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Nanomedicine: Nanotechnology, Biology, and Medicine
9 (2013) 39–54nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

Review Article

Interaction of inorganic nanoparticles with the skin barrier: current status and critical review

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Received 20 November 2011; accepted 12 April 2012

Abstract

Integration of nanotechnology with biology leads to various advantages in applied pharmaceutical and medical sciences. In that regard, the behavior of nanoparticles (NPs) in relation to the skin, an important biological barrier, has been the target of several recent studies. Yet the potential ability of NPs to penetrate into the underlying viable tissue lies at the center of debate. This review briefly highlights the current applications of inorganic NPs, then discusses the current status of their skin penetration in view of the vast variation among the experimental setups in use. Determinants of particle penetration, adopted approaches for enhanced penetration, the underlying mechanism, as well as qualitative and quantitative analysis of NPs present in the skin are also within the scope of this review article. We emphasize analyzing the data generated from experiments on human skin, the “gold standard” for assessment of in vitro skin penetration. Based on this, we include some recommendations for future research.

From the Clinical Editor: Transdermal application of inorganic nanoparticle-based medications is of growing interest in nanomedicine research. This critical review discusses the knowns and the unknowns of this field, providing insightful recommendations for future research. © 2013 Elsevier Inc. All rights reserved.

Key words: Inorganic nanoparticles; Nanoparticle penetration; Particle penetration enhancement

Nanomaterials in the size range of ~1–100 nm offer new functions and applications in relation to bulk materials, primarily because of their high surface-to-volume ratio. Additionally, unusual physical, chemical, and biological properties can emerge in materials at this nanoscale dimension. These properties may significantly differ from those of bulk materials and single atoms or molecules.¹ However, there is no guarantee that unique properties exist below the size boundary of 100 nm.² In most cases these unusual new properties are attributed to inorganic nanomaterials. Gold in the bulk state, for instance, is an excellent conductor of heat and electricity, but no heat transfer reaction occurs on directing light onto it. Gold nanoparticles (AuNPs) can, however, absorb light and transform it into heat, acting like miniature thermal scalpels that can kill unwanted cells in the body, such as cancer cells.³ Titanium dioxide (TiO₂) and zinc oxide (ZnO), used as sunscreen agents, have the disadvantage of being visible, masking the skin with a white color. TiO₂ and ZnO nanoparticles (NPs) are transparent and are thus preferred to microparticles in sunscreen products.⁴ On the other hand,

polymeric nanosystems usually acquire properties that are essentially an interpolation of that of the same material at the larger scale. It is also relatively easier to prepare inorganic NPs in such a size range than polymeric NPs. Last, but not least, inorganic NPs have recently been the subject of several applications in nanomedicine and drug delivery.⁵ Fine-tuning the surface properties of inorganic NPs by surface functionalization has further extended their applications.⁶ Selected applications of some inorganic NPs are highlighted in the next section, and relevant review articles are cited to provide further details.

Inorganic NPs and their pharmaceutical and biomedical applications

Quantum dots (QDs)

QDs are nanocrystals composed of a semiconductor material (e.g., CdS). QDs have attracted widespread interest in biology and medicine because of their unique electronic and optical properties in relation to organic dyes and fluorescent proteins, including size- and composition-tunable emission wavelength, improved signal-to-noise ratio, and higher photostability. Therefore, QDs have rapidly emerged as a new class of fluorescent probes for biomolecular and cellular imaging.^{7,8}

No conflict of interest was reported by the authors of this paper.

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1549-9634/\$ – see front matter © 2013 Elsevier Inc. All rights reserved.
<http://dx.doi.org/10.1016/j.nano.2012.04.004>

Surface functionalization of QDs and bioconjugation to biomolecules via a covalent linkage was subsequently adopted to enhance the dispersibility of QDs, reduce toxicity and provide better in vivo and cellular targeting. This has further allowed the use of QDs in medical diagnostics, especially in cancer diagnosis^{7–10} Further development of QDs might enable their application in tracking drug delivery and monitoring the efficacy of therapeutics noninvasively in real time.⁸

Silica NPs

Nanosized silica particles are another type of bioprobe that have also been reported to be ideal protein hosts. This is due to their high chemical, physical, and mechanical stability, large surface area, good dispersibility in aqueous solution, and relative inertness. Silica NPs have a high surface silanol concentration, which facilitates a wide variety of surface reactions and the binding of biomolecules.¹¹ Therefore, they have attracted widespread interest in applications in bioanalysis and in diagnosis, especially in the diagnosis of cancer cells. Silica NPs have also been considered good candidates for drug and gene delivery.^{11,12}

Silver NPs

Silver compounds have been well known for centuries as antimicrobial agents and are widely exploited in the treatment of bacterial infections encountered in burns, open wounds, and chronic ulcers.¹³ Recently, however, silver NPs showed higher antimicrobial efficiency compared to silver salts because of their extremely large surface area, with diameters generally smaller than 100 nm containing 20–15,000 silver atoms, providing better contact with microorganisms. Thus, on cellular or tissue exposure to silver NPs, the active surface of silver NPs would be large compared to silver compounds, and would thereby exhibit remarkably unusual physicochemical properties and biological activities.^{14–17} They interact with bacteria and produce electronic effects, which enhance the reactivity of NPs.¹⁷ Thus, the bactericidal effect of silver NPs was proved to be dependent on both size¹⁵ and shape¹⁸ because of differing interaction with the microorganism. Several dressings for wound healing incorporating silver NPs as a topical antibacterial agent have already found their way to the market, including Acticoat (Smith & Nephew, London, United Kingdom) and Silverlon (Argentum Medical, Lakemont, Georgia). They have the advantage of providing a more controlled and prolonged release of nanocrystalline silver to the wound area. This mode of silver delivery allows less frequent dressing changes, thereby reducing the risk of nosocomial infection as well as the cost of care and further tissue damage, and definitely improving patient compliance.¹³

Gold nanoparticles (AuNPs)

Applications of AuNPs in biology and life sciences constitute a fast-growing field. These bioapplications can be classified into four areas: labeling, delivery, hyperthermia, and sensing.¹⁹ Similar to silver NPs, AuNPs have unique optical properties. They have the ability to resonantly scatter visible and near-infrared light upon the excitation of their surface plasmon oscillation. The scattered light intensity is sensitive to the size and shape of particles.²⁰ Therefore, they are useful as biosensors and diagnostic agents, especially in cancer diagnosis.^{21,22} Based

on their physical properties, AuNPs cause local heating when they are irradiated with light in the range of 800–1200 nm. El-Sayed's group²³ has shown the potential use of AuNPs in photothermal destruction of tumors. Recently, AuNPs have emerged as a delivery system for various payloads,^{24,25} either drug molecules^{26–28} or large biomolecules such as proteins,²⁹ DNA,^{30,31} and RNA.³²

Magnetic NPs

NPs with a magnetic core allow for the magnetic manipulation of the particles in the presence of an external magnetic field. In this size range, below 100 nm, there is a fundamental change in the magnetic structure of ferro- and ferrimagnetic materials. Their superparamagnetic moment with high magnetic saturation value has resulted in explorations of the use of magnetic NPs as targeting agents in a number of applications including drug and gene delivery, in addition to tumor diagnostics and therapeutics. Another important therapeutic application is hyperthermia, which involves heating organs or tissues resulting in tumor cell necrosis.^{33–37} Functionalization of magnetic NPs has further extended their in vivo applications based on higher biocompatibility and reduced toxicity, in addition to adopting additional targeting approaches in coating the surface with biofunctional molecules and thus enhancing the targeting efficiency of the final developed system.^{36,37}

Titanium dioxide (TiO₂) and zinc oxide (ZnO) NPs

TiO₂ and ZnO NPs are currently used in many sunscreen formulations as ultraviolet (UV) filters to protect against UV-induced skin damage. TiO₂ and ZnO have the advantage over other chemical agents that they do not undergo any chemical decomposition on exposure to UV radiation. Moreover, they offer a wider range of protection against UVA and UVB types of radiation, compared to other organic compounds. Currently in the cosmetics industry TiO₂ and ZnO are included as nanosized particles, because in this form they are transparent and more esthetically acceptable to consumers.^{4,38} This is in addition to the size-dependent antibacterial activity of ZnO NPs.³⁹

Significance and scope of the review article

One could thus far conclude that inorganic NPs have found several applications, especially in cancer diagnosis and therapy, and more recently in drug and gene delivery. This potential for applications has triggered the investigation of the NP interaction with the various biological barriers. An excellent biological barrier, the skin, has been addressed in several recent studies regarding NP penetration.

Skin, a unique barrier composed of several highly organized and heterogeneous layers, also includes a number of appendages such as hair follicles, and sweat and sebaceous glands. Skin is composed of three layers from outside to inside: epidermis, dermis, and hypodermis. However, from a penetration perspective only epidermis and dermis are important. The outermost layer of the epidermis is the stratum corneum (SC), to which the main barrier function of the skin is attributed.^{40,41} Nevertheless, topical and transdermal drug delivery systems have always been a

main target of many researchers, avoiding the numerous problems encountered with the oral route. Although the mechanism is still unclear, NPs were shown to enhance the skin penetration of several active agents.^{42,43}

Hence, studying the skin penetration of inorganic NPs is crucial for the following reasons:

- Design of potential topical and transdermal nanocarriers and biomedical diagnostic agents

Understanding the behavior of NPs when they contact the skin surface and their interaction with the different skin layers would ultimately lead to the design of the “ideal” carrier or diagnostic agent in terms of the physicochemical parameters of the NPs (e.g., size, shape, surface chemistry), in addition to other factors (e.g., formulation and environmental factors) influencing skin penetration of NPs.

- Health risk analysis

The public could come in contact with nanomaterials intentionally on applying topical cosmetic preparations containing NPs, or non-intentionally through the handling of several products used in our daily life that contain NPs (e.g., sports clothes, surface-cleaning agents, computer devices, paints).^{17,38} Environmental exposure comprises water or even air contaminated with NPs. This is in addition to NP exposures for people handling NPs in research and industry. In hospitals, physicians and patients may also come in contact with NPs (e.g., silver-impregnated medical devices such as surgical masks and implantable devices).¹⁷ The occasions for exposure to NPs are thus increasing constantly.

Among inorganic NPs, the extent of skin penetration of TiO₂ and ZnO NPs was investigated intensively by several research groups^{44–62} because of their high pharmaceutical value; they are frequently employed as inorganic physical sun blockers.^{4,38} Given the growing commercial and scientific interest, their safety and effectiveness as well as their regulatory status have been repeatedly reviewed in literature; for more detailed information please refer to the review articles by Smijs and Pavel,⁶³ Schilling et al.,⁶⁴ and Newman et al.³⁸ In this review article, however, recent research on the interaction of inorganic NPs with the skin barrier is discussed and analyzed in an attempt to answer still open questions: (i) How do inorganic NPs, in the size range < 100 nm, as well as submicron particles, interact with the skin barrier? (ii) Do they have the ability to penetrate the SC into the viable deeper skin layers (DSLs)? And if so, (iii) what is the possible mechanism of skin penetration? Finally, (iv) what are the factors contributing to their penetration?

But first it is important to define two important terms used in our review: skin *penetration* and skin *permeation*. The former term indicates transport of an agent—here NPs—across the SC into DSLs. The latter, however, stands for transport across the entire skin barrier into the receptor solution (in vitro) or to the systemic circulation (in vivo).⁶⁵

Current dilemma in the status of skin penetration of inorganic NPs

Investigating the ability and the possible mechanism of particle penetration through skin is a recent area of research receiving

great interest of researchers for the reasons already mentioned. Starting from the year 2004, the number of studies focusing on skin penetration of inorganic particles is generally increasing. To the best of our knowledge, the total number of research articles in this field is 40 (excluding replicate studies common in skin penetration/permeation experiments), in which 125 different penetration/permeation experiments (different in particle type and size, skin type, skin treatment, etc.) were conducted. Yet the ability of inorganic NPs to overcome the barrier function of the SC into DSLs is a point of debate among researchers. Different outcomes were reported for particle penetration as shown in Table 1. About 49% of all these experiments were reported to result in particle penetration with or without adopting a mechanical or a chemical enhancement approach or a combination thereof. Some of these studies (five) showed even particle permeation of the whole skin thickness either in vitro^{66,67} or in vivo^{59,66,68}. The other 51 % of these experiments, however, resulted in neither passive particle penetration nor particle penetration on physical or chemical skin treatment (discussed below under “Approaches Adopted to Enhance Skin Penetration of Inorganic NPs”). Further analysis of the reported outcomes over the years 2008–2010, witnessing most of the conducted particle skin experiments, one could observe an increase in the frequency of research articles reporting particle penetration, increasing from ~38% in 2008 to ~67% in 2010, relative to the total number of research articles reported in that year. This could be a result of implementing even more approaches for enhancing skin penetration in addition to the improvements of the analytical techniques for detection of NPs inside the skin (discussed under “Qualitative and Quantitative Analysis of Inorganic NPs in the Skin”). Unfortunately, the feasibility of publishing studies showing particle penetration, being more attractive to the research community, could be also a contributing factor. Nevertheless, to further add to this dilemma, the size range of inorganic particles, regarded as the primary determinant of skin penetration, is overlapping for particles reported to penetrate the SC (4 nm to 1.5 µm) and particles that could not (4 nm to a few microns).

Overall, the reported experimental setups regarding skin type, as well as environmental and mechanical conditions, revealed a high diversity. This might be the basis of the current controversy among researchers on whether NPs do or do not penetrate the SC into DSLs, as highlighted in the following points:

- *Animal vs. human skin.* Although excised human skin is regarded as the “gold standard” for in vitro skin penetration studies especially in human dermal risk assessment,⁶⁹ it was only used in 51% of the experimental setups used in particle penetration studies (35% in vitro and 16% in vivo). However, 47% of skin penetration experiments of inorganic NPs were conducted on animal skin (pig, mouse, and rat skin) in vitro (31%) or in vivo (16%) (Figure 1). This is apparently due to the limited availability of human skin, driving most of the research laboratories to depend on animal skin. However, structural and morphological differences between human and animal skin, especially in terms of the density of the hair follicles, thickness of SC and total skin, the amount of skin lipids, in addition to variations among animal species, could

Table 1

Overview of the skin penetration studies of various inorganic particles applied to different skin types under differing experimental conditions

Particle	Diameter (nm)	Skin type (in vitro/in vivo)	Additional enhancement approach	Reported outcome	Ref
QD-COOH	4	Human (in vitro)	– Massage Tape-stripping Tape-stripping + massage	No penetration No penetration No penetration Penetration	95
Fe ₂ O ₃ -TMAOH	4.9 ± 1.3	Human (in vitro)	–	Penetration	84
AuNPs	10	Human (in vitro)	– Dermaportation by pulsed electromagnetic field	No penetration Penetration	89
AuNPs-alkylated mercapten [†]	~6	Human (in vitro)	–	Penetration	77,78
AuNPs-lecithin	~6		–	Penetration	
AuNPs-cetrimide [†]	~15		–	Penetration	
AuNPs-citrate	~15		– CHCl ₃ /methanol Tape-stripping	No penetration Penetration Penetration	
TiO ₂	10–50	Human (in vivo)	–	Penetration [‡]	44
AuNPs	~12.9	Human (in vitro)	– Dermabrasion	Penetration Penetration	117
AuNPs	~15	Human (in vitro)	– Urea Tween 80 SLS DMSO	No penetration No penetration Negligible penetration No penetration Penetration	82
ZnO	15–40	Human (in vitro)	–	No penetration	45
TiO ₂	20	Human (in vivo and in vitro)	–	No penetration No penetration	46
TiO ₂ TiO ₂ in sunscreens with and without ZnO	20	Human (in vitro)	–	No penetration	47
ZnO	20–30	Human (in vivo and in vitro)	–	No penetration	48
TiO ₂	20–70	Human (in vitro)	–	No penetration	49
ZnO	<200		–	No penetration	
AgNPs	25 ± 7.1	Human (in vitro)	– Abrasion	Low penetration (both), but higher for damaged skin	110,111
ZnO	30	Human (in vitro)	–	No penetration	50
TiO ₂ (hydrophobic)	20- to 100-nm aggregates	Human (in vivo)	–	No penetration	51,52
TiO ₂ (amphiphilic)			–	No penetration	
TiO ₂ (hydrophilic)			–	No penetration	
TiO ₂ platelets	Size not given (nm range)	Human (in vitro)	–	No penetration	53
ZnO platelets	116.8 length × 57.5 width		–	No penetration	
AuNPs	Size not stated (nm range)	Human (in vitro)	Electrophoresis	No penetration	76
TiO ₂	Size not given (nm range)	Human (in vivo, healthy)	–	No penetration	54
		Human (in vivo, psoriatic)	–	No penetration	

Table 1 (continued)

Particle	Diameter (nm)	Skin type (in vitro/in vivo)	Additional enhancement approach	Reported outcome	Ref
QD-PEG, QD-PEG-amine, QD-COOH	12.65–29.35 nm (at different pH values)	Human (in vitro)	– Tape-stripping	QD-PEG penetration–pH 8.3 Penetration	118
QD-PEG-amine	7 ± 2	Reconstructed human skin	–	No penetration	71
AuNPs	4.6 ± 1.5	Porcine, full-thickness (in vitro) Porcine, dermatomed (in vitro)	Ultrasound and SLS Ultrasound and SLS	Penetration Penetration	92
QD-PEG, QD-PEG-amine, QD-COOH	15–45(spherical and ellipsoid)	Porcine (in vitro)	–	Penetration	80
Polymer-coated QDs	20	Porcine (in vitro)	Ultrasound with or without SLS	Penetration (increased with SLS)	91
AgNPs (uncoated) AgNPs (carbon coated)	20, 50, and 80 25 and 35	Porcine (in vivo)	–	No penetration	119
TiO ₂ with different coatings	35 uncoated, 35 coated, 10 × 100, 250 ^{ll}	Porcine (in vitro)	– Tape-stripping Hair removal	No penetration No penetration Penetration for 35-nm coated	55
PEG-coated QDs	Nail-shaped:5.78 width × 8.4 length 39 ± 1 hydrodynamic diameter	Porcine, dermatomed (in vitro)	–	No penetration	105
TiO ₂ (four formulations)	45–150 length, 17–35 width (lanceolate shape)	Porcine (in vitro)	–	Penetration	56
ZnO TiO ₂ agglomerates	80 up to 200	Porcine (in vitro)	–	No penetration	57
Au particles	900 ± 600	Porcine (in vitro)	Ballistic delivery	Penetration	100
TiO ₂ (uncoated submicron sized, uncoated nano- or Al(OH) ₃ , dimethicone/methicone co-polymer-coated nanosized)	207 ± 53 [¶] , 30 ± 8 [¶] , and fibrils of 57 ± 18 length and 15 ± 5 width [¶]	Porcine (in vivo)	–	No penetration	58
QD-COOH	4.1	Mouse (in vitro and in vivo)	–	Permeation	66
Fe ₂ O ₃	4.6–10	Mouse (in vitro)	Blade incision, 1 µm width	Penetration	106
ZnO	10	Mouse (in vitro)	– OA, EtOH, and OA-EtOH	No penetration Penetration	102
AuNPs	11.6	Mouse (in vivo)	–	Penetration	73
QD-COOH	~20 and ~33 nm ⁸⁶ ; ~12–20 nm ⁷⁴	Mouse (in vivo)	– UV exposure	Low penetration for both but higher on UV exposure	74,86
QD-PEG	37	Mouse (in vivo)	– Acetone-pretreated Tape-stripped Dermabraded	No penetration No penetration No penetration Permeation	68

(continued on next page)

Table 1 (continued)

Particle	Diameter (nm)	Skin type (in vitro/in vivo)	Additional enhancement approach	Reported outcome	Ref
DT-QD-COOH conjugate	Size not stated (nm range)(nail-shaped)	Mouse (in vivo)	Hyperthermia	Penetration [§]	87
QD-COOH	6 ± 2	Rat (in vitro)	–	No penetration	96
			Flexion	No penetration	
			Tape-stripping	No penetration	
			Abrasion	Penetration	
AuNPs	15, 102, 198	Rat (in vitro)	–	Permeation	67
TiO ₂	4, 10, 21, 25, 60, 90	Porcine (in vitro, 1 day)	–	No penetration	59
	4, 60	Porcine (in vivo, 30 days)	–	Penetration	
	10, 21, 25, 60	Mouse (in vivo, 60 days) ^{††}	–	Permeation	
TiO ₂	width 20 × length 100	Human (in vitro)	–	No penetration	60
		Porcine (in vitro)	–		
TiO ₂	20–100	Porcine (in vitro)	–	No penetration	61
		Human skin grafted on	–	No penetration	
		SCID mouse (in vivo)	–	No penetration	
TiO ₂	Size not given (commercial formulation)	Human foreskin grafted on	–	No penetration	62
		SCID mouse (in vivo)	–		

“Skin penetration” indicates transport of NPs, across the SC (stratum corneum) into DSLs (deeper skin layers). “Skin permeation” stands for transport across the whole skin thickness into the receptor solution, in vitro or to the systemic circulation, for in vivo experiments.

AgNPs, silver nanoparticles; AuNPs, gold nanoparticles; DMSO, dimethyl sulfoxide; DT, diphtheria toxoid; EtOH, ethanol; Fe₂O₃, iron oxide nanoparticles; OA, oleic acid; QDs, quantum dots; SCID, severe combined immune deficient; SLS, sodium lauryl sulfate; TiO₂, titanium dioxide nanoparticles; TMAOH, tetramethylammonium hydroxide; ZnO, zinc oxide nanoparticles.

[†] Dispersed in toluene.

[‡] Non-statistically significant higher Ti levels in the deeper layers vs. background levels of Ti normally present in the skin (unexposed control).

^{||} These reported sizes are the primary sizes; however, aggregation was reported for these nanodispersions.

[¶] These sizes are not the primary size diameters but are those determined by TEM of the particles in skin after application.

[§] No proof for penetration was provided by the authors (only fluorescent images without depth information).

^{††} Different pathological lesions were observed in several organs, especially the skin and liver.

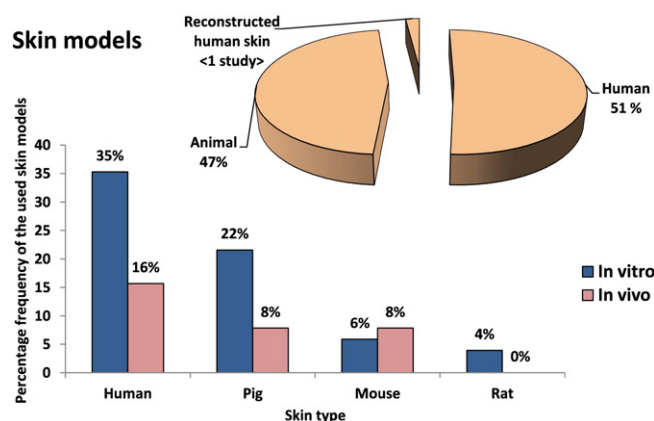


Figure 1. In vitro and in vivo skin penetration and permeation experiments in the literature classified according to the skin type: human, reconstructed human, and animal (pig, mouse, and rat) skin. Percentage frequency of using each of these skin models in examining NPs penetration, either in vitro or in vivo, is indicated.

certainly result in different penetration profiles.^{69,70} Furthermore, only one study was conducted on an in vitro–reconstructed human skin model using 7-nm QDs.⁷¹ However, no penetration was reported. This is even though these models are generally known to overestimate drug

flux across human skin as a result of the lower barrier properties relative to human skin.^{69,72} All this makes it quite difficult to compare the data generated by different laboratories to reach a conclusion regarding the current status of skin penetration of inorganic NPs.

- Among the different animal species, there are hairy animals used for conducting particle penetration experiments. Therefore, hair removal is often indicated to be able to apply a formulation. Removal of hair by clipping, applying cosmetic-grade hair removal cream, or even by shaving before the penetration experiment possibly has an effect on the barrier function of the skin used. Consequently, particle penetration might also be affected. For instance, 11.6-nm AuNPs could penetrate mouse skin *in vivo* after hair was clipped.⁷³ On the other hand, no penetration was reported for QDs having nearly the same size, ~12 nm, through hairless mouse skin *in vivo*.⁷⁴ Recently, Senzui et al.⁵⁵ have shown the effect of hair removal on penetration of TiO₂ NPs through pig skin. Particles were shown not to penetrate through intact or stripped skin; however, penetration was observed on hair removal, possibly by entering empty hair follicles. Therefore, hair removal should be taken into consideration especially when assessing the safety of NPs. This also implies that the dermatomed skin of thickness 200–400 μm , recommended by the Organisation for Economic Co-operation and Development according to the guideline 428 for *in vitro* testing of skin absorption,⁷⁵ could possibly overestimate the skin penetration/permeation of NPs. Overestimation is especially the case for particles likely to accumulate in the hair follicles, because the hair follicle is cut on splitting the dermis and the NPs can then diffuse into the dermis/receptor solution.⁵⁵
- *Skin exposure time* to particles also differed greatly among studies. Penetration of particles was tracked over a period of as short as a few hours (e.g., 1 hour,⁷⁶ 3 hours,⁷³) up to several days (60 days).⁵⁹ However, unlike monitoring penetration of drug molecules especially lipophilic molecules, it is usually not practically feasible to generate appropriate pharmacokinetic parameters for particles, including the flux for normalizing the effect of exposure time, due to absence or a scarce concentration of particles penetrating into the skin. This further limits the ability to analyze data based on different studies raising an analytical problem in determination of the amount of NPs present in the skin in typical penetration/permeation experiments. This will be discussed in detail later under “Qualitative and Quantitative Analysis of Inorganic NPs in the Skin.”
- Different results were also observed on using *flow-through diffusion cells*⁶⁷ vs. *static Franz diffusion cells*^{77,78} for *in vitro* skin penetration of 15-nm AuNPs, although the experimental setup was also very different among these studies. Flow-through diffusion cells offer the advantage of continuous perfusion of the dermis side of the skin with fresh receptor fluid maintaining sink conditions for diffusion⁷⁹; thus, higher fluxes might be expected for these experiments. However, because of the minute NP penetration, this is expected to have a small effect on the results.
- The *application dose* and *volume*, and the *diffusion area* are important factors that would significantly affect the results of any penetration experiment. For instance, earlier

we have reported on the inability of citrate-stabilized AuNPs to cross the whole depth of the SC of human skin into DSLs.^{77,78} This was contrary to the findings of Sonavane et al.,⁶⁷ who showed permeation of AuNPs, prepared by the same method and of almost the same size, through the full skin thickness after only 1 hour of skin exposure. However, they used a volume of donor solution per square centimeter of skin surface (4 mL donor volume and 6.3 cm² diffusion area) that is 2.2 times more than that used in our study (0.5 mL and 1.76 cm²). This is in addition to using rat skin of different permeation and structural characteristics than human skin.

- Different *skin treatment approaches*, physical or chemical, were adopted for inducing or enhancing skin penetration of NPs. This would rather limit the ability of a study-with-study comparison, which will be discussed in detail under “Approaches Adopted to Enhance Skin Penetration of Inorganic NPs.” This is in addition to other factors such as formulation factors (e.g., surface coatings, vehicle), which differ greatly from one study to another. These factors were shown as important determinants of particle penetration as detailed in the next section.

Factors affecting skin penetration

In addition to the aforementioned experimental variations among different studies, several factors were systematically studied and found to contribute significantly in the skin penetration by NPs. Critical determinants of NP skin penetration include physicochemical attributes of the NPs (size, surface charge, surface chemistry, and physical state of the nanodispersion on coming in contact with the skin surface), formulation factors (vehicle), and experimental factors (concentration and skin exposure time to NPs).

Physicochemical attributes of the NPs and formulation factors

The physicochemical attributes of NPs and the nature of the dispersing vehicle are key factors governing their skin penetration. Sonavane et al.⁶⁷ showed size-dependent skin permeation of AuNPs through rat skin. Greater permeation for 15-nm AuNPs compared to 102-nm and 198-nm AuNPs was described through rat skin, and the permeability coefficient was reported to decrease on increasing the particle size. A similar size-dependent penetration phenomenon was also shown for smaller AuNPs through human skin, where 6-nm particles could penetrate to a greater extent than 15-nm AuNPs.⁷⁷

The effect of particle surface charge and shape on penetration through skin was the focus of a study by Ryman-Rasmussen et al.⁸⁰ They studied the effect of various coatings imparting different surface charges (cationic, anionic, and neutral) on the skin penetration of two types of QDs of different size and shape: spherical QDs of 4.6-nm core/shell diameter and ellipsoid QDs with 12 nm (major axis) by 6 nm (minor axis) core/shell diameter. Following skin exposure, confocal examination of longitudinal skin sections showed penetration of the spherical NPs into the viable epidermis and dermis after only 8 hours, regardless of the surface charge. Similar behavior

was reported for cationic and neutral ellipsoid NPs. However, 24 hours of contact of the anionic ellipsoid QDs with the skin surface were required for their penetration into DSLs. Based on these findings, the authors proposed a shape dependency for particle penetration, especially in that the two anionic spherical and ellipsoid QDs had a more-or-less similar hydrodynamic diameter, 14 and 18 nm, respectively. Faster skin penetration for cationic and nonionic ellipsoid QDs relative to anionic ellipsoid QDs could be attributed to the negative surface charge of the pig skin used in the latter study; the isoelectric point of pig skin is ~ 4.4 .⁸¹ Aside from the surface charge, the hydrophobicity of the particle surface was recently shown to favor skin penetration of AuNPs through human skin.⁷⁷ In the latter study we showed that citrate-stabilized ~ 15 -nm AuNPs could not penetrate human skin after 24 hours of skin exposure. However, surface modification having cetrimide on the outer surface (i.e., a hydrophobic surface) resulted in skin penetration into DSLs. A similar pattern was observed for smaller ~ 6 -nm AuNPs, in which surface modification of hydrophobic alkyl thiolated AuNPs using lecithin yielded relatively hydrophilic AuNPs with lower skin penetration ability under the same experimental conditions.⁷⁷

Further, tracking of the physical state of the particles during or after contact with the skin barrier is also required for better interpretation of skin penetration experiments, being an important contributor. Recently we studied the skin penetration of different types of AuNPs.⁷⁷ Although all AuNP dispersions showed physical stability at the beginning of the skin experiment, one particle type was aggregated after 24 hours of skin contact and could not penetrate the SC into DSLs. Aggregation of applied particles over time limits the availability of the individual particles that would have a higher probability of penetrating the resilient barrier.

Besides the physicochemical parameters, the vehicle nature could affect either the barrier state of the skin, thus favoring more penetration,⁷⁷ and/or even the physical state of NPs,⁸² a determinant of skin penetration of NPs as discussed above. For instance, we studied the effect of toluene in comparison to water as a dispersion medium for AuNPs on particle penetration through human skin.⁷⁷ Toluene was shown to have an effect on the barrier function of the SC by means of lipid extraction. However, there were no drastic changes in the intercellular lipid composition on toluene application under the experimental conditions used. Ceramides, known as the most important component of the SC multilamellar lipid structure with definite physicochemical properties necessary for the barrier function of the skin,⁸³ were not extracted by toluene. Further results of our study indicated that the vehicle did not have a major effect on the penetration of the prepared NPs.

Obviously the understanding of particle penetration is still limited. The difficulty of studying single effects of particle parameters on the penetration behavior is due to the inability to tune particle synthesis adequately. It is difficult to change one parameter (size, surface charge, etc.) without changing others (e.g., dispersibility). On the other hand, one would be inclined to question the necessity of such “perfect” studies based on nanoparticulate systems allowing for univariate parameter control, because the determinants of skin penetration

parameters are very much integrated and skin penetration is usually a complex mechanism, dependent not only on the effect of the individual parameters alone but also on the interplay between them.

Experimental factors

Concentration of the nanodispersion applied and skin exposure time could greatly influence the skin penetration of NPs as shown in our recent study.⁷⁷ We applied alkyl-terminated AuNPs on human skin with two different concentrations of gold (90 and 437 $\mu\text{g/mL}$), for exposure times of 0.5, 2, 6, and 24 hours. Both the amount of AuNPs penetrating into the DSLs and the penetration depth increased with higher concentration and longer skin exposure time. Some other studies investigated skin particle penetration at different time intervals. For instance, Baroli et al.⁸⁴ studied the penetration of magnetic NPs through human skin after 3, 6, 12, and 24 hours, where particle penetration was reported starting from 6 hours’ skin exposure time. Based on these studies, generalized overestimated conclusions indicating safety of NPs on topical application or the opposite case based on studying a single concentration is inappropriate. Figure 2 sketches the main factors affecting skin penetration of inorganic NPs.

Although more studies are still required at the basic level to advance our understanding and to gain a deeper insight into the mechanism and determinants of skin penetration of NPs, several approaches have been adopted by researchers in an attempt to enhance skin penetration of inorganic NPs via breaching the barrier function of the skin. These approaches, however, did not always ensure an increase or even occurrence of skin penetration of particles.

Approaches adopted to enhance skin penetration by inorganic NPs

There is no study that systematically investigates the possibilities of enhancement of particle penetration. Some approaches were, however, adopted to enhance skin penetration of inorganic NPs. Most of these approaches were physical methods. Yet, the use of chemical enhancers was also explored.

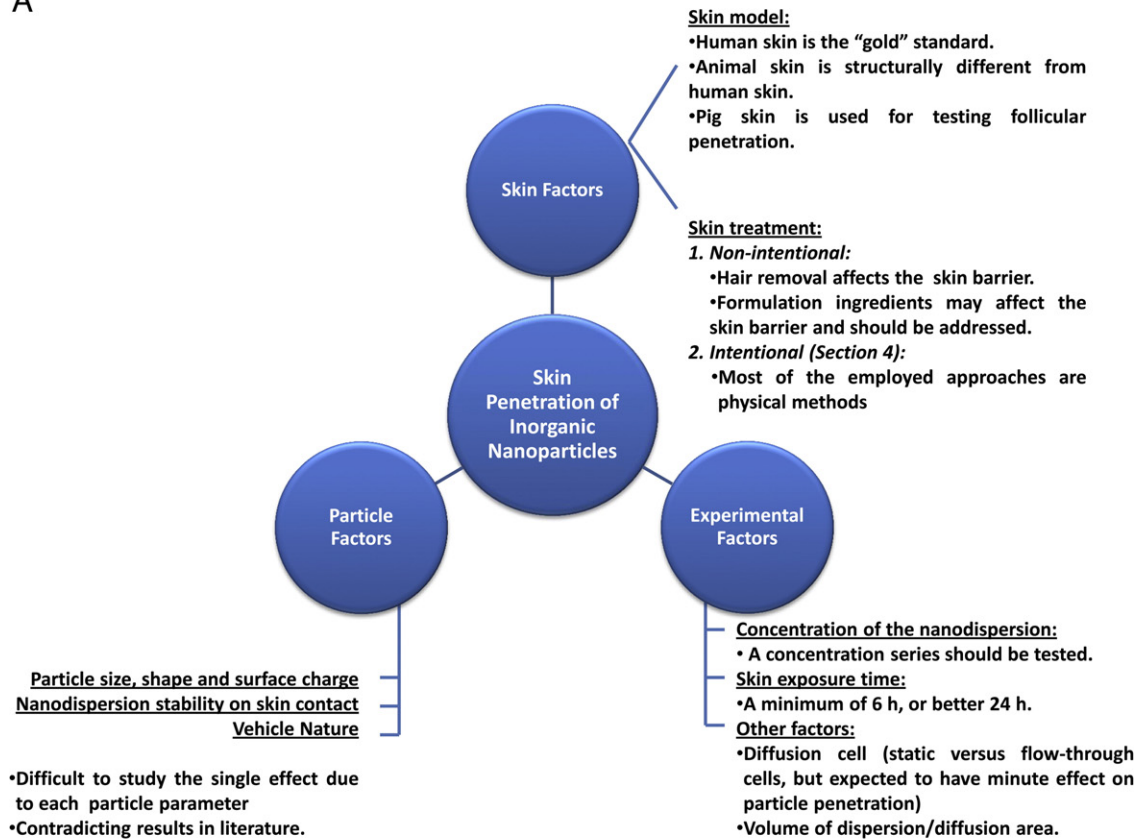
Physical/mechanical enhancement

UV exposure

UV radiation is known to have a deleterious effect on the skin barrier function by causing biophysical and morphological changes of the SC lipids.⁸⁵ As mentioned earlier, inorganic NPs composed of TiO_2 and ZnO are currently used in sunscreen formulations.⁴ Therefore, studying particle penetration into the skin was of the utmost importance for these topically applied particles.^{74,86}

Increased penetration of QDs as model particles through damaged skin was observed, by the effect of UV radiation compared to intact mouse skin,^{74,86} although in both cases low levels of penetration were qualitatively detected. However, according to the authors, penetration was detected mostly in areas with defects in the SC or around hair follicles; they referred to this as another possible mechanism for the transport of particles in addition to the SC intercellular weakening effect of UV radiation.

A



B

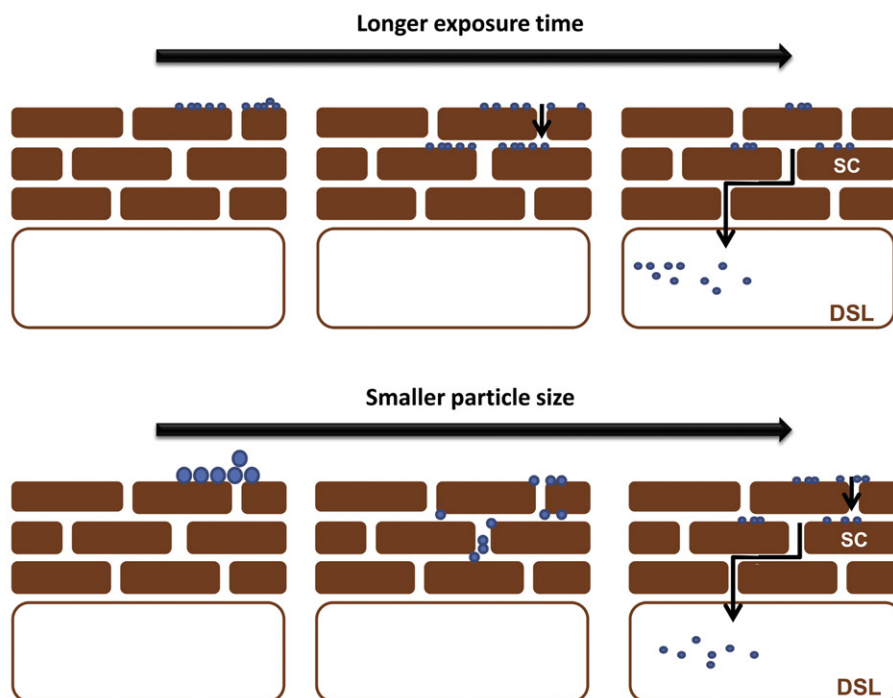


Figure 2. (A) A sketch showing the main factors affecting skin penetration of inorganic NPs according to the available literature: skin exposure time to NPs and the NP size. (B) The longer the exposure time and the smaller the particle size, the greater the probability of penetration of the stratum corneum (SC) into the viable deeper skin layers (DSLs). The limits for this latter effect have not been ascertained, with the literature showing overlapping size ranges for particles that penetrate the skin barrier and particles that do not.

These results should raise public concern regarding the use of NP-based formulations intended for topical use. However, one should also consider the effect of the surface properties of the particles applied on skin penetration when extrapolating these results to commercial sunscreens containing TiO₂ and ZnO NPs.

Hyperthermia

Upadhyay⁸⁷ explored the application of mild local hyperthermia for transdermal delivery of diphtheria toxoid (DT) vaccine. DT-conjugated QDs were applied to mice *in vivo* with concomitant application of pads for local hyperthermia. According to the author, this led to skin transport of the QD-labeled vaccine. However, it should be noted here that the evidence the author provided for skin penetration was based on top-view fluorescence images of QDs in skin specimens with no depth information. However, the unlabeled vaccine was found, when applied topically to mice, to induce an immune response as effective as intramuscular injection of the vaccine. Nevertheless, care should be taken construing the results of this study with respect to particle penetration.

Iontophoresis

Iontophoresis provides a mechanism to enhance the penetration of hydrophilic and charged molecules across the skin by application of constant current.⁸⁸ Similarly, a transdermal-delivery chip system was used to deliver negatively charged citrate-stabilized AuNPs across human skin.⁷⁶ At zero voltage, AuNPs were localized on the surface of the SC. On application of 6 V, AuNPs were shown to pass through the intercellular routes of the SC. Further transport to the DSLs was not shown. The results obtained from this particular study are quite questionable, however, as the experimental setup was not fully explained in terms of the particle size, surface charge, concentration, and volume of the applied dispersion.

Dermaportation

Dermaportation is a novel transdermal drug delivery technology that uses pulsed electromagnetic fields to enhance the transport across the skin. A preliminary experiment by Krishnan et al.⁸⁹ showed enhanced penetration of 10-nm AuNPs into epidermal human skin sheets. A potential mechanism proposed by the authors for enhanced transport is the formation of transient pores through which particles can diffuse more easily.

Sonophoresis

Sonophoresis, defined as the application of ultrasound, particularly at low frequency (20–100 kHz), has been shown to greatly enhance the skin permeability of a variety of drugs.⁹⁰ Based on this, low-frequency sonophoresis was also applied for enhancing the skin penetration of 20-nm QDs.⁹¹ Application of ultrasound on porcine skin increased the frequency of the formation of scattered and separated lacunar spaces in the SC, which are assumed to eventually lead to higher connectivity of these voids and formation of porous networks within the SC. This has induced significant transport of QDs through the lipid regions of the SC into the viable tissue. However, QDs were also found on a few occasions in the corneodesmosomes and even occasionally inside the corneocytes.⁹¹ Simultaneous application of this technique and sodium lauryl sulfate (SLS) induced a

similar but more pronounced effect on the SC ultrastructure and consequently higher QD penetration. The same combined approach was recently exploited by Seto et al.⁹² to enhance the skin penetration of smaller particles (~4.6-nm AuNPs) through porcine skin.

Tape-stripping and dermabrasion

Because the skin barrier function resides primarily in the SC, its total or partial removal by tape-stripping or dermabrasion can disrupt the skin barrier enhancing transport across the skin. Tape-stripping has been commonly used to enhance drug delivery across the skin, and to obtain information about the SC function. Moreover, tape-stripping has been proposed by the US Food and Drug Administration as a part of the standard method to evaluate the bioequivalence of topical dermatological dosage forms.^{93,94} Earlier, we also reported on the penetration of ~15-nm AuNPs through human skin that had been previously tape-stripped for complete removal of the SC⁷⁷ as compared to no penetration for untreated skin specimens. However, tape-stripping of the SC before application of inorganic NPs did not always lead to satisfactory results. For instance, ~4-nm,⁹⁵ 6-nm,⁹⁶ and ~37-nm⁶⁸ QDs could not penetrate tape-stripped human (20 tape-strips), rat (10 tape-strips), and mouse (5–20 tape-strips) skin, respectively. Abrasion is another technique that involves the removal or disruption of the upper skin layers to facilitate the skin permeation of several drugs.^{97–99} Dermabrasion is often used in clinical practice by dermatologists as a facial resurfacing technique in the treatment of acne, scars, hyperpigmentation, and other skin blemishes.⁹⁷ Based on this, the effect of abrasion on skin penetration of rigid inorganic NPs was examined. Dermabrasion of excised rat skin⁹⁶ and mouse (*in vivo*)⁶⁸ resulted in skin penetration and permeation of 6-nm and 37-nm QDs, respectively, whereas no penetration was reported through intact skin in these studies.

Skin flexion and massage

The impact of mechanical stress on the barrier function of the skin with regard to skin penetration of inorganic NPs was further examined via flexion and massage. Skin flexion is a method that simulates flexing movements such as repetitive wrist bending. Zhang and Monteiro-Riviere⁹⁶ studied the effect of mechanical flexion on skin penetration of ~6-nm QDs. However, no penetration was reported after flexing the skin on an automated apparatus for 60 minutes. A more clinically acceptable manual approach, massaging for a short time (5–10 minutes), was later explored by Gratieri et al.⁹⁵ to drive QDs to penetrate into the DSLs of human skin. However, QDs could be found in the DSLs only after massaging of tape-stripped skin for 10 minutes.

Ballistic bombardment

Ballistic bombardment with particles, a needle-free technique of targeting cells within defined layers of the viable epidermis, was used by Kendall et al.¹⁰⁰ A hand-held supersonic device was used to impact porcine skin at a high rate with submicron AuNPs, 900 ± 600 nm diameter, resulting predictably in skin penetration. Environmental relative humidity and temperature were shown to affect particle penetration using this method.

Chemical enhancement

Chemical permeation enhancers are defined as agents that promote drug diffusion through the SC and the epidermis. These agents have been extensively studied and used as enhancers in favoring transdermal drug permeation. They work mainly by temporarily reducing the barrier function of the SC allowing for more drug transport. However, their efficacy with regard to the delivery of high-molecular-weight drugs remains somehow limited.¹⁰¹ Few studies have focused on the use of these enhancers for skin delivery of inorganic NPs. Kuo et al¹⁰² studied the effect of chemical enhancers, oleic acid and ethanol, on the penetration of ~10-nm ZnO NPs through mouse skin. They showed enhanced transport values for NPs in the presence of oleic acid, ethanol, and oleic acid–ethanol mixture. Gopee et al⁶⁸ have also pretreated mouse skin with acetone but observed no penetration of QDs. More recently, we investigated the penetration of NPs through human skin in the presence of some selected chemical enhancers from different classes, urea (amides), SLS (anionic surfactant), polysorbate 80 (nonionic surfactant), and dimethyl sulfoxide.⁸² The NPs-vehicle interaction and the stability of the NPs in the applied vehicle were shown to be a critical parameter in transdermal delivery of NPs.

As a conclusion so far, many studies have focused on treating the skin physically or chemically for enhancing the skin penetration of inorganic NPs based on the previous knowledge and understanding of transdermal drug delivery. However, some of these have shown contradictory results on using the same enhancing approach. In other cases, unsatisfactory results were obtained. This could be again attributed to the different experimental setups used in different studies. Nevertheless, the basic knowledge on the potential mechanism is still somehow missing or at least not yet clear.

Mechanism of skin penetration

The exact contribution of the relevant parameters for potential skin penetration of inorganic NPs is still unknown. Studying the skin architecture could provide a possible explanation for the skin penetration of NPs. The intercellular lipids in the SC arrange themselves in a head-to-head and a tail-to-tail manner. The lipophilic pores are formed by tail-to-tail configuration of the lipids. On the other hand, the aqueous pores are hydrophilic regions delimited by lipid heads.¹⁰³ Although the aqueous pores provide a possible route for penetration of hydrophilic penetrants, the penetration of water and polar molecules is reported to be poor.¹⁰⁴ Based on this, one could question whether the penetration of inorganic NPs is dependent on these pores. Nevertheless, from a purely dimensional point of view, agents smaller than the diameter of either the lipidic intercellular route or aqueous pores can penetrate the SC into the DSLs. Even larger agents may enter the skin appendages (hair follicles and pilosebaceous pores or sweat gland pores) but still have to penetrate the respective tissue.¹⁰³

In a study by Zhang et al,¹⁰⁵ transmission electron microscopic (TEM) examination showed the localization of most PEG-coated QDs in the intercellular lipids of the outermost SC layers, leading these workers to conclude that NPs penetrate the skin via the intercellular pathway. They suggested theoret-

ically that as the outer PEG coating is a soft coating, it thereby allows the particles to squeeze through the intercellular spaces. In contrast, Lee et al¹⁰⁶ showed that on application of iron oxide NPs (4.6–10 nm) on skin after superficial skin incision, 1 μ m width, particles were found to distribute in both the intercellular and intracellular spaces of the SC and viable epidermis near the area of incision, but only in the intracellular spaces of the viable epidermis at longer distances. Recently, we studied the effect of weakening the barrier function of human skin by removal of the epidermal lipids or even total removal of the SC via tape-stripping.⁷⁷ Citrate-stabilized 15-nm AuNPs could not penetrate through intact human skin. Removal of the epidermal lipids resulted in penetration of the tested AuNPs, indicating that the intercellular lipids are the main barrier for skin penetration of NPs. Complete removal of the SC resulted, however, in about 28-fold further increase in penetration into DSLs. This would rather indicate that the barrier function of the SC to particle penetration relies not only on the intercellular lipids but on the whole microstructure of the SC with its tortuous intercellular aqueous and lipidic channels.⁷⁷

Finally, the follicular pathway is also a possible mechanism for skin penetration of inorganic NPs; however, the skin appendages occupy only a small fraction (about 1/1000) of the entire skin surface.¹⁰⁷ It has been hypothesized by Lademann et al¹⁰⁸ that the hair and the hair follicle might act as a pumping system pushing the NPs into the hair follicles when the hair is moving (in vivo). Hair movement could be simulated in vitro by massaging excised skin. Based on this, medium-sized NPs (~400–700 nm) could diffuse deeper into the porcine hair follicles rather than smaller or larger particles, allowing for selective follicular targeting by size modification.^{108,109} Lekki et al⁶⁰ have studied the possible role of the follicular pathway in the percutaneous uptake of TiO₂ NPs of 20 nm width and 100 nm length. Although particles were observed as deep as ~400 nm inside the follicle, no particles were observed in the surrounding viable tissue. Overall, NP penetration into the appendages is apparently not sufficient for increased skin penetration.

Besides studying the mechanism of skin penetration of inorganic NPs, it is important to know whether NPs potentially penetrate the skin only as a result of the “nano” effect (i.e., passive penetration) or under the influence of other factors.

Penetration of inorganic NPs through human skin: dissecting the nano effect

Whether particle penetration is studied for risk assessment or drug delivery purposes, the basic knowledge of the underlying mechanisms is an essential aspect. For NPs the size is the most prominent parameter. Hence the contribution and importance of the particle size has always faced several difficulties.

Following in vivo studies on human volunteers (difficult to perform), excised human skin is regarded as the “gold standard” for in vitro skin penetration studies, especially in human dermal risk assessment.⁶⁹ Therefore, the reported data using human skin was carefully analyzed in an attempt to arrive at a conclusion about the feasibility of skin penetration by NPs in real-case scenarios. To our knowledge, there are 22 different studies

investigating human skin penetration by inorganic NPs. Of these, 14 studies reported no particle penetration into the DSLs using either in vitro^{45–50,53,54,60,61} or in vivo^{46,48,51,54,76} human skin, as well as human skin grafted in severe combined immune-deficient mice.^{61,62} In three other studies, skin penetration was induced only by skin treatment such as massage of tape-stripped skin,⁹⁵ dermaportation using pulsed electromagnetic field,⁸⁹ or the use of chemical penetration enhancers.⁸² For the remaining studies, the skin was not treated physically or chemically before or concomitant with the application of the particulate formulations. However, the applied nanodispersion included some ingredients used in the synthesis of NPs that could have an effect on the skin integrity, hence favoring particle penetration. For instance, some particles were coated with trimethylammonium hydroxide⁸⁴ (corrosive to skin) or docusate sodium⁸⁴ (anionic surfactant), or dispersed in absolute alcohol (99%).^{110,111} The effect of formulation ingredients such as dodecanethiol, *tert*-butylamine-borane, and thioglycolic acid (possible skin irritants), and lecithin and cetrimide (penetration enhancers) were highlighted in our recent study.⁷⁷ Even if the amounts used do not allow the skin penetration of NPs, this should be regarded as a contributing factor. In the latter study⁷⁷ we thoroughly investigated the effect of toluene used as a vehicle for some applied AuNPs, showing that it has an influence on the penetration of NPs. As a conclusion, it is very difficult to dissect a single effect of bringing inorganic materials to the nanorange on their behavior with the skin barrier. This is presumably due to an interplay of multivariate factors including the physicochemical attributes of the NPs as well as the formulation, environmental, and mechanical factors. The challenge would be to have a system that is tunable in size in the respective range of interest, providing the same chemistry and hence the same properties regarding dispersibility and skin interaction.

Qualitative and quantitative analysis of inorganic NPs in the skin

The rapid development of sensitive analytical techniques in the past decades has enabled researchers to monitor and accurately quantify the amount of drugs present in the skin after penetration/permeation experiments, and study the factors that either hinder or enhance their penetration. Among these techniques, high-pressure liquid chromatography provides a convenient method with a suitable limit of detection for accurate drug quantitation. On the other hand, quantitation of NPs often represents a great analytical challenge; the sparse concentration of NPs able to penetrate the skin with regard to the detection limit, as well as the integrity of the particulate nature, limits the available techniques or requires combination of at least two approaches of the available techniques. Table 2 lists the currently employed techniques with some examples from the literature.

As seen in Table 2, so far monitoring skin penetration of NPs is mostly based on qualitative microscopic visualization. This includes histology, scanning electron microscopy (SEM), TEM, fluorescence microscopy, and confocal and multiphoton microscopy. Of all these methods, development of both confocal and multiphoton laser scanning microscopy was considered a great

addition to the field, allowing researchers to obtain three-dimensional information on the distribution of NPs in different skin layers via optical sectioning. This would avoid artifacts due to mechanical sectioning and sample preparation. However, this is limited by the loss of laser power and the loss of resolution with depth inside the skin tissue.^{112–114}

Further attempts were made with the aim of establishing a quantitative approach that would allow for a better understanding of skin penetration of NPs and provide a sound scientific basis for diverse biomedical applications and health risk assessment. This involves adopting quantitative analytical methods such as inductively coupled plasma optical emission spectroscopy, inductively coupled plasma mass spectroscopy, and atomic absorption spectroscopy. However, these techniques suffer the disadvantage of not detecting the particles themselves but their elemental composition. This would raise doubts whether these analyzed atoms or ions originate from the NPs themselves or from raw salts or chemical ingredients used in particle synthesis. This is in addition to possible interference from trace elements in biological materials (skin), such as zinc. Other approaches involved intensity measurement of fluorescence⁸⁷ and confocal⁹⁵ images or even manual counting of the number of fluorescent spots per field in confocal images.¹¹⁵ Recently, we established a combined multiphoton imaging–pixel analysis approach for semiquantitation of AuNP populations in different skin locations in terms of pixels, from which the weighed number of particles could be calculated.¹¹⁶ These values could be used to estimate the number of AuNPs penetrating into the SC and DSLs in the same skin penetration experiment and correlate data among different experiments. This latter method offers a relatively higher degree of accuracy and more freedom in selecting the optical settings; this is discussed in detail elsewhere.¹¹⁶ However, an in vivo noninvasive quantitation would be the final goal for any analytical technique.

This leads us to an auxiliary question: what would be a good model for studying skin penetration of NPs? From the analytical perspective, away from the clinical significance (mentioned earlier), inorganic NPs—especially QDs and gold and silver NPs—could be tracked inside the skin more effectively than polymeric particles. This represents a further reason why most of the studies concerned with skin penetration of NPs were conducted using inorganic particles rather polymeric ones. Another technical reason for this is the relative ease of preparing inorganic NPs with respect to polymeric NPs with a size <100 nm down to ~4 nm.

Standpoint and recommendations for future directions

Applications of inorganic NPs in pharmaceutical and biomedical fields have been established and are increasing progressively. Yet, the behavior of NPs with respect to the skin barrier is still in question with several conflicting results reported in the literature. In an attempt to solve this dilemma some points should be taken into consideration as recommendations for future investigations:

- Human skin should be used as the first-choice “gold standard” skin model for in vitro penetration experiments.

Table 2

Analytical methods used for monitoring particle penetration in the skin barrier

Analytical method	Examples from literature	Comments
Microscopic visualization		
Light microscopy of stained skin samples	73,100	Advantage: easy technique Disadvantage: artifacts on staining and mechanical sectioning
SEM	48,55,84	Advantage: high resolution Disadvantage: artifacts on mechanical sectioning
TEM	58,61,67,76,86,91,106,119	Advantage: high resolution for electron-dense materials Disadvantage: artifacts on mechanical sectioning
Fluorescence microscopy	74,87	Advantage: higher selectivity, availability Disadvantage: no depth information
Confocal laser scanning microscopy	68,80,86,91,95	Advantage: optical sectioning Disadvantage: loss of laser power with depth in the skin specimen, expensive
Multiphoton laser scanning microscopy	48,77,78,82,102,114,116	Advantages: intrinsic optical sectioning, less scattering by the tissues, and less phototoxicity than confocal Disadvantage: loss of laser power with depth in the skin specimen, expensive
MPM-FLIM	50,78,89	Advantages: can study the effect of NPs on skin metabolism by measuring autofluorescence of the endogenous fluorophores without the need of extrinsic labels Disadvantage: loss of laser power with depth in the skin specimen, more expensive
Nuclear imaging	47,120 (ion microscopy and autoradiography) ⁶⁰	Includes particle-induced x-ray emission, scanning transmission ion microscopy, and Rutherford backscattering Disadvantages: determination of the elemental composition of the particles, not the particle themselves, thus an interference possibility
Other analytical techniques		
ICP–optical emission spectrometry	66,84	Advantage: quantitative techniques
ICP-MS	45,55,58,68,92	Disadvantage: determination of the elemental composition of the particles, not the particles themselves, thus an interference possibility
Atomic absorption spectroscopy	57,59,110,111	

ICP-MS, inductively coupled plasma–mass spectrometry; MPM-FLIM, multiphoton microscopy–fluorescence-lifetime imaging; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

In parallel, more studies should be conducted on finding a correlation between in vitro and in vivo animal experiments and human studies with regard to penetration for several particles of different physicochemical parameters. This would allow better prediction of in vitro absorption when using these validated animal models.

- At the level of the experimental setup, development of a more-or-less “universal” model with technical guidelines for testing skin penetration/permeation of NPs is required. This also involves incubation times and concentration, which are critical for sufficient and detectable penetration.
- Both the experimental conditions and the characteristics of the NPs should be fully described; publications with missing information such as size have occasionally been published.
- The physical state of the applied nanodispersion, whether composed of individual particles or aggregated ones, should be carefully analyzed in parallel with the skin penetration/permeation study rather than solely depending on the primary size of the NPs.
- Formulation ingredients should always be considered when discussing the results of the penetration/permeation experiments. Similarly, skin preparation before the experiment, such as shaving of the hair, should be considered as a critical parameter influencing the barrier function of the skin model and thus the results of the penetration/permeation experiment.
- Last but not least, data obtained should be well presented to allow the reader to extract the same conclusion made by the authors. For instance, Upadhyay⁸⁷ has reported penetration of a DT-QD conjugate through mouse skin in vivo using local thermia. However, only images captured by conventional fluorescence microscope were presented, with no depth information. As another example, the fluorescence-lifetime imaging microscopy–multiphoton images shown by Krishnan et al.⁸⁹ for AuNPs in human epidermis are also

somehow unclear. It is not stated by the authors whether these images do show depth information of AuNPs in the SC and the underlying viable epidermal layers or not. This would unfortunately leave the reader in a state of uncertainty about the reported results.

Nevertheless, investigation of the skin penetration/permeation of NPs, especially inorganic NPs, with the aim of designing ideal topical/transdermal carriers or for health risk analysis, is a very recent area of research that holds great promise especially in development of new analytical approaches and higher sensitivity techniques that allow tracking and quantitation of minute concentrations of NPs present in the skin. A further challenge facing future studies, however, could be the ability to dissect the nano effect on NP penetration through the skin barrier, as the resultant penetration indicated in several studies was not exclusively due to bringing the material into the nanosize range, but also due to other concomitant formulation and environmental factors, in addition to the barrier state of the skin.

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