Safety assessment of cosmetic products containing nanomaterials. Current research trends and challenges

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Summary

Nanomaterials (NMs) may exert biological effects that differ from their macroscale counterparts. The combination of small particle size, large surface area, and ability to generate reactive oxygen species has been demonstrated to be a key factor in induction of cell injury following exposure to certain engineered NMs. Nanostructure can be associated with modification of biological properties and toxicological effects of ingredients used in cosmetics. However, the issue is controversial. Contrasting results have been obtained with widely used agents such as TiO_2 and ZnO.

From a regulatory perspective, there are two major uncertainties that impact on safety assessment strategies for nanotechnology-derived products. The first is as to whether nano-sized particles have to be considered new chemicals, or whether the use of existing materials at the nano-level should be considered a "new use" of an existing chemical. Addressing this question is of crucial importance to define adequate strategies and establish whether NM-tailored testing methods should be added to conventional toxicity testing protocols to comply with regulatory demand. A second issue is whether the classical toxicity testing methods and strategies that are currently used in the hazard evaluation of macroscale chemicals are adequate when applied to NMs.

NMs used in cosmetics can be divided into two groups: labile nanoparticles which disintegrate upon application to skin into their molecular components (e.g. liposomes microemulsions, nanoemulsions) and insoluble nanoparticles such as TiO_2, fullerenes and quantum dots. It is currently believed that while conventional risk assessment methodologies may be adequate for labile nanoparticles, specific toxicological and physicochemical parameters must be investigated to establish safety characteristics of insoluble particles. At the present time, the production and commercialisation of manufactured NMs do not trigger additional safety testing. However, the European regulation of chemical substances (REACH), which effectively shifts responsibility from authorities to industry to assess safety of chemical substances, is likely to represent a significant challenge in resolving the conflict between progress and protection of cosmetics containing NMs.

Riassunto

I Nanomateriali (NM) possono causare effetti biologici diversi rispetto a quelli prodotti dai corrispondenti composti esistenti su scala macrometrica. Studi sperimentali hanno dimostrato come la combinazione di tutte le peculiari caratteristiche chimico-fisiche, quali la dimensione estremamente ridotta delle particelle combinata ad un'ampia area di superficie, e l'abilità di generare specie reattive all'ossigeno e indurre stress ossidativo, sia un fattore chiave nel determinare fenomeni di insulto cellulare dopo esposizione a certi NM ingegnerizzati. La nanostruttura di questi materiali di nuova sintesi, come nel caso di vari ingredienti utilizzati nella produzione di cosmetici, è spesso associata a modificazioni delle proprietà biologiche e degli effetti tossicologici.

Comunque, l'argomento è ancora controverso; risultati sperimentali contrastanti sono stati ad esempio ottenuti con due composti "nano" ampiamente utilizzati nel mercato della cosmesi: titanio biossido (TiO_2) e zinco biossido (ZnO).

Dal punto di vista regolatorio, esistono a tutt'oggi due grosse incertezze sulle strategie di studio per valutare la sicurezza dei prodotti ottenuti con nanotecnologie. La prima è se una particella con dimensione "nano" debba essere considerata come un nuovo composto chimico, o se, piuttosto, l'utilizzo di materiali, già esistenti, su scala nanometrica debba essere considerato semplicemente un "nuovo utilizzo" di un composto chimico pre-esistente. Affrontare questo problema è di fondamentale importanza per poter definire strategie di studio adeguate e stabilire se nuovi test messi a punto ad hoc per i NM debbano essere aggiunti ai tradizionali metodi d'indagine per la caratterizzazione tossicologica, al fine soddisfare le emergenti esigenze regolatorie.

La seconda questione è se i test tossicologici tradizionali e le strategie correntemente utilizzate nella valutazione della pericolosità dei composti chimici su scala macrometrica siano adeguati allorché applicati ai nuovi NM.

I NM utilizzati nella cosmesi possono essere fondamentalmente suddivisi in due gruppi: le nanoparticelle labili, che, subito dopo l'applicazione sulla pelle, si scindono nei loro componenti molecolari (ad es. liposomi, microemulsioni, nano-emulsioni) e le nanoparticelle insolubili come il TiO_2, i fullereni ed i quantum dots. Si ritiene che, mentre per le nanoparticelle labili le convenzionali metodologie di "risk assessment" siano adeguate, specifici parametri tossicologi e fisico-chimici dovrebbero essere invece considerati ed attentamente studiati al fine di stabilire le caratteristiche di sicurezza per le nanoparticelle insolubili.

A tutt'oggi, nonostante la vasta produzione e commercializzazione di NM ingegnerizzati, non si è ancora provveduto alla messa a punto e applicazione di nuovi test adeguati per la determinazione della sicurezza di questi prodotti nanostrutturati. Ciononostante, le recenti direttive europee, con il nuovo regolamento destinato ad aumentare la sicurezza nell'ambito della produzione e dell'utilizzo di sostanze chimiche (REACH), che effettivamente sposta la responsabilità del "safety assessment" delle sostanze chimiche, sia pre-esistenti sia di nuova produzione, dagli organi regolatori direttamente alle imprese, sembra rappresentare un significativo passo avanti nella risoluzione del conflitto fra progresso e protezione nell'ambito del mercato dei prodotti cosmetici contenenti NM.

INTRODUCTION

Cosmetics and pharmaceutical products

That a cosmetic must be inactive with no biological or physiological interactions with cells and tissues is an ambiguous concept often contrasting with scientific evidence (31). The simplest cosmetic emulsion composed of oils and water is known to induce physiological skin modifications and is effective in increasing biological activity or promoting absorption of other active ingredients.

Experimental and clinical studies of cosmetic products have indicated a range of biological activities that are compatible with their observed (desired or adverse) effects. In principle, the ability to modify a biological function may be interpreted as an indicator of possible pharmacological activity. However, accurate studies of the mode of action and evaluation of effects occurring at various levels of biological complexity may valuably contribute to distinguish a cosmetic from a pharmaceutical product (31).

Application of *omics* and other special molecular techniques offer new opportunities to characterise biological effects of cosmetics and determine whether these biological activities do not reflect unacceptable toxicity and are compatible with the intended cosmetological applications. In the last years, molecular biology techniques have increasingly been used to assess the mode of action of ingredients that are currently found in the cosmeceutical marketplace such as retinoids, B vitamins, peptides, antioxidants, and polyhydroxy acids. These methods can also be used to define the biological effect profile of other emerging topical agents such as peptides, growth factors, nanotechnology-derived products, and a range of products proposed for natural skin defence, lightening and depigmentation. Findings obtained by these techniques clearly indicate that the boundaries between cosmetics and dermatological products are fading away rapidly (57). Certain cosmetics have been proposed in the treatment of minor skin disorders and mild skin abnormalities based on the hypothesis that they may be effective as adjuvant of physiological processes by mechanisms not implying direct pharmacological action. These dermatological applications should be decided case by case based on prudent evaluation of safety and risk-benefit issues.

In minor skin disorders, cosmetic treatment has been proposed as a valuable alternative to anti-inflammatory agents, antibiotics and other potentially dangerous drugs (56, 55, 30, 26, 31). Nanotechnology is expected to make it even less marked the difference of certain cosmetic products from pharmaceuticals.

Nanomaterials and cosmetol ogical applications

A future in which the ability to understand and control matter at the nanoscale is expected to represent a revolution in technology, medicine and industry that will benefit society (32, 50, 34). Today, NMs are present in a broad range of consumer products including colloidal health drinks, carbon fibre sport equipment, electronic products, as antibacterial components of toys, and cooking products. NMs have also entered numerous personal care products on the market, including sunscreens and cosmetics (43). Common nano-ingredients in cosmetic products include metal oxides such as titanium dioxide (TiO_2) and zinc oxide (ZnO), nanoemulsions and nanoencapsulated delivery systems (Table I).

However, concern has been expressed about the potential of adverse and unanticipated toxic effects of NMs on human health, apparently due to their unique and tunable chemical, mechanical, electrical, optical, or magnetic properties. These physico-chemical properties can lead to

NMs used in cosmetics can be classified into two general groups: (i) labile nanoparticles (e.g. liposomes, microemulsions, nanoemulsions), which disintegrate upon application to skin into their molecular components, (ii) insoluble or persistent particles such as TiO_2, fullerenes, and quantum dots. It is primarily for the insoluble particles that health concerns related to dermal contact and possible absorption arise. Should they become systemically available, translocation/transportation and eventual accumulation in secondary target organs may occur. This feature does become important with repeated application of cosmetic products.

According to the EU's statement in 2008 (European Commission, 2808), "current legislation covers to a large extent risks in relation to nanomaterials; these risks can be dealt with under the current legislative framework".

This concept, however, has become a matter of considerable debate among experts in the nano-risk community (5). In particular, many have questioned how the safety can be assessed given important knowledge gaps, as mentioned above, (Table II).

With respect to NMs used in cosmetics, it is generally believed that, for the labile NPs, conventional risk assessment methodologies based on mass metrics may be adequate whereas other metrics, such as number, surface area and size distribution of particles, should be adopted for testing insoluble particles.

In toxicological studies, it is crucial to assess transfer of nanoparticles across biobarriers and their possible uptake by tissues and cells following exposure by the intended route (dermal). Exposure via inhalation (occupational setting), ingestion (accidental exposure), conjunctival and mucosal surfaces may should be considered as relevant aspects for risk characterisation.

There are two controversial issues that significantly impact on regulatory aspects and safety assessment strategies for NMs used in cosmetology. The first is as to whether these nano-sized particles have to be considered as "new chemicals", or whether the use of existing materials at the nano-level should be considered a "new use" of an existing chemical.

Addressing this question is of crucial importance to define adequate testing strategies and establish whether NM-tailored studies should be integrated into conventional toxicity assessment to comply with regulatory demand.

A second issue is whether the classical toxicity testing methods and strategies that are currently used in the hazard evaluation of macroscale chemicals are adequate when applied to NMs.

In vitro toxicology has developed significantly in recent years, based on the 3Rs strategy: refinement, reduction, replacement put forward by Russell and Burch, 1959. *In vitro* assays are expected to become an essential component of toxicological and risk assessment research paradigms for chemicals, including pharmaceuticals, consumer products, and fine and ultrafine particulates. *In vitro* (cell culture) testing is also an essential element in all tiered approaches currently proposed for toxicity assessment of NMs.

In perspective, a great power is attributed to high-throughput systems that can be used for rapid and cost-effective screening of chemical hazards and identification of toxicity mechanisms evaluated at subcellular and molecular levels (4, 15). However, despite considerable growing application of *in vitro* systems to NM toxicity assessment (6, 24), large data gaps and methodological uncertainties remain with respect to evaluation of NMs in cosmetic products. In particular, only a limited number of validated *in vitro* methods exist that are applicable for regulatory purposes in risk assessment of cosmetics.

The validated *in vitro* assays currently in use are listed in Commission Regulation EC No 440/2008 of 30 May 2008, laying down test methods pursuant to Regulation EC No

1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) that came into force in June 2007.

Use of these methods is required for the safety assessment of cosmetic ingredients, according to the recent EU Cosmetic Product Regulation (EC 1223/2009), aiming at the complete phasing out of animal testing as indicated in the last 2010/63/EU. Validated alternative methods are currently available for assessing acute and short-term effects but not repeated-dose or long-term toxicity (Table III).

Notably, all these methods were originally developed for application to safety assessment of traditional chemicals and therefore still require rigorous scrutiny and validation studies to determine their general applicability to nanotoxicology. Other *in vitro* assays that could become a valuable tool for testing of NMs include the bovine cornea opacity permeability screening test (BCOP) to assess eye corrosives and irritants, the isolated chicken eye test (ICE), as well as IRE (isolated rabbit eye test) and HET-CAM (hen's egg test - chorioallantoic membrane). These approaches are not yet recognized officially by regulatory bodies for quantitative risk assessment.

To be acceptable for comprehensive and scientifically sound toxicity assessment of NMs, an *in vitro* testing strategy should take into careful consideration a set of pivotal toxicological endpoints, namely (i) penetration across physiological barriers (e.g. skin, lung, oral route), (ii) uptake and translocation, (iii) biochemical and functional cytotoxicity, (iv) induction of cellular stress with emphasis on oxidative stress and inflammation, (v) mutagenicity/genotoxicity.

TABLE III

Validated in vitro testing methods applicable to nanotoxicology.

Enpoint	*Methods*
– Skin irritation	Human skin models (Episkin™)
– Skin corrosion	– Transcutaneous Electrical Resistance Test (TER) – Human skin models (Episkin™ and Epiderm™
– Skin absorption	Franz cell using human/pig skin
– Phototoxicity	3T3 NRU
– Genotoxicity/Mutagenicity	– Bacterial reverse mutation test – Gene mutation test in *Saccharomyces Cerevisiae* – Sex-linked recessive lethal test in *Drosophila melanogaster* – Mammalian cell gene mutation test – Micronucleus test – Mammalian chromosome aberration test
– Embryotoxicity	– EST (embryonal stem cell test) – MM (micromass assay) – WEC (whole embryo culture)

A variety of techniques have been proposed in studies of dermal skin penetration of chemicals (49). However, due to the specific characteristics of NMs, doubts have been raised about the applicability of these techniques to topical formulations of cosmetics containing NMs, such as liposomes.

Useful information from *in vitro* studies can be obtained by morphological methods examining the post-treatment skin, such as bright-field microscopy, high resolution transmission electron microscopy and autoradiographic methods (1, 2). The requirements for testing the mutagenicity/genotoxicity potential of NPs do not differ from those regarding other agents. However, the peculiar properties of NPs may require further considerations.

Genotoxicity of NPs can be assessed *in vitro* using mammalian cells provided that exposure of the nucleus at relevant times for each assay is demonstrated. Validated *in vivo* assays can also be used, even though current experience with these assays applied to NM-containing cosmetics is limited and, in addition, it is difficult to establish whether the model would cover the expected target organ(s) for the NP of interest.

The EU Directive EC 1223/2009, adopted from 30 November 2009, is the first piece of supranational legislation that incorporates rules relating specifically to the use of NMs in any commercial products. The Directive (provisions of which will be applicable from 11 July 2013) confirms the ban of animal testing for the safety assessment of cosmetics, in accordance to the 7th Amendment.

An exception from this rule, which should be justified by specific regulatory needs, is the provision regarding strictly controlled *in vivo* assays of acute- and repeated-dose toxicity, reproductive toxicity and toxicokinetics. Since no alternatives are yet available, these studies are permitted, provided that limited numbers of laboratory animals are used. Experiments must be designed in order to minimize suffering of animals (2010/63/EU), after taking into account (i) any existing human and/or animal data on toxicity and toxicokinetics of the test substance or related materials, (ii) possible structure activity relationships (SAR), and (iii) results from *in vitro* or ex vivo tests. On these grounds, a tiered toxicity testing strategy has recently been developed in our laboratories as a preliminary research tool applicable to engineered nanomaterials (Table IV) (9).

TABLE IV

A proposed tiered toxicity testing strategy as a preliminary research tool applicable to engineered nanomaterials.

- Physico-chemical characterisation
- Evaluation of existing toxicological data
- In *silico* (SAR modelling, read-across, computational data gap filling, etc)
- *In vitro* (cell/tissue cultures)
- *In vitro* ex vivo
- Limited, justifiable *in vivo* testing
- Overall product evaluation and risk-benefit analysis

In summary, a number of validated methods may be used for toxicity assessment of NMs in cosmetics, according to the guidelines proposed by various European organisations such as the Scientific Committee for Consumer Safety (SCCS), ECVAM (European Centre for the Validation of Alternative Methods) and OECD (Organisation for Economic Cooperation and Development) guidelines.
However, despite a large body of research in this field, no methods are still available which could reliably predict the reactions of the intact organism exposed the cosmetics containing man-made nanoparticles.

CONCLUSIONS

Based on current information, some NMs that have been adopted by the cosmetics industry raise little concern. Other could present risks because of their new properties, such as resistance to degradation or tendency to cause oxidative stress. The growing production and commercialisation of engineered NMs poses the urgent need to develop effective testing strategies that can be applied for risk assessment purposes.
The challenge is developing a battery of tiered *in vitro* assays that can replace or reduce the application of existing *in vivo* protocols to fully understand the effects and mechanisms of toxicity of NMs.
The European regulation of chemical substances (REACH), which effectively shifts responsibility from authorities to industry to assess safety of chemical substances, is certainly a real incentive to promote scientific research aimed at protecting consumers against possible risks associated with NM-containing cosmetics.

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