emulsions.

Furthermore, the physical and chemical properties of the nano-transporters may be very difficult to describe as these parameters may be problematic to examine as the nano-transporters in several cases may be an integrated part of the cosmetic product and due to its labile nature, it may be difficult to isolate and examine particles on its own.

These, requirement may very well be less demanding for the more persistent nano-transporters. However, specific cut-off criteria for the persistency of the materials would be necessary to apply.

7.3.4 Practicability/ Feasibility Regarding Implementation and Enforcement

Introducing regulative criteria and requirements for labile nanomaterials with dynamic properties would be a huge challenge for the compliance/documentation as well as for the enforcement of the regulation as the regulated items may be considered as "moving targets". Thus, such provisions would be very difficult to implement and enforce.

If only the most persistent nano-transporters should be subjected to the nanomaterial provisions this would cause increased efforts for implementation and enforcement compared to the solid/ persistent nanomaterials having less dynamic properties.

So, even though the characterisation of biodurable and solid nanoparticles is already highly challenging, the situation is likely to be even more difficult for biosoluble nano-transporters.

7.4 Recommendation

From the above discussion it can be concluded that only marginal – if any - achievements regarding human health protection would be obtained if the labile nano-transporters intended for rapid degradation in skin were to be included by the provisions for nanomaterials in the cosmetic regulation. Also, inclusion of these labile materials in the nanomaterial definition would be far from a straight forward process as suggestions for such inclusion most probably would be considered as very controversial.

Furthermore, human health protection in relation to the use of labile nano-transporters in cosmetics is considered to be most adequately addressed by the general provisions regarding use of chemical substances in cosmetic products.

For the more persistent nano-transporters (e.g. nanospheres and nanocapsules) there might be an issue for covering these by the nanomaterial provisions.

As indicated in the discussion above, there are both *pros* and *cons* for covering these materials with the nanomaterial provisions. However, one argument may be a foreseen increased use of these type of nano-transporters in cosmetic applications.

One way to include these materials would be to consider these materials by including a relevant cut-off criterion for persistency in the nanomaterial definition, as it seems that the term persistency in any case will be a matter for further clarification in the upcoming revision of the nanomaterial definition.

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Appendix 1: Sources Evaluated – Computational Results

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Appendix 2: Summary Tables of Nano-Transporter Containing Products

TABLE 13: NON EXHAUSTIVE LIST OF PRODUCTS USED IN COSMETICS ON THE MARKET

(adapted from Lohani *et al.* 2015 (Lohani, Verma et al. 2014))

Product	Proposed use	Manufacturer	Marketing claims
Hydra Flash Bronzer Daily Face moisturizer	Moisturizer	Lancôme	Nanocapsules of pure vitamin E provide powerful antioxidant protection. A light touch of self-tanner ensures a natural, healthy glowing skin
Hydra Zen Cream	Moisturizer	Lancôme	Containing Nanoencapsulated Triceramides, Hydra Zen helps restore perfect comfort and softness and renew skin's healthy look. Protected from signs of daily stress and fully hydrated, your skin is beautifully soft and smooth all day long
Nano-In hand and nail moisturizing Serum and foot moisturizing serum	Moisturizer	Nano-Infinity Nanotech	Fine crystals of ZnO NP will go straight into skin tissue to prevent hand and nails from being hurt and restore skin health
Lancome Renergie Microlift	Antiwrinkle	Lancôme	Formulated with colloidal silica and soy protein NP to provide the closest possible face-lift effect
RevitaLift Anti-wrinkle and firming face and neck contour cream	Antiwrinkle	L'Oreal	The Revitalift formula enriched with Pro-Retinol A, a powerful antiwrinkle agent, which is encapsulated in nanosomes. Nanosomes penetrate deep into the epidermis to work at the heart of wrinkles.
Revitalift Double lifting	Antiwrinkle	L'Oreal	It contains nanosomes of Pro-Retinol A. RevitaLift Double Lifting is a unique dual action treatment that instantly retightens skin and effectively fights wrinkles.
Eye tender	Antiwrinkle	Kara Vita	It contains nanospheres, delivers 13 bioactives including proven, wrinkle-reducing peptides to stimulate fibroblasts, build collagen, brighten skin, and reduce inflammation for a younger, healthier appearance.
Eye contour Nanolift	Antiwrinkle, Antiaging	Euoko	It is based on nanocapsules technology. Lifting nanocapsules join seven other immediate and long-term fighters of fine lines, wrinkles, and puffiness. It provides instant and long term smoothness, gives the eye area more radiance, and diminishes the appearance of dark circles and puffiness.
Soleil Soft-Touch anti-wrinkles Sun Cream SPF	Antiwrinkle sunscreen	Lancôme	It contains vitamin nanocapsules which help to preserve skin's youth effectively. SPF 15 offers optimal protection against the sun. It contains exclusive ingredients to guarantee

Product	Proposed use	Manufacturer	Marketing claims
15			a long-lasting effect.
Nano Gold Firming Treatment	Antiaging	Chantecaille	Infinitely small nanoparticles of pure gold are bound to silk microfibers to firm and tone skin, while delivering incredible anti-inflammatory, healing, and age defying power.
Nanosphere Plus	Antiaging	DermaSwiss	A stem cells revolutionary antiaging therapy Nanosphere Plus serum has been specially formulated to allow natural stem cells to preserve and protect skin cells. Using the cells from a rare Swiss apple (Uttwiler Spatlauber), Nanosphere Plus protects longevity and combats chronological aging.
Zelens Fullerene C-60 Night Cream	Antiaging	Zelens	Fullerene C-60 is a naturally occurring microscopic form of carbon which was found to have remarkable antioxidant properties.
Clearly It ! Complexion Mist	Antiacne	Kara Vita	This nanosphere technology-based product tackles acne conditions and balance sebum production. Nanosphere time-released bioactives stimulate capillary activity for all-day detoxifying results.
DiorSnow Pure UV Base SPF 50	Sunscreen	Dior	Contains nano-UV filters for ultraprotection against the damaging effects of UVA and UVB rays.
Soleil Instant Cooling Sun Spritz SPF 15	Sun protection spray	Lancôme	Contains vitamin nanocapsule. Instant cooling sun spray SPF 15 immediately offers a sensation of freshness. SPF 15 provides optimal protection against the sun.
Fresh As A Daisy Body Lotion	Body lotion	Kara Vita	This lotion uses nanospheres to quickly penetrate, moisturize, and nourish all types of skin.
Cosil Nano Beauty Soap	Cleanser	Natural Korea	Silver nanoparticles are highly effective as disinfectant and guarantee protection of skin.
Cosil Whitening Mask	Face mask	Natural Korea	Made with nanocolloidal silver used for the effect of getting rid of germs from your face, compressing pores, soothing the skin condition, and keeping your skin radiant and soft.
Nanorama-Nano Gold Mask Pack	Face mask	LEXON NanoTech	It contains pure nanosized gold that is highly effective in penetrating small pores and disinfecting skin, helps to reduce pore size, and prevents and treats acne. It is well known that nanogold is very effective disinfectants.
Primordiale Optimum Lip	Lip treatment	Lancôme	Delivers 100 % botanically pure vitamin E via nanocpasule technology to reduce lip bleeding and feathering due to fine lines and wrinkles.
Lip Tender	Lip moisturizer	Kara Vita	Ten bioactive ingredients are precisely calculated to work with lyphazomes, delivering a 4- in-1 formula and bringing long-lasting hydration for fast and dramatic lip repair.

Product	Proposed use	Manufacturer	Marketing claims
Nano Cyclic Cleanser Silver	Cleanser	Nano Cyclic	Cyclic cleanser is a scientifically balanced blend of nanosilver and natural ingredients. It kills harmful bacteria and fungi, treats acne, exfoliates dead skin on all parts of the body, diminishes age spots, deodorizes the body, and fights wrinkles.
LifePack Nano	Face gel	Pharmanex	LifePack Nano is a nutritional antiaging program formulated to nourish and protect cells, tissues, and organs in the body with the specific purpose of guarding against the ravages of aging. Nano-encapsulation increases bioavailability coenzyme Q10 by 5-10 times.

TABLE 14. SUMMARY OF THE COSMETIC AND COSMECEUTICAL PRODUCTS THAT USE CARRIER SYSTEMS TO IMPROVE PERFORMANCE.

Reproduced from Li *et al.* 2011 (Li, Wu et al. 2011)

Trade name	Ingredient	Use	Name of brand	Type of carrier system used
Capture®	-	Anti-aging	Dior	Liposome
Advanced Night Repair Protective Recovery Complex Serum	-	Anti-aging	Estee Lauder	Liposome
Clean It® Complexion Mist	-	Acne-controlling	Kara vita	Liposome
Royal Jelly Lift Concentrate	-	Anti-wrinkle	Royal Jelly	Liposome
Niosome Plus®Daily Treatment	-	-	Lancôme	Niosome
Rénergie® line roducts (Flash Lifting Serum, Microlift Serum, Microlift Cream, Microlift Eye Cream	Nanoparticles of silica and proteins	Skin-tightening, anti-wrinkles	Lancôme	Nanoparticles
Nano Gold® Energizing Cream	24-karat gold, a natural protein	Anti-aging Anti-inflammation	Neiman Marcus	Nanoparticles
Nanorama™—Nano Gold® Mask Pack	Nano-gold, amino acid, collagen, coenzyme Q10	Skin tightening Anti-wrinkles	Lexon Nanotech Inc ™	Nanoparticles
Oleogel®	Coenzyme Q10, primrose, Vitamin A,E	Antioxidation, anti-aging	Dermaviduals	Nanoparticles
Platinum Silver Nanocolloid® line products (Platinum Silver Nanocolloid Milky Essence, Platinum Silver	Botanicals and coenzyme Q10Nanocolloid Cream)	Anti-wrinkles, anti-aging	DHC Skincare	Nanoparticles
RevitaLift® line (RevitaLift Double Lifting Serum, Intense Lift Treament Mask)	Pro-Retinol A, vitamin A	Skin-tightening, anti-wrinkles	L'Oreal nanoparticles	Nanosomes,
Rutína® Nano-force Moisturizer	Hyaluronic acid derivative	Skin-	KOSÉ	Nanotechnology

Trade name	Ingredient	Use	Name of brand	Type of carrier system used
		moisturizing		
Rutína® Nano-white Serum	-	Skin-whitening	KOSÉ	Nanotechnology
The Makeup Dual Balancing Foundation SPF 17	-	Makeup	Shiseido	Nanoparticles
Skin Forever®—Extreme Wear Flawless Makeup SPF 25	-	Makeup	Dior	Nanotechnology
Snow Pure® UV Base SPF 50	-	Makeup	Dior	Nano UV filters
Hydra flash® Bronzer Daily Face Moisturizer	Vitamin E	Self-tanning Skin-moisturising	Lancôme	Nanocapsules
Hydrazen® Cream	Triceramides	Skin-moisturising	Lancôme	Nanosphere
Elixir Skin Up® Cream	Titanium dioxide	Makeup foundation	Shiseido	Nanoparticles
Bioperformance Crème Super Régénérante Absolue®	Gamma linolenic acid	-	Lancôme	Nanocapsules
Platinéum®	Hydroxyapatite	Anti-aging	Lancôme	Nanoparticles
Gold Future®	Gold	Anti-free radical	Helena Rubinstien	Collodial
Happylogy® Glowing Skin Essence	Pro-endorphins complex	Anti-wrinkle	Guerlain	Nanoemulsion
Precisone ® Calming Emulsion	-	-	Chanel	Nanoemulsion
Coco Mademoiselle Fresh Moisture Mist ®	-	Skin-moisturizing	Chanel	Nanoemulsion

Assessment of Nano-enabled Technologies in Cosmetics

This report reviews the available literature on nano-enabled technologies for cosmetic products, specifically addressing soluble nano-transporters. Accompanying this report is an appraised database (web link can be found in Appendix 1) summarising the literature which formed the basis of this review which addresses the following areas: Types and uses of soluble nano-transporters in cosmetic applications; assessment of the extent of dermal absorption/ penetration of nano-transporters; evidence of dermal/ systemic toxicity arising from interactions with nano-transporters; identification of nano-specific characteristics that may influence dermal absorption/ toxicity of nano-transporters; assessment of the specific research areas that require more knowledge and to discuss to which extent provisions on nanomaterials in the cosmetic regulation are to be applied for nano-transporters.

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