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XXVII CICLO DEL DOTTORATO DI RICERCA IN
SCIENZE E TECNOLOGIE CHIMICHE E
FARMACEUTICHE

IN VITRO AND IN VIVO EVALUATION OF SILVER
NANOPARTICLES PENETRATION THROUGH
HUMAN SKIN

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ABSTRACT

The skin is one of the larger organs of the human body and plays an important role in water homeostasis, having sensorial properties, structural functions and acting as a primal barrier (Blank et al, 1984). It can be an important route of uptake for many chemicals. Percutaneous absorption has been debated in several studies since the last century, but recently has gained new relevance due to the advent of the “nanoparticles world”. Human exposure to nanoparticles takes place both from anthropic and natural sources. Since nanoparticles have dimensions compatible with the dermal route it is important to evaluate the possibility of uptake under real life scenarios.

Silver nanoparticles (AgNPs) are increasingly applied to a wide range of materials for biomedical use since AgNPs enable a large release of silver ions that are responsible for a broad spectrum of antimicrobial activity. These materials are usually applied directly to human skin, in most cases to impaired skin, which has a reduced barrier function. Consequently this could lead to enhanced systemic uptake potentially leading to adverse effects.

The main objectives of this thesis are: (i) evaluating the penetration of silver from different AgNP materials under exposure scenarios which are as close as possible to the “in use” scenario; (ii) definition of the experimental factors which might influence the results e.g. storage of the skin; (iii) optimization of the analytical methods for the measurement of silver in different biological samples.

It is known that silver can permeate the skin, both intact and damaged; however, there are no data available on silver permeation through skin grafts commonly used in burns recovery. Therefore in this thesis silver penetration was evaluated using fresh, cryopreserved, and glycerol preserved human skin grafts after exposure to a suspension of AgNPs in synthetic sweat in a Franz diffusion cell apparatus for 24 h. Silver permeation profiles revealed a significantly higher permeation through glycerol preserved skin compared with both fresh and cryopreserved skin: 24-h silver flux penetration was 0.2 ng cm\(^{-2}\) h\(^{-1}\) (lag time: 8.2 h) for fresh skin, 0.3 ng cm cm\(^{-2}\) h\(^{-1}\) (lag time: 10.9 h) for cryopreserved skin, and 3.8 ng cm\(^{-2}\) h\(^{-1}\) (lag time: 6.3 h) for glycerol preserved skin. Permeation
through glycerol preserved skin is significantly higher compared to both fresh and cryopreserved skin. This result might have important clinical implications for burns treatment with products containing AgNPs. Another important result is that permeation through cryopreserved and fresh skin does not differ significantly. This finding justifies the use of cryopreserved skin in *in vitro* experiments.

Once the permeation of silver through human skin grafts had been evaluated and a suitable skin storage protocol found for in vitro studies, the second objective of this thesis was to characterize the silver species released from commercial Ag-textiles and to determine the *in vitro* percutaneous absorption of silver released. A preliminary screening of eight different Ag-textiles was performed in order to choose, for further studies, materials with significative release of silver and whose use could have a relevant social impact. The materials selected were two different wound dressings and a textile garment for children affected by atopic dermatitis. The *in vitro* percutaneous penetration was determined soaking three samples of each material in artificial sweat contained in the donor compartments of Franz cells. The characterization of silver species in the textiles was made by Scanning Electron Microscopy with integrated Energy Dispersive X-Ray spectroscopy (SEM-EDX) and Atomic Force Microscopy (AFM).

The concentration of silver in soaking solutions and in the skin was determined by an Electro Thermal Atomic Absorption Spectrometer (ET-AAS) and by Inductively Coupled Plasma Mass Spectrometer (ICP-MS).

All investigated materials contained silver nanoparticles of different size and morphology and, after soaking, the presence of silver chloride clusters on the textile surface was detected. Released silver concentrations in the bathing solution ranged from 21 to 104 µg/g (w/w). Silver and silver chloride sub-micrometrical aggregates were identified both in epidermis and dermis. This result suggests that precipitation phenomena occur in the skin. Moreover silver detection in all skin strata could lead to systemic absorption. Therefore, it would be important to evaluate also long-term and
repeated exposure. Since no significant differences were found between the three materials tested in *in vitro* conditions, the silver pajama was selected for further *in vivo* studies.

Data on *in vivo* uptake of Ag after dermal exposure are limited partly because of the lack of suitable analytical approaches for the determination of Ag in biological matrices. A further aim of this thesis was the development of an analytical approach to evaluate the *in vivo* uptake of silver after wearing the nanosilver garment. After *in vivo* dermal exposure to Ag containing textile material under "in use" exposure scenarios, the outermost layers of the skin (*Stratum Corneum, SC*) were sampled by using adhesive tapes. Different extraction and dissolution procedures of Ag from biological samples have been evaluated; the extracted solutions were analyzed by ICP-MS. The developed method results in a limit of detection (LOD) of 2 ng Ag per removed *SC* layer. The method allows the measurement of the Ag concentrations at different depths of the *SC* enabling the deduction of the percutaneous penetration kinetics. The sensitivity of the method permits measurement of the ultra-trace concentrations of Ag in urine before and after dermal exposure; LOD is 0.010 µg Ag / L urine.

The approach described above was then used to determine *in vivo* percutaneous penetration of silver after repeated dermal exposure in healthy individuals and patients with atopic dermatitis. Furthermore the inflammatory effect of silver in the skin after dermal exposure to silver was assessed. Healthy control subjects (n=15) and patients with mild and moderate atopic dermatitis (AD) (n=15) wore respectively a sleeve containing 3.6% silver and a placebo (i.e. silver free) sleeve on their lower arms for 8 hours during 5 consecutive days. The percutaneous penetration has been deduced from the silver concentration-time profiles in the *SC* layers collected by adhesive tape from the forearm skin after the 1<sup>st</sup> and 5<sup>th</sup> exposure. Furthermore the strips were analyzed by SEM-EDX and AFM to evaluate the presence of silver aggregates and nanoparticles eventually penetrated. Systemic uptake was assessed by determining silver concentrations in urine samples collected before and after five days of exposure. Inflammatory response was assessed by comparing the *SC* levels of IL-1α and IL-1RA between exposed and non-exposed skin sites after five days of wearing the sleeves. The silver concentration in the tape strips and urine samples was determined by ICP-
MS as previously indicated. Steady state flux into the skin in healthy subjects and AD patients was respectively 2.3 (1.2-3.8) and 2.0 (0.8-4.1) *10^{-6} mg Ag/cm^2/h. On the tape strips sampled from silver exposed arms, silver aggregates with a wide dimensional range were found. SEM-EDX revealed the presence of silver aggregates in the range of 150-2000 nm in all the strips sampled, with a decreasing number starting from the outer layer to the inner one. AFM revealed similar aggregates and highlighted structural differences between AD and healthy subjects. No silver was detected on the strips sampled from the placebo. EDX revealed that some of the silver aggregates contained also sulphur and chloride. The urine silver concentrations showed no increase after exposure to silver either in healthy individuals or AD patients. Furthermore, exposure to silver did not lead to changes in the profiles of IL-1\(\alpha\) and IL-1RA.

The presence of aggregates with sub-micrometrical dimensions is probably due to in vivo precipitation of silver ions permeated through the SC and to aggregation of permeated nanoparticles. The presence of sulphur in aggregates suggests the binding of silver to proteins i.e. penetrated Ag ions which have high affinity for SH group will react with these functional groups where they are present in the SC proteins. AFM revealed aggregates partly covered by lipidic layers suggesting an intercellular pathway. Interaction of Ag with SC proteins and formation of aggregates might facilitate formation of a reservoir of Ag in the SC layers. Aggregates might slowly release Ag, so the actual exposure time might be longer. On the other side, the size of the aggregates is too large for diffusion and they will be removed from the skin by normal desquamation, so the formation of the aggregates might be also advantageous as it decreases the absorbed amount of Ag.

In vivo data on the percutaneous penetration and excretion of silver in urine revealed that dermal absorption of silver after the wearing of the silver containing sleeve is lower than the current reference dose as proposed by the US Environmental Protection Agency (EPA). Dermal exposure to the nanosilver garment did not lead to altered expression of inflammatory IL-1 cytokines in the skin.
In this thesis a maximum exposure of five days has been evaluated. The in vivo data obtained under this scenario revealed that dermal absorption of silver after wearing the garment is low and not likely to lead to systemic toxicity. Otherwise these findings highlight the need to evaluate both the systemic effects after a prolonged exposure in subjects with damaged skin and the fate of the silver species retained in time.

Riassunto

La cute è uno degli organi più estesi del corpo umano e gioca un importante ruolo nella regolazione dell’idratazione corporea, ha proprietà sensoriali, funzioni strutturali e agisce come prima barriera contro gli agenti esterni (Blank et al, 1984). Può costituire un’importante via di uptake per molte sostanze. L’assorbimento percutaneo è stato oggetto di studio in numerosi lavori fin dallo scorso secolo, ma ha recentemente riscosso nuovo interesse a causa dell’ascesa del “mondo delle nanoparticelle”. L’esposizione umana alle nanoparticelle può avvenire sia per cause antropiche che naturali. Dal momento che le nanoparticelle hanno dimensioni compatibili con quelle della via cutanea è importante valutare la possibilità di uptake in scenari di esposizioni reali.

Le nanoparticelle di argento (AgNPs) sono sempre più spesso applicate a un’ampia gamma di materiali a scopo biomedico, proprio perché sono in grado di rilasciare una considerevole quantità di ioni argento che sono responsabili di un’attività antibatterica ad ampio spettro. Questi materiali nanoparticellati sono solitamente applicati a diretto contatto con la cute umana, nella maggior parte dei casi a cute lesa con una ridotta capacità di agire da barriera. Soprattutto nel caso della cute lesa, questo tipo di esposizione potrebbe portare a un incremento dell’uptake sistemico di argento con potenziali effetti collaterali.

I principali obiettivi di questa tesi sono dunque: (i) valutare la permeazione dell’argento da parte di differenti materiali al nanoargento, simulando scenari di esposizione che siano il più possibile realistici; (ii) definire i fattori sperimentali che potrebbero influenzare i risultati degli esperimenti
in vitro, come ad esempio la metodologia di conservazione della cute; (iii) l’ottimizzazione dei metodi analitici per la quantificazione dell’argento in diverse matrici biologiche.

E’ noto dalla letteratura che l’argento sia in grado di permeare la cute, sia intatta che lesa; d’altra parte, non sono disponibili dati riguardo alla permeazione dell’argento attraverso le più comuni tipologie di cute utilizzate come impianti per la cura di ustioni gravi. Petanto in questa tesi la permeazione dell’argento è stata valutata, attraverso il metodo delle Celle a Diffusione di Franz, esponendo campioni di cute fresca, cute crioconservata e cute glicerolata a una sospensione di AgNPs in sudore sintetico per 24 ore. Studiando i profili della permeazione dell’argento nel tempo, risulta evidente una maggiore permeazione attraverso cute glicerolata: il flusso di permeazione dell’argento a 24-h è di 0.2 ng cm\(^{-2}\) h\(^{-1}\) (lag time: 8.2 h) per la cute fresca, 0.3 ng cm\(^{-2}\) h\(^{-1}\) (lag time: 10.9 h) per la crioconservata, e 3.8 ng cm\(^{-2}\) h\(^{-1}\) (lag time: 6.3 h) per la glicerolata. La permeazione attraverso cute glicerolata è significativamente più alta sia rispetto alla cute fresca che a quella crioconservata. Questo risultato potrebbe avere delle importanti implicazioni cliniche per il trattamento delle ustioni con prodotti nanoargento. Un ulteriore importante risultato è che la permeazione attraverso cute crioconservata non differisce significativamente da quella fresca. Ciò giustifica l’utilizzo di cute crioconservata nel caso di esperimenti in vitro.

Una volta valutata la permeazione cutanea attraverso i diversi modelli di cute e determinato quindi il modello più idoneo per gli studi in vitro, il secondo obiettivo di questa tesi è quello di caratterizzare il rilascio di argento da parte di alcuni tessuti al nanoargento, disponibili in commercio, e determinare l’assorbimento percutaneo in vitro dell’argento da essi rilasciato. E’ stato effettuato uno screening preliminare di otto diversi tessuti all’argento in modo da scegliere, per successivi studi, materiali che fossero in grado di rilasciare un quantitativo elevato di argento e il cui utilizzo avesse una certa rilevanza sul piano sociale. I tessuti selezionati sono stati due diverse garze per la cura di cute lesa (ustioni o tagli) e un pigiama ideato per bambini affetti da Dermatite Atopica. L’assorbimento percutaneo in vitro è stato determinato immergendo 3 pezzi di ciascun materiale nel sudore sintetico contenuto nelle celle donatrici di Franz. La
caratterizzazione dell’argento presente nei tessuti è stata effettuata mediante Scanning Electron Microscopy with integrated Energy Dispersive X-Ray spectroscopy (SEM-EDX) e Atomic Force Microscopy (AFM). 

La concentrazione dell’argento nelle soluzioni donatrici e nella cute è stata determinata mediante un Electro Thermal Atomic Absorption Spectrometer (ET-AAS) e un Inductively Coupled Plasma Mass Spectrometer (ICP-MS).

Tutti i tessuti analizzati contenevano AgNPs di diverse dimensioni e morfologia e, in seguito all’immersione in sudore, è stata rilevata la presenza di clusters di AgCl sulla superficie delle fibre. Le concentrazioni di argento in sudore sintetico raggiungevano tra i 21 e i 104 µg/g (w/w). Sono stati inoltre rilevati microaggregati di Ag e di AgCl sia nell’epidermide che nel derma utilizzati per l’esperimento. Le dimensioni di questi aggregati suggeriscono che la loro formazione avvenga a causa di fenomeni di precipitazione proprio tra gli strati cutanei. Inoltre, il fatto che l’argento sia stato trovato anche negli strati più interni della cute vascolarizzata (derma) suggerisce la possibilità di assorbimento sistemico dell’argento permeato. Alla luce di questi risultati sarebbe quindi opportuno valutare anche l’esposizione ripetuta e prolungata nel tempo. Poiché non sono state rilevate significative differenze tra i tre materiali testati, il pigiama al nanoargento è stato selezionato per la successiva sperimentazione in vivo.

I dati riguardanti l’uptake in vivo dell’argento in seguito a esposizione cutanea sono limitati, in parte a causa della mancanza di metodi analitici adeguati per la determinazione dell’argento in matrice biologica. Un ulteriore obiettivo di questa tesi è dunque lo sviluppo di un metodo analitico per la quantificazione dell’uptake in vivo su soggetti che abbiano indossato il tessuto al nanoargento. Campioni dello strato più esterno dell’epidermide (Stratum Corneum, SC) sono stati prelevati, mediante tapes adesivi, da volontari che avevano indossato il tessuto al nanoargento secondo scenari realistici. Diverse tipologie di estrazione dell’argento dai tapes adesivi sono state confrontate; le soluzioni estraenti sono state analizzate per la quantificazione dell’argento mediante ICP-MS. Il metodo descritto in questa tesi ha come limite di detezione (LOD) 2 ng di Ag.
per campione di SC. Il metodo permette di misurare la concentrazione di Ag a diversi spessori di SC permettendo di ricavare le cinetiche di permeazione dell’argento. La sensibilità del metodo permette inoltre di determinare la concentrazione dell’argento in ultra trace nelle urine dei soggetti prima e dopo l’esposizione cutanea (LOD=0.010 µg Ag / L in urina).

Il metodo sopra descritto è stato quindi applicato per determinare l’assorbimento percutaneo in vivo in seguito a ripetuta esposizione di soggetti sani e di pazienti affetti da dermatite atopica. Inoltre, è stato valutato l’effetto infiammatorio dell’argento permeato nella cute. Soggetti sani (n=15) e pazienti affetti da una lieve forma di dermatite atopica (n=15) hanno indossato una manica di tessuto contenente il 3.6% di argento, su di un avambraccio, e, sull’altro, un tessuto placebo (senza argento) per le 8 ore notturne, per 5 giorni consecutivi. La permeazione dell’argento è stata valutata analizzando l’andamento della concentrazione in funzione della profondità di SC prelevato dall’avambraccio, dopo la prima e la quinta esposizione. Inoltre, i campioni di SC sono stati analizzati mediante SEM-EDX e AFM per valutare la presenza di aggregati o nanoparticelle di argento eventualmente penetrate. L’uptake sistemico è stato verificato determinando la concentrazione di Ag nelle urine raccolte prima e dopo i cinque giorni di esposizione. Il quadro infiammatorio è stato valutato comparando i livelli di interleuchine IL-1α e IL-1RA nella cute tra siti esposti e non esposti dopo i 5 giorni di esposizione. L’argento è stato quantificato con i metodi descritti in precedenza. Il flusso di argento attraverso lo SC al raggiungimento dello stato stazionario in soggetti sani e nei pazienti era rispettivamente di 2.3 (1.2-3.8) e 2.0 (0.8-4.1) *10⁻⁶ mg Ag/cm²/h. Sui tape strips campionati dagli avambracci esposti all’argento, sono stati trovati aggregati di argento in un ampio range dimensionale. Il SEM-EDX ha rilevato la presenza di aggregati nel range 150-2000 nm in tutti i campioni prelevati, con un numero descrescente partendo dagli strati cutanei più esterni a quelli più interni. L’AFM ha confermato la presenza di questi aggregati e ha inoltre evidenziato le differenze strutturali tra i soggetti sani e quelli affetti da dermatite atopica. Non è stato riscontrato argento nei campioni derivanti dalla cute esposta al placebo. L’EDX ha rivelato che alcuni aggregati di argento
contenevano inoltre zolfo e cloro. I livelli urinari di argento non hanno subito variazioni significative in seguito all’esposizione né nei casi né nei controlli. Infine non sono state riscontrate differenze nei livelli di interleuchine in seguito all’esposizione al tessuto contenente nanoargento.

La presenza di aggregati con dimensioni sub-micrometriche è probabilmente dovuta a una precipitazione in vivo di ioni argento permeati attraverso lo SC e all’aggregazione delle nanoparticelle permeate. La presenza di zolfo negli aggregati è probabilmente dovuta alla chelazione dell’argento da parte dei tioli delle proteine nello SC. L’AFM ha inoltre mostrato la presenza di un sottile strato lipidico sulla superficie degli aggregati suggerendo una penetrazione attraverso gli spazi intercellulari. L’interazione dell’Ag con le proteine dello SC e la formazione di aggregati potrebbe facilitare la creazione di una riserva di ioni Ag⁺ negli starti cutanei. Gli aggregati potrebbero lentamente rilasciare Ag, rendendo l’esposizione effettiva più lunga. D’altra parte, la misura degli aggregati è troppo grande perché possano ulteriormente diffondere e potrebbero venire rimossi dai normali processi di desquamazione; perciò la formazione degli aggregati potrebbe anche essere svantaggiosa per un ulteriore assorbimento di Ag.

I dati riguardanti l’assorbimento percutaneo in vivo e l’escrezione urinaria di Ag mostrano che il quantitativo di argento assorbito per via cutanea (secondo questo scenario di esposizione) è inferiore alla dose di riferimento corrente proposta dall’US Environmental Protection Agency (EPA). Inoltre, l’esposizione cutanea al tessuto contenente nanoargento non ha alterato il quadro infiammatorio delle citokine nella cute.

In questa tesi è stata testata un’ esposizione che non supera i 5 giorni consecutivi. I dati ottenuti secondo questo scenario espositivo hanno rivelato che l’assorbimento cutaneo dopo aver indossato il tessuto in esame è basso e non dovrebbe realisticamente portare a tossicità a livello sistemico. D’altra parte questi risultati evidenziano la necessità di valutare sia gli effetti sistemici in seguito a un’esposizione più prolungata nel tempo, soprattutto in soggetti con cute danneggiata, sia il destino nel tempo delle forme di argento trattenute nella cute.
1. BACKGROUND

1.1. The barrier property of the skin

The skin can be divided into three main components: the stratum corneum (SC), the epidermis and the dermis. The SC is responsible for two of the major functions of the skin: it forms the main permeability barrier and maintains water homeostasis. The epidermis has a thickness of typically 50–150 µm and is principally constituted of keratinocytes which differentiate as they migrate toward the SC (Fig.1). The dermis, with a thickness of 600–3000 µm, is a strong connective tissue, mostly comprised of collagen, with a variety of specialized structures (Rawlings et al, 1994).

**Fig.1.** Typical structure of the epidermis and critical steps in the formation of the stratum corneum. Adapted from Rawlings AV, Scott IR, Harding CR, Bowser PA. Stratum corneum moisturization at the molecular level. J Invest Dermatol 1994: 103: 731–740

1.1.1. The Stratum Corneum
The human SC is made of ~10 to 20 cellular layers and is ~10 µm thick when dry, although it may swell upon hydration to several times this value (Marks, 1994). The SC consists of terminally differentiated, anucleated keratinocytes that are called corneocytes. These cells have keratin as the major protein, cross-linked protein envelope, and an extracellular lamellar lipid network comprised mostly of ceramides, free fatty acids and cholesterol. It is possible to describe the keratinized cells as a sponge that holds a surface film material derived from sebaceous and sweat glands that blend in with the products of the keratinizing epidermis. This is an extremely complex substance, hydrophilic and lipophilic, homogenized by cholesterol and wax alcohols. The surface film, whose proper name is natural moisturizing factor (NMF), contains proteins (mostly derived from filaggrin), carbohydrates, lipids, acids, salts and their derivatives. Initially it was thought that the only role of NMF was the property of preventing the loss of water and acting as humectant. At the present time it is known that NMF, by maintaining free water in the SC, also facilitates critical biochemical events (Awlings and Arding, 2004; Burke et al, 1966).

The corneocytes are held together by two proteins junctions, lectin and corneodesmosomes, that maintain cellular shape and regular packing. Desquamation is due to the eventual degradation of the corneodesmosomes by proteolytic enzymes (Mendelsohn et al, 2006). The SC is usually depicted by a “bricks and mortar” model (Fig.2). The corneocytes (bricks) are embedded in a mixture of fatty acids, ceramides, lipids and cholesterol (mortar). On the right side of Fig.2 is shown the “domain mosaic model”: a more elaborated model that considers supramolecular organization of the mortar envelope (Forslind, 1994).
Fig. 2. Left: a cartoon of corneocyte distribution within the lipid matrix of the stratum corneum. Elongated corneocytes (thickness \(\sim 0.5-0.8 \, \mu m\)) overlie each other and together with the stacked bilayers of the lipid matrix provide a tortuous route for the permeation of exogenous substances. Right: a schematic of the conformationally ordered (bottom) and disordered (top) lipid phases thought to simultaneously exist in the lipid matrix of the stratum corneum. R. Mendelsohn et al. / Biochimica et Biophysica Acta 1758 (2006) 923–933

In this model the lipids are mainly segregated into ordered orthorhombic and hexagonally packed lipid domains (depicted in the right hand panel of the figure). Around this ordered domain, there are “grain boundaries” where lipids with a shorter chain are located in a conformationally disordered phase (liquid crystalline).
It is thought that hydrophobic species may diffuse in the disordered regions. Structural transformations of the lipids located in these regions, induced by permeation enhancers, do not affect the structure of the more ordered phases. R. Mendelsohn et al. / Biochimica et Biophysica Acta 1758 (2006) 923–933

1.2. The percutaneous absorption theory

As defined in Environmental Health Criteria 235, percutaneous absorption is a global term that describes the transport of chemicals from the outer surface of the skin both into the skin and into systemic circulation. The SC represents the main physical barrier for any substance permeating throughout the skin. This means that passing the SC represents the rate limiting step for the diffusion process. It is thought that the permeation through the SC occurs mainly by passive diffusion via three possible routes: the transcellular, the intercellular and the appendage routes (Fig.3). Nanoparticles are thought to penetrate the skin through two possible routes: the intercellular route, following the lipid channels between the corneocytes to deeper skin layers and the appendage route (hair follicles, sweat glands).
Fig. 3. The three possible routes for skin penetration are here depicted: 1-A hair follicle, 1-B sweat glands, 2-A intercellular route, 2-B transcellular route.

**Inter-cellular route.** The intercellular route is the most used for most substances. The dimensional parameter limits the permeation through the lipid channels, whose diameters have been estimated by van der Merwe et al. (2006) to be 19 nm and by Baroli et al. (2007) to be 75 nm. The small molecules, and potentially also the nanoparticles, could freely move through these lipophilic channels but the diffusion rates depend on their chemical-physical properties (lipophilicity, molecular weight or volume, solubility and hydrogen bonding ability).

**Hair follicles.** Hair follicles cover only 0.1% of the human skin surface area, but because of their complex vascularization and deep penetration into the skin represent an important route. The penetration of solid particles (as liposomes and nanoparticles) through the follicular appendages has been demonstrated (Schaefer and Lademann, 2001). Penetration of particles of 300-600 nm through hair follicles is reported (Lademann et al, 1999; Lieb, 1992; Vogt et al, 2006 ), but factors other
than the dimensions seem to limit the penetration through this route. Lademann (2007) showed that
titanium dioxide particles did not penetrate beyond the follicles, but that follicles could be an
efficient reservoir for nanoparticle-based drug delivery.

Transcellular route.

Originally, the transcellular route was thought to be preferred by polar solutes. However
histochemical and theoretical evidences showed that diffusion through intercellular lipids was more
likely for most solutes. Recently there was a reaffirmation of the importance of the transcellular
route, even for lipophilic solutes, by Wang et al. (2009), but the transcellular route remains
controversial.

1.2.1 Theoretical aspects of diffusion

Fick’s first law describes the diffusion of compounds across a membrane (Crank, 1975):

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

The flux of a compound \( (J, \text{mass/cm}^2 \text{per second}) \) at a given time and position is proportional to the
differential concentration change \( \partial C \) over a differential distance \( \partial x \) (i.e. the concentration gradient
\( \partial C/\partial x \)).

Fick’s second law describes the concentration of a compound within a membrane:

\[ \frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \]
This is a derived form of the differential mass balance in the skin combined with Fick’s first law. This derivation is possible thanks to the following assumptions: that the compound does not bind, the compound is not metabolized, and its diffusion coefficient does not vary with position or composition (Crank, 1975).

Fick’s first law can be applied to describe the diffusion processes in the individual layers of the skin, which are treated as pseudo-homogeneous membranes (Scheuplein and Blank, 1971; Dugard, 1977).

For a membrane of thickness \( h \), the flux at steady state (\( J_{ss} \)) is given by:

\[
J_{ss} = \frac{D (C_1 - C_2)}{h} \quad \text{[Equation 1]}
\]

where \( C_1 \) and \( C_2 \) are the concentrations of the chemical in the membrane at the two faces (i.e. at \( x = 0 \) and \( x = h \)). \( D \) is an effective diffusion coefficient when this formula is applied to the stratum corneum. In this case, \( h \) is the thickness of the stratum corneum, and the concentration at \( x = h \) is zero or very small (which is sometimes called sink conditions). Also, the concentration of chemical at \( x = 0 \) is in local equilibrium with the vehicle (i.e. \( C_1 = K_m \cdot C_v \), in which \( K_m \) is the pseudo-homogeneous partition, or distribution, coefficient between the stratum corneum and the vehicle and \( C_v \) is the vehicle concentration).

Under these conditions, Equation 1 becomes:

\[
J_{ss} = \frac{D \cdot K_m \cdot C_v}{h} \quad \text{[Equation 2]}
\]

The steady-state flux across the skin is sometimes written in terms of the permeability coefficient (\( K_p \)) as follows:
\[ J_{ss} = K_p \cdot C_v \] [Equation 3]

Comparing Equations 2 and 3,

\[ K_p = K_m \cdot \frac{D}{h} \] [Equation 4]

The partition coefficient \( K_m \) is unitless, but it represents the ratio of concentrations in the stratum corneum and vehicle in units of mass/volume (Cleek and Bunge, 1993).

The steady-state flux \( J_{ss} \) and the permeability coefficient \( K_p \) can be extrapolated from an \textit{in vitro} experiment when the infinite dose conditions are applied. The flux gradually approaches a steady-state value (\( J_{ss} \)), whilst the cumulative amount penetrating the skin increases. Plotting the concentration of the penetrated substance as a function of time, the slope of the linear portion of the graph represents the steady-state flux \( J_{ss} \) (Scheuplein and Blank, 1971; Crank, 1975; Dugard, 1977). The time required for the permeation rate across a membrane to reach 95% of the steady-state value is approximately 2.3 times the lag time. The lag time (\( t_{lag} \)) is the time intercept of the linear portion of the graph in Figure 4.
Fig. 4. The steady state flux (Jss) and the permeability coefficient (Kp) can be obtained plotting the concentration of the penetrated substance per surface unit against time.

As indicated by Equation 3, Kp is the ratio of Jss and the vehicle concentration Cv. Thus, estimates of steady-state flux and permeability coefficients should include data only from times greater than the time to reach steady state. Including data for times before the steady state is established will lead to a false estimate, usually an underestimate, of the permeability coefficient and lag time (Shah, 1993; Schaefer and Redelmeier, 1996; Geinoz et al., 2004). In reality, depletion of the donor phase, the use of non-sink receptor conditions, and a deterioration of the skin over time can occur and result in inaccuracies in steady-state flux and lag time estimations.

The maximum flux ($J_{\text{max,ss}}$) of a solute through a membrane occurs for a pure solid or a saturated solution of a chemical in a vehicle when $C_2$ is 0 (Higuchi, 1960; Roberts et al., 2002). At equilibrium, a saturated solution of a chemical in a vehicle will be in equilibrium with the saturated concentration of solute in the stratum corneum ($S_{\text{sc}}$). The maximum flux $J_{\text{max,ss}}$ is therefore given by Equation 5, which is derived from Equation 1. It is to be noted that $J_{\text{max,ss}}$ is also related to the
permeability coefficient of a solute in a given vehicle $K_{p,v}$ and the solubility of the solute in that vehicle $S_v$.

$$J_{\text{max,ss}} = S_{sc} \cdot \frac{D}{h} = K_{p,v} \cdot S_v \ [\text{Equation 5}]$$

In principle, fluxes that are higher than maximum can be observed in intrinsically unstable systems, such as supersaturated solutions.

### 1.3. The nanoparticles and the skin

Nanoparticles, thanks to their peculiar properties, are increasingly applied to several fields such as textile, biomedical, renewable energy, food and agriculture, environment, electronics. The reason for the great achievement of nanoparticles in material sciences is the same that originates great alarm with regard to nanoparticles absorption by the human body. Since a nanoparticle has at least two dimensions in the range 1-100 nm (IUPAC), it’s easy to imagine that their uptake by the human body could be strongly increased compared to that of micrometric particles (Fig. 5). Nanoparticles could penetrate the human body via three possible routes: inhalation, oral and dermal uptake. Once absorbed, the potential interactions at the nanometric scale are unlimited.
Fig. 5. Comparison of different objects at the nanometric scale

Nanotoxicology, “is the study of the adverse effects of engineered nanomaterials (ENMs) on living organisms and the ecosystems, including the prevention and amelioration of such adverse effects.” (Oberdorster, 2010). This discipline has recently emerged and its importance is continuously increasing with several journals devoted to this topic. The main toxicological risks related to nanoparticles are due to the strong catalytic surface that they have. Thanks to their dimensions, nanoparticles could pass the cellular membranes and, since the nanoparticles surface is highly reactive, they can generate radical species increasing oxidative stress. Nanoparticles, moreover, could interact with the metabolic pathways and, once they have entered the cells, could disrupt the DNA double helix or bind to proteins and enzymes with a huge number of consequent potential side effects.

In the nanotoxicological field, the evaluation of dermal absorption has high relevance. Indeed, dermal absorption of nanoparticles is of interest for at least two reasons: on the one hand
penetrating the SC could be an important route for drug delivery (this is the case of nanocarriers and nanomedicine), on the other hand skin uptake of nanoparticles could lead to systemic absorption and adverse health effects.

### 1.3.1 The silver nanoparticles as topical agents

Silver nanoparticles (AgNPs) are a well known source of silver ions, which are responsible for a broad spectrum of antibacterial activity. For this reason AgNPs are increasingly applied to several materials (ceramic, polymeric, fibers, glass) to reduce the bacterial colonies and to avoid resistance mechanisms (Rai et al. 2009; Law et al. 2008; Silver et al. 2006; Kim et al. 2007; Thomas et al. 2007).

One of the major advantages of using silver as biocide is that it exhibits low toxicity to mammalian cells (Zhao and Stevens, 1998). The minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of AgNPs 5-10 nm towards *Staphylococcus aureus* ATCC25923, methicillin-sensitive *S. aureus* (MSSA), and methicillin-resistant *S. aureus* (MRSA) were examined in a study of Ansari et al. (2011). The values of MIC and MBC of Ag-NPS against all clinical isolates of MSSA, MRSA and *S. aureus* were found at very low concentrations of AgNPs (in the range of 12.5-50 µg/ml and 12.5-100 µg/ml, respectively). These results confirmed the previous findings of Fernandez et al. (2008). Martinez et al. (2008) and Ayala-Nunez et al. (2009) found slightly different concentrations but this is related to the dimensional parameter of the particles tested. Indeed, the antibacterial effect is known to be related to the ability to release ions and, therefore, to the dimensions of AgNPs.
1.4. Nanoparticles uptake: healthy and diseased skin

The percutaneous uptake of different kinds of nanoparticles has been evaluated both *in vitro* and *in vivo* mostly in case of intact human skin (Baroli, 2010; Labouta et al, 2013). Due to the great number of variables affecting dermal absorption of nanoparticles, the data found in literature are often contradictory.

1.4.1. Healthy skin uptake of nanoparticles

In literature several works on nanoparticles, polymeric or inorganic nanoparticles dermal absorption were found. Most papers suggest that NP only permeate the superficial layers of the SC, probably due to the lesser cohesion that outer corneocytes have (Labouta et al, 2013). Only a few papers showed that epidermal penetration and dermal absorption are possible (Baroli, 2010). Typically, epidermal permeation seems to be associated with some enhancing factors as, for example, in the case of mechanical stress, relatively harsh vehicles application or where the skin is artificially damaged.

1.4.1.1 Metal oxide nanoparticles and intact skin

Zinc oxide (ZnO) and titanium dioxide (TiO₂) are the most studied forms of metal oxide nanoparticles because of their wide use in sunscreen formulations. Many *in vitro* and *in vivo* studies
on TiO$_2$ and ZnO skin permeation are reported, showing in most cases no penetration of SC or the presence of particles only in the outer layers.

A recent review examined the disposition of metal oxide particles following application to healthy skin, concluding that transdermal penetration of intact particles does not occur (Lane, 2011).

1.4.1.2. Metal nanoparticles and intact skin

Among metal nanoparticles silver and gold are probably the most studied. Larese et al. (2007, 2009) evaluated the percutaneous absorption of silver nanoparticles (<50 nm) in human skin with Franz diffusion cells. The authors reported that silver nanoparticle absorption through intact skin was low but detectable; the presence of silver nanoparticles was only demonstrated in the lower layers of the SC. These results are consistent with those of Samberg et al. (2010): after 14 days of topical dosing (in vivo porcine skin) TEM confirmed that silver nanoparticles (<50 nm) could penetrate only the superficial layers of the SC.

Baroli et al. (2007) evaluated the permeation of iron nanoparticles (4.9–23.3 nm) in full-thickness human skin in vitro. Iron nanoparticles were found below the viable epidermis and within and proximal to the hair follicles, but the particles could not pass the skin barrier.

A study based on rat skin showed the penetration of different sized AuNP (15 nm, 102 nm and 198 nm) (Sonavane et al, 2008). Gold was found to permeate the skin with kinetics linked with the size of the applied particles. The 15 nm particles were found to aggregate by TEM in deeper skin layers, whilst the larger particles reach the viable epidermis and dermis. Similarly to these results Labouta et al. (2011) found that gold nanoparticles (6-15 nm) were able to diffuse into the skin, underlining both the effects of particle size and polarity. Graf et al. interestingly found that bigger gold particles (gold core particles of about 94-160 nm, with silica shells; silica particles of about 298 nm diameter, coated with a gold shell) were observed in the superficial layers of the SC and hair follicles. No
deeper penetration was observed. Another study, performed in in vivo conditions with rabbits, confirmed gold nanoparticles permeation. A study of Krishnan et al. (2010) showed that 10 nm gold particles were not able to penetrate human skin (in vitro). Otherwise Larese et al. (2011) found, in in vitro conditions, that gold nanoparticles (<15 nm) are able to penetrate and permeate the skin in greater amount than other kinds of nanoparticles.

1.4.2. Diseased skin uptake of nanoparticles

The risk that chemicals, pathogens, bacteria and particles penetrate into the human body increases whenever the barrier property of the skin is compromised. There could be different reasons for SC impairment as for example lesions, burns, dermatitis or environmental conditions such as dry weather.

According to the nanotechnology report of the EU Scientific Committee on Cosmetics and Non-Food Products (SCCNP), the data on skin penetration of nanoparticles through diseased skin are limited. There is a gap in the clinical literature since the penetration of nanoparticles has not been quantified in subjects with clinically relevant skin diseases like psoriasis and dermatitis. Hypothetically, nanoparticles could penetrate the lesions caused by these diseases more efficiently due to: altered SC, inflammation and increased keratinocyte turnover. Since an impaired skin could be exposed to nanoparticles even for therapeutical reasons, some important skin diseases will be shortly analyzed hereafter. Among the most common adult skin diseases are psoriasis (6.6%) and atopic dermatitis (6.9%) (Plunkett et al, 1999).
1.4.2.1. **Psoriatic skin**

Psoriasis is a widespread skin disorder due to a T-cell mediated autoimmune disease, leading to keratinocyte hyperproliferation. It is known that psoriasis has genetic predisposition that can be further aggravated by external factors, but the exact etiology of the disease remains unknown (Gadhially et al, 1996). In addition to incomplete keratinization and scaling dermatosis, an alteration of tight junctions (which are important elements of the epidermal barrier system) has been shown in psoriasis (Fig.6). This led to the hypothesis of an increased percutaneous absorption of nanoparticles as for example zinc oxide nanoparticles present in sunscreen formulations.

![Fig. 6 Comparison between healthy and psoriatic skin.](image)

A study of Prow et al. (2011) shows that zinc oxide nanoparticles (35 nm) do not penetrate healthy and diseased skin differently. Zinc oxide particles were concentrated in lesion furrows but did not
penetrate the viable epidermis. The lack of nanoparticle penetration could explain why the application of zinc oxide sunscreens to subjects with psoriasis does not give rise to an increase in systemic zinc levels (Prow et al, 2011).

1.4.2.2. Atopic Dermatitis

Atopic dermatitis is a familial, chronic inflammatory skin disease that commonly presents itself during early infancy and childhood but can persist or start in adulthood. Pruritus, scratching, and chronic, relapsing, or both eczematous lesions are major hallmarks of the disease (Boguniewicz and Leung, 2011). In infants and young children, there is a characteristic pattern of involvement of the face, neck, and extensor skin surfaces. The understanding of AD is constantly undergoing revision as more data become available on the role of IgE-bearing Langerhans cells, atopic keratinocytes, monocytes/macrophages, eosinophils, and mast cells and their interaction with IL-4–, IL-5–, and IL-13–producing TH2, regulatory T, and TH22 lymphocytes (Schultz-Larsen and Hanifin J, 2002). There is a complicated interaction between these cells and their products and susceptibility genes and the host environment, which leads to the clinical findings that characterize AD (Fig.7). Filaggrin is an important protein which is responsible for structural function and hydration of the SC. Reduced levels of filaggrin may lead to increased penetration of irritants and allergens through the skin, and subsequent inflammation (Jakasa et al, 2011; Visser et al, 2013). Approximately 20–30% of patients with AD carry a filaggrin mutation (Brown and McLean, 2012; Barker et al, 2007). However, not all the AD patients with reduced skin barrier are filaggrin carriers and the majority of heterozygous filaggrin carriers never develop AD (Greisenegger et al, 2010; Lesiak et al, 2011; Palmer et al, 2006; Weidinger et al, 2007; O’Regan and Irvine 2010).
Skin colonization with *Staphylococcus aureus* is a characteristic feature of atopic dermatitis with more than 90% of patients being colonized (Abeck and Mempel, 1998). Extracellular matrix proteins are important for the adherence of *S. aureus* to human keratinocytes. The bacterium interferes in the inflammatory process of atopic dermatitis in various ways; among these the ability to release superantigens in a high percentage of clinical isolates is of great importance. As the colonization correlates significantly with the severity of eczema, anti-staphylococcal treatment measurements are widely used. In cases of atopic dermatitis exacerbation with wide-spread weeping lesions, a systemic antibiotic treatment is warranted.

![Fig. 7. Filagrin mutations and Atopic Dermatitis. AD patients with filaggrin mutations have an increase in pH which can contribute to infection and inflammation and an increased risk of bacterial invasion. Moreover, AD patients have a reduced amount of natural moisturizing factors and ceramides which contribute to dry skin, and disorganized keratin filaments causing epidermal fragility.](image)

**1.4.2.3. Burns**

Burned skin is increasingly treated with silver nanoparticles formulations, in the clinical field as in common life. Depending on the gravity of the burn, the skin could be completely or partly
damaged. Cryopreserved and glycerolated skin are commonly used for the preparation of grafts in burns recovery (Castagnoli et al., 2010). Wound dressings containing nanosilver are usually applied also on this kind of skin allograft.

Rigo et al. (2013) have recently showed the *in vivo* aggregation and agglomeration of AgNPs in dermis after application of a nanosilver containing textile (Acticoat™ Flex 3) to a real partial thickness burn patient. A great quantity of Ag NPs was found in the cytoplasm of the fibroblasts, but since the study was conducted on only one patient, it is important to better evaluate if the uptake through burned skin could lead to systemic adverse health effects.

1.5. *In vitro methods for dermal absorption evaluation*

*In vitro* methods are commonly used to estimate the penetration and the permeation of chemicals into the skin. These methods can utilize non-viable skin to measure penetration and permeation but fresh skin is needed to evaluate skin metabolism. The permeability properties of the SC last after excision from the body, allowing a good agreement between *in vivo* and *in vitro* experiments. *In vitro* experiments are usually more convenient than *in vivo* ones, reducing time and costs, originating better reproducibility of results, and less restricted parameter variations, in addition to ethical reasons.

1.5.1 *Franz Diffusion cells*

The Franz diffusion cell is one of the most widely used systems for *in vitro* skin permeation studies (Friend, 1992). Franz-type diffusion cell systems are relatively simple in design. In the donor chamber is usually put the chemical of interest dissolved (or dispersed in case of solid particles) in
an aqueous solution, as for example synthetic sweat. The skin sample is placed between the donor and the receptor compartment. The receptor fluid (usually saline solution) beneath the skin is manually sampled by removing aliquots periodically for analysis (Bronaugh, 1985). The Franz cell is kept at 32°C (to reproduce *in vivo* conditions) by heated water circulating in a glass jacket. During the experiment the receiving solution is continuously stirred (Fig.8).

**Fig.8. A schematic drawing of a Franz Diffusion Cell**

### 1.5.2 Skin models for *in vitro* experiments: fresh, cryopreserved and glycerolated

Several kind of skin can be used in *in vitro* experiments. Human skin is the “gold standard” and has to be preferred for risk assessment purposes. Since there is reduced availability of human fresh skin, stored skin from human and laboratory animal sources can be used. Further, the use of human skin is subject to national and international ethical considerations (ECETOC, 1993).

Typical human *in vitro* experiments with viable skin involve the use of female abdominal and/or breast skin obtained at autopsy or from cosmetic surgery (Dressler, 1999). Non-viable skin from several anatomical sites of male and female cadavers has also been used. Although viable skin (Bronaugh, 2004b) is preferred, especially for metabolism studies, non-viable skin can also be used
for certain tests. OECD Test Guideline 428 (OECD, 2004c) allows a number of skin types and methods of skin preparation for the measurement of dermal absorption \textit{in vitro}:

1) Full-thickness skin, incorporating the stratum corneum, viable epidermis, and dermis

2) Dermatomed skin, in which the lower dermis has been removed

3) Epidermal membranes, comprising the viable epidermis and the stratum corneum

4) Stratum corneum alone

For risk assessment purposes studies full-thickness skin is preferred. It has been recommended that before and in some cases after the experiment, the barrier integrity of the skin should be checked by physical methods, such as transepidermal water loss or transcutaneous electrical resistance (Davies et al., 2004; OECD, 2004c; USEPA, 2004a). In literature there are several studies in which cryopreserved human skin is used. The recommendation of testing skin integrity is even more important when stored skin is evaluated.

In common life not only intact skin could be exposed to chemicals. For this reason, in literature many \textit{in vitro} experiments evaluating damaged skin permeation are found (Larese et al., 2009; Baroli, 2010). Usually, in \textit{in vitro} conditions, only the outer layers of stratum corneum are damaged, following a certified protocol, in order to avoid overestimations. Glycerolated skin is a common skin graft use in burn recovery (Castagnoli et al, 2010). It is known that glycerol-preserved dermis maintains intact tissue morphology and is comparable to fresh and cryopreserved dermis (same content of collagen fibers and elastic properties) even if it does not contain any viable cell. Otherwise, the SC of glycerol-preserved allografts is weaker than cryopreserved and fresh skin(). Since silver nanoparticles are commonly used in wound and burn treatment to reduce the risk of infections, it is important to estimate glycerolated skin permeation as well as that of cryopreserved skin.
1.6 In vivo methods for dermal absorption evaluation

In vivo experiment with humans is the “gold standard” for percutaneous absorption determination. Experiments with laboratory animals, usually rodents, are also allowed. The main disadvantage of using laboratory animals is that they have different skin permeability and systemic disposition compared with humans. Moreover the conduct of any in vivo study has ethical issues, thus discomfort of the animals has to be avoided.

Typically, in vivo experiments can give information not only on the cutaneous uptake but also on the systemic absorption of the test substance. As stated by OECD (2004a, b), the main advantage in performing an in vivo study rather than an in vitro study is that the in vivo study uses a physiologically and metabolically intact system.

1.6.1 Studies with human volunteers

As previously declared, studies with human volunteers are the most trustworthy for the assessment of percutaneous absorption of chemicals through human skin. Their use is limited and their conduct is closely regulated mainly for technical and ethical reasons (Declaration of Helsinki, 1964 and updates: World Medical Association, 2004; and the International Conference on Harmonization Guideline for Good Clinical Practice: ICH, 1996). Several kinds of in vivo studies are found in literature as for example biomonitoring of occupational exposure, cutaneous microdialysis, whole body autoradiography, skin biopsy and tape stripping. The comparison between in vitro and in vivo data often highlights important factors for dermal absorption.
1.6.1.1 The tape stripping technique

One of the most widely used techniques for the investigation of the skin is the tape stripping of stratum corneum (Bashir et al., 2001; Choi et al., 2004; Loffler et al., 2004). Main advantages of this method are that it is simple, inexpensive, and minimally invasive. Thus tape stripping can be applied to both humans and laboratory animals, with low discomfort.

A tape-stripping experiment provides the exposition of a delimited area of skin to a substance for a fixed period of time. Then, the stratum corneum of the exposed skin site is removed sequentially by successive application of adhesive tape.

Several studies allowed the standardization of the procedure (Choi et al. 2004; Loffler et al., 2004; Bashir et al., 2001; Lademann 2009) showing the most relevant factors that affect the fraction of stratum corneum removed by the tapes.

There are three different methodologies based on the tape-stripping technique. The first method has been reviewed by the United States Food and Drug Administration (USFDA, 1998). It is based on the determination of the total amount of substance in the stratum corneum strips collected during uptake and elimination phases. For drug uptake assessment, the product is applied at multiple sites, and the stratum corneum is removed from each site just after the removal of the applied substance at sequentially increasing times.

To assess elimination, the substance is applied to multiple sites and maintained for a period of time. The substance is then removed, and the SC is sampled at different times. The results are plotted as the amount/surface area vs. time. This method is typically used to determine drugs penetration into the SC (Shah et al., 1998). In spite of the simplicity of this method, it has not yet been sufficiently validated; there has been criticism concerning its robustness, reliability, and reproducibility for bioequivalence testing.

The second approach is based on the determination of the concentration profile of a chemical in the SC depending on the relative SC depth. The depth of the SC for each strip can be estimated from
the mass of the SC stripped off from each consecutive tape. The concentration of substance found at a certain depth is then divided per the protein amount sampled in each strip. The data on concentration–stratum corneum depth profile were fitted into a solution of Fick’s second law of diffusion. From this, the partition coefficient of a chemical between the stratum corneum and a vehicle \((K_m)\) and diffusivity \((D)\) were derived, enabling deduction of the chemical permeability coefficient \(K_p\) (e.g. Pirot et al., 1998; Alberti et al., 2001; Reddy et al., 2002). A limitation of the method is that it assumes the skin as a homogeneous membrane, and this is not always observed (Mueller et al., 2003).

Finally, the third tape-stripping method provides for the application of the chemical for a single exposure period and then for the determination of the amount of chemical in collected tape strips (Dupuis et al., 1984; Rougier et al., 1999; Mattorano et al., 2004).

### 1.7. The analytical techniques for nanoparticles determination

The physical-chemical factors affecting dermal absorption and toxicity of particles are:

- Shape
- Dimensions
- Charge
- Hydrodynamic range
- Surface area
- Zeta potential
- Solubility
- Bulk composition
The comprehension of dermal absorption mechanisms cannot overlook a good characterization of the nanoparticles under examination. In table 1 the most common analytical techniques for nanoparticles characterization are reported (University of Essex, for Nanocap).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Measures</th>
<th>Sample</th>
<th>Sensitivity</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmission Electron Microscopy (TEM)</td>
<td>particle size and characterization</td>
<td>&lt; 1 μg has to be prepared as a thin film and be stable under an electron beam and a high vacuum</td>
<td>down to 1nm</td>
<td>additions to TEM can provide more information e.g. Scanning Transmission Electron Microscopy (STEM), High-Resolution TEM (HRTEM) or in-situ measurements as Environmental TEM</td>
</tr>
<tr>
<td>Scanning Electron Microscopy (SEM)</td>
<td>particle size and characterization</td>
<td>sample must be conductive or sputter coated, easier to prepare than TEM sample</td>
<td>down to 1nm</td>
<td>can be used in-situ as Environmental SEM</td>
</tr>
<tr>
<td>Atomic Force Microscopy (AFM)</td>
<td>particle size and characterization</td>
<td>samples must adhere to a substrate and be rigid and dispersed on the substrate. The appropriate substrate must be chosen. Air or liquid samples</td>
<td>1nm - 8μm</td>
<td>a form of Scanning Probe Microscopy (SPM). Requires less time and cost than SEM and TEM</td>
</tr>
<tr>
<td>Photon Correlation Spectroscopy (PCS)</td>
<td>average particle size and size distribution</td>
<td>sample must be a very dilute suspension</td>
<td>1nm - 10μm</td>
<td>based on Dynamic Light Scattering, an extension of the technique is Photon Cross Correlation Spectroscopy (PCCS) for high concentration opaque suspensions giving particle size and stability of nanoparticles</td>
</tr>
<tr>
<td>Nanoparticle Surface Area Monitor (NSAM)</td>
<td>human lung-deposited surface area of nanoparticles</td>
<td>aerosol, concentrations 0 to 10000μm²/cm³, temp 10 - 35°C</td>
<td>down to 10nm</td>
<td>similar to an Electrical Aerosol Detector (EAD).</td>
</tr>
<tr>
<td>Condensation Particle Counter (CPC)</td>
<td>number concentrations of particles</td>
<td>aerosol, concentrations 0 to 100,000 particles/cm³, can be in a flow, higher temps to 200°C possible</td>
<td>2.5 to 3,000nm</td>
<td>can be used for a flow, hand held models available</td>
</tr>
<tr>
<td>Differential Mobility Analyzer</td>
<td>particle size distribution</td>
<td>aerosol</td>
<td>down to 3nm</td>
<td>can be combined with other techniques to create Tandem DMA or DMPS</td>
</tr>
<tr>
<td>Scanning Mobility Particle Sizer (SMPS)</td>
<td>particle size distribution</td>
<td>aerosol, can be a concentrated sample of 1,000,000 - 2,400,000 particles/cm³</td>
<td>3 - 1,000nm</td>
<td>uses an electrosstatic classifier and a CPC, can also add DMA</td>
</tr>
<tr>
<td>Nanoparticle Tracking Analysis (NTA)</td>
<td>particle size and size distribution</td>
<td>500μl suspension, temp 5 - 50°C, wide range of solvents can be used</td>
<td>10 - 1,000nm</td>
<td>use with DLS or PCS</td>
</tr>
<tr>
<td>X-Ray Diffraction (XRD)</td>
<td>average particle size for a bulk sample</td>
<td>larger crystalline samples (&gt;1μm) required</td>
<td>down to 1nm</td>
<td>can identify individual crystals</td>
</tr>
<tr>
<td>Aerosol Time of Flight Mass Spectroscopy</td>
<td>particle size and composition</td>
<td>aerosol</td>
<td>100 - 3,000nm</td>
<td>the efficiency of this method is less for smaller particles.</td>
</tr>
<tr>
<td>Aerosol Particle Mass Analyzer (APM)</td>
<td>particle mass</td>
<td>aerosol sample with particle density approx 1g/cm³</td>
<td>equivalent to 30 - 50μm</td>
<td>gives only mass information and is not dependent on particle size or shape</td>
</tr>
</tbody>
</table>

Table 1. Measurement Techniques For Nanoparticles

Hereafter the two main techniques used in this study for nanoparticles characterization are discussed in detail.

1.7.1. SEM-EDX

The scanning electron microscope (SEM) was designed primarily for producing electron images, but today it is also possible to use it for element mapping, and even point analysis, if an X-ray spectrometer is added. The information is derived from the interactions between accelerated
electrons emitted by the SEM source and the atoms constituting the sample. The dissipation of energy of the impact produces a variety of signals. These signals are: secondary electrons (that actually produce SEM images), backscattered electrons (BSE), diffracted backscattered electrons (that are used to determine crystal structures and orientations of minerals), photons (characteristic X-rays that are used for elemental analysis and continuum X-rays), visible light (cathodoluminescence–CL), and heat. Secondary electrons show morphology and topography on samples, while backscattered electrons are useful for illustrating contrasts in composition in multiphase samples. X-rays are produced by inelastic collisions of the incident electrons with the electrons contained into the shells of atoms in the sample. The relaxation process of electrons to lower energy states yields X-rays with a fixed wavelength. Thus, characteristic X-rays are produced for each element of the solid sample. SEM analysis is a "non-destructive" method since the x-rays generated do not lead to degradation of the sample: this means that it is possible to analyze the same objects time after time. In figure 9 a schematic description of the main components of a SEM is illustrated.
SEM-EDX is suitable for solid conductive materials; otherwise a sputtering with a conductive film is required. One of the advantages of the technique is thus the absence of pretreatment or a relatively simple procedure with reducing possibilities of artifacts. The use of SEM-EDX for metal nanoparticles analysis is mostly limited by the spatial resolution: usually this technique does not allow the detection of particles with dimensions <50 nm.

1.7.2. AFM

The AFM consists of a cantilever that ends with a sharp tip (Fig.10). The cantilever is typically made of silicon (or silicon nitride) with a tip radius of curvature on the order of nanometers. Anytime that the tip of the cantilever gets close to the sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to Hooke's law. The forces
originated by this interaction include mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces and so on. One of the most common ways to measure the deflection is to use a laser spot reflected from the top surface of the cantilever into an array of photodiodes.

To avoid the risk that the tip collides with the surface, in most cases, a feedback mechanism is employed to adjust the tip-to-sample distance to maintain a constant force between the tip and the sample. There are many modes to operate with AFM. In general, possible imaging modes are divided into:

- **Contact mode:** the tip deflection is used as a feedback signal. In contact mode, the force between the tip and the surface is kept constant during scanning by maintaining a constant deflection.

- **Dynamic mode:** the cantilever is oscillated at either its resonant frequency (frequency modulation) or just above (amplitude modulation) where the amplitude of oscillation is typically in a range between 10 to 100 nanometers.

It is possible to image the samples measuring: the position of the cantilever, the amplitude modulation and the phase.
A topographic image can be built from the difference in z position of the cantilever. Another way to collect information from the same scan of the sample is the amplitude (or feedback error) that better represents edges and variations in the profile of the sample surface.

Collecting the phase signal it is possible to obtain information about mechanical properties (hardness, viscosity).
2. AIM OF THE THESIS

In view of the widespread presence of AgNPs in the European Community due to their use in healthcare products as well as cosmetics, food preservatives etc., this thesis examines the skin penetration of silver released from AgNP-containing products.

The evaluation of the percutaneous absorption of released silver goes through different steps, from preliminary in vitro experiments to the realization of an in vivo study on human volunteers.

In the third section of this thesis the significance of the skin storage protocol for percutaneous absorption of silver has been evaluated by means of the Franz in vitro method. Two different protocols of skin storage, commonly used in burn recovery, were compared to fresh human skin. This preliminary step was performed using a solution of free AgNPs placed in contact with each sample of skin for 24 hours.

After determination of the most suitable storage protocol, in the fourth section of the thesis the in vitro method was applied to study the permeation of silver released from AgNP textiles. First of all a selection of AgNP-containing products was made and their silver release was assessed: 8 different materials were soaked in synthetic sweat. From these three materials were selected for further experiment, both for their high release of silver and for the impact that their use was expected to have on consumers’ health. The materials used in this thesis were two wound dressings and a garment for the treatment of children affected by Atopic Dermatitis (AD). The Franz apparatus was set up putting three samples of each silver material in the donor compartment. The released silver permeated the skin and silver aggregates (in the range 150-2000 nm) were found in all the skin samples after 24 hours exposure to the materials tested. Due to the finding of silver aggregates in all the skin compartments (both in epidermis and dermis), it was important to verify if in in vivo conditions these results would be confirmed. Since no significant differences were found concerning the permeation of silver released from the three materials, the silver garment for AD patients was selected for further experiments. This choice was based on the major impact that this
garment is expected to have in the treatment of children with *Staphylococcus Aureus* superinfections: evaluating the safety of this material is thus very important. Furthermore, in the literature there are only some papers reporting silver permeation through intact skin and a few regarding burned skin. Therefore, it was important to determine the permeation not only through intact skin but also through atopic skin, which is the final target of the material tested.

The protocol of the *in vivo* study was determined from the analysis of the results of a preliminary pilot study on a few volunteers. In the fifth part of this thesis a pilot study on human volunteers, regarding the skin permeation of silver released from the garment for the treatment of AD patients, is reported. Thanks to this pilot study, the analytical methods to be used to quantify the silver permeation and the protocol for risk assessment evaluation were determined. The analytical methods used in the *in vivo* study for silver quantification are thus extensively described in section 5.

In conclusion, the sixth section of this thesis reports the *in vivo* study on healthy and atopic human volunteers, with particular attention being paid to the comparison with the results of the previous in vitro work described in section 4.

In summary, the main objectives of this thesis are: (i) the definition of the experimental factors which might influence the results e.g. storage of the skin; (ii) the optimization of the analytical methods for the measurement of silver in different biological samples; (iii) the evaluation of silver penetration from different AgNP materials under exposure scenarios which are as close as possible to the “*in use*” scenario.
3. DETERMINATION OF THE PROPER SKIN MODEL FOR PERCUTANEOUS ABSORPTION PURPOSES

3.1. Background

As described in section 1.5.2., there are several allowed kinds of skin models available for *in vitro* experiments. Fresh human skin, immediately used after excision, represents the “gold standard”. Since the use of fresh human skin is limited both for practical and ethical reasons, there are several studies on the skin penetration of a chemical performed with different procedures. Unfortunately, often different methods generate different results. The discrepancies in results of studies concerning the percutaneous absorption of metal NPs are indeed mostly due to the lack of standardized protocols, which allows differences in techniques and methods, laboratory conditions, and so on. Differences in thickness, viability, location, and hair density of skin used in *in vitro* experiments generate a great variability in data. The guidelines for skin absorption evaluation report only few indications about the requisites that have to be satisfied. Furthermore, many aspects related to the nature of metal NPs seem to affect their permeation through the skin, for example, bulk composition, dimensions, surface area, ionization of the bulk component, and functionalization of the surface. AgNPs are the most common application of nanotechnology to the biomedical field because of their strong release of silver ions that is responsible for a broad spectrum of antimicrobial activity. Wound dressing containing AgNPs are used to treat burns, wounds, and scars in hospitals and are already available in shops. These products are used in close contact with the skin, and it is reported in the literature that the silver released from these products can penetrate the skin, mainly if damaged. A previous *in vitro* study of Larese (2009) revealed the permeation of silver through human skin after AgNP (polyvinylpyrrolidone-coated) exposure: Larese compared silver permeation through intact and damaged skin showing that it strongly increases with the damaging of the SC. Transmission electron micros-copy (TEM) analysis of the skin revealed the
presence of NPs in just a few sections of the SC and upper epidermis. In this thesis, percutaneous absorption of AgNPs has been evaluated on stored human skin following three different protocols (fresh, cryopreserved, and glycerol preserved) commonly used for the preparation of grafts in burns recovery.

The first part of this thesis has the aim of estimating silver permeation through human skin by means of the comparison among different skin storage protocols. This comparison could be useful to better understand how to reproduce, in in vitro conditions, a real scenario for the clinical application of AgNP products. For this purpose, we applied the protocol adopted in the European project EDETOX (Evaluations and predictions of DErmal absorption of TOXic chemicals), already used by our group to study the in vitro dermal absorption of silver and gold NPs.

### 3.2 Materials and methods

#### 3.2.1. Chemicals

All chemicals used were of analytical grade. Urea, lactic acid, sodium chloride, sodium hydrogenphosphate, potassium dihydrogenphosphate, nitric acid (69% v/v), and hydrochloric acid (36.5–38% v/v) were purchased from Sigma Aldrich (Milan, Italy) and ammonium hydroxide (25%) from J. T. Baker (Milan, Italy). Reagent-grade water was produced with a Millipore purification pack system (MilliQ water).

#### 3.2.2. Nanoparticles characterization

Polymer-coated AgNPs (content of silver: 25% w/w; polyvinylpyrrolidone: 75% w/w) were supplied by NanoAmor Materials Inc. (Houston, TX, USA). AgNPs dispersed in synthetic sweat
were characterized to obtain NP size and morphology on a TEM (EM208; Philips, Eindhoven, The Netherlands; operating at 200 kV) with a high-definition acquisition system based on a side-mounted TEM camera OSIS Morada and an iTEM software platform (Olympus Soft Imaging Solutions GmbH, Münster, Germany). A solution of AgNPs was analyzed by means of an atomic absorption spectrometry graphite furnace (GF-AAS, 4100 ZL, Perkin Elmer, Waltham MA, USA, with autosampler AS/71) to assess the declared percentage of silver while the composition of polymer coating was revealed by means of an FT-IR Spectrum 100 (Perkin Elmer, Waltham, MA, USA; FT-IR, Fourier transform infrared) fitted with attenuated total reflection (ATR) (Ge/Ge) accessory.

3.2.3. Preparation of skin samples

Split-thickness cadaver skin grafts, harvested from different cadaveric donors, after the authorization of the local ethical committee, were preserved with two different methods: glycerolization and cryopreservation. The two methods, hereafter described, have been previously validated by the Skin Bank of CTO Hospital in Turin *:

1) Glycerolization protocol

Glycerolization was performed on skin grafts (legs and arms skin, number of donors (N) was 2, one male and one female) with the following procedures: skin was placed in 50% glycerol, amikacin (1 mg ml⁻¹), and ampicillin (600 µg ml⁻¹) and then incubated for 3 h at room temperature. The glycerol concentration was then first increased to 70% and then to 85%. The skin grafts were agitated gently for 3 h at 33°C at each step. After glycerolization, the skin grafts were stored at 4 °C. Glycerol was
removed from the grafts by sequential washing in sterile 0.9% NaCl solution at 37 °C before testing.

2) Cryopreservation protocol

Skin allografts (back skin, N = 3, one male and two females) were cryopreserved using 10% dimethyl sulfoxide (DMSO) as cryoprotectant and placed in sterile packets inside aluminum heat sink boxes. Freezing was done in three steps: first 15 min in ice, then 1 h at -20 °C, and at -80 °C until use. Thawing was done rapidly by immersion of the packets in a 37 °C water bath; once the frozen cryoprotectant solution had nearly all melted, the pouches were removed from the water bath and opened; the skin grafts were then eluted, first in 40% dextran and then in a solution with Roswell Park Memorial Institute medium (RPMI) 1640 and 10% albumin. Lastly, they were put into fresh NaCl 0.9% solution before clinical use. The skin graft used in experiment 3 (arm skin) was obtained from a donor (male) provided by CTO Hospital Skin Bank and immediately used after excision. From each skin specimen, 4 cm x 4 cm pieces were cut and mounted separately on the diffusion cells, which were previously washed first with nitric acid (69% v/v), second with diluted nitric acid (6% v/v), and finally rinsed three times with Milli-Q water. The technique used to evaluate skin graft viability was the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay with tetrazolium salts. This assay is based on the conversion of the tetrazolium salt into formazan crystals that are soluble in 2-methoxyethanol and measured with a spectrophotometer at a wavelength of 570 nm. The quantity of the formazan product measured is directly proportional to the number of living cells in the sample. Viability index of skin is expressed as the ratio between the optical density (OD) of the skin samples and their weight in grams. The percentage of the viability index is the ratio of the fresh sample considered as 100% viable and the value of the same specimen after storage in glycerol or cryopreservation as previously described. A cryopreserved sample after thawing has a mean percentage of 64% viability; glycerol preserved grafts, instead,
show negative value of viability, and are considered nonviable grafts with intact tissue morphology. Skin integrity was tested before and after each experiment using electrical conductivity by means of a conductometer (Metrohm, 660, Metrohm AG Oberdorfstrasse, 68 CH-9100 Herisau, Switzerland) operating at 300 Hz and connected to two stainless steel electrodes [19]. The conductivity data in mS were converted into kΩ cm\(^{-2}\). Cells with a resistance <3.95 ± 0.27 kΩ cm\(^{-2}\) were considered to be damaged and rejected as suggested by Davies et al. [20].

3.2.4. In vitro diffusion system

Samples of human fresh, cryopreserved, and glycerol preserved skin were tested by means of a Franz diffusion cell apparatus to evaluate percutaneous absorption of AgNPs according to the Franz method [21]. The mean volume of the receptor compartment was 14.0 mL and was maintained at 32 °C by circulating thermostated water in the jacket surrounding the cell. This temperature value was chosen in order to reproduce the hand’s physiological temperature at normal conditions. The physiological solution used as the receptor fluid was prepared by dissolving 2.38 g of Na\(_2\)HPO\(_4\), 0.19 g of KH\(_2\)PO\(_4\), and 9 g of NaCl into 1 L of Milli-Q water (final pH = 7.35) in order to reproduce the saline concentration of the blood stream. The solution in each cell was continuously stirred using a Teflon-coated magnetic stirrer. Postmortem skin grafts were harvested from four different multiorgan donors with a mean age of 56 years (range 26–70). Each piece of skin (donor sites: back, arms, and legs) was clamped between the donor and the receptor compartment; the mean exposed skin area was 3.29 cm\(^2\) and the average membrane thickness was 0.7 mm for fresh skin, 0.7 mm for cryopreserved skin, and 0.7 mm for glycerol preserved skin. Thickness was measured by means of a high-precision micro-caliper with an accuracy of 0.01 mm and repeatability expressed as relative standard deviation (RSD) of 5%. Three independent experiments were performed as follows:
• Experiment 1 (fresh skin): The AgNP solution was prepared just before the experiment; 400 mg of AgNPs was dispersed by sonication in 100 mL of water. In the donor phase, 0.75 mL of AgNPs solution was diluted 1:1 in synthetic sweat (pH 4.5) in order to reproduce in vivo conditions. The synthetic sweat solution was made of 0.5% sodium chloride, 0.1% urea, and 0.1% lactic acid in Milli-Q water; pH 4.5 was adjusted with ammonium hydroxide. The total silver amount in the donor phases (113 mg cm$^{-2}$) was confirmed after the end of the experiment. The experiment was performed using seven samples of intact fresh skin.

• Experiment 2 (cryopreserved skin): In this experiment, seven samples of intact cryopreserved skin were tested following the procedure of experiment 1.

• Experiment 3 (glycerol preserved skin): The experimental conditions were the same applied in experiment 1 with the exception that in this case the experiment was performed using five samples of glycerol preserved skin.

• Blanks: For each experiment, two cells were added as blank. The blank cells were treated just as the other cells were with the exception that no AgNPs were introduced to the exposure chamber.

### 3.2.5. Sampling procedure

At selected intervals (2, 4, 8, 12, 21, 24 h), 1.5 mL of the dermal bathing solution was collected for next analyses and immediately replaced with an equal volume of fresh physiological solution. At 24 h, the dermal bathing solution and the donor phase of each diffusion cell were recovered for the analysis.
3.2.6. Skin content evaluation

At the end of each experiment, the skin grafts were removed from the diffusion cells and separated into epidermis and dermis by heat shock immersion in water at 60 °C for 1 min. All the skin compartments were collected and stored individually in the freezer at -25 °C for the following digestion and analysis. At the time of the analysis, the skin membranes were dried for 2 h at room temperature, cut into sections, and put into glass tubes with 2 mL of HNO₃ 69% v/v for digestion. They were heated at 80 °C for 8 h and then diluted to a final volume of 10 mL with Milli-Q water for the analysis using an inductively coupled plasma mass spectrometer (ICP-MS).

3.2.7. Analytical measurements

ICP-MS 7500 CE Agilent Technologies Inc., Santa Clara, CA, USA instrument (with integrated autosampler) was used to determinate the total silver concentration in the receiver phases, while a GF-AAS (Perkin Elmer 4100 ZL, with auto-sampler AS/71) equipped with Zeeman effect background correction and end-capped graphite tubes (transversely heated graphite atomizer, THGA) was used in order to quantify silver in the donor phases and in the solutions resulting after mineralization of skin samples. The calibration curves were obtained using standard solutions at 5, 10, 25, 50, and 100 µg L⁻¹ and a solution of 0.1% Mg(NO₃)₂ and 1 g L⁻¹ 1 of Pd(NO₃)₂ was added in every sample as matrix modifier for GF-AAS analysis. A seven-point standard curve was used for ICP-MS measurements (0.01, 0.05, 0.1, 0.5, 1, 5, and 10 µg L⁻¹, ion mass 107 u.m.a.). The limit of detection of silver was 0.005 µg L⁻¹ for ICP-MS and 1.5 µg L⁻¹ for GF-AAS; the precision of the measurements, expressed as repeatability, was calculated from the percentage of relative standard deviation for the analysis (RSD %) which was always <5%.
3.2.8. Data analysis

Silver concentration data (µg cm\(^{-3}\)) in the receptor solution were converted to the total amount that penetrated (µg cm\(^{-2}\)) with a correction for dilution due to sample removal. Data analysis was performed using the statistical software IBM SPSS Statistic 20 for Windows. Data were reported as median ± 25–75° percentile. The difference between independent data was assessed by means of the Kruskal–Wallis test. A p value <0.05 was considered as the limit of statistical significance.

3.3. Results

3.3.1. Nanoparticles characterization

TEM measurements revealed that AgNPs were as small as 19 ± 5 nm (number of measured NPs: 100). In figure 1 TEM two images of AgNPs dispersed in synthetic sweat are reported. Silver content of AgNPs was consistent with the composition declared by the producer: a solution containing 1.0 mg/L of nanoparticles contained 0.250 ±0.005 mg of silver. The polymer composition (polyvinyl pyrrolidone) was confirmed by ATR-FTIR analysis.
3.3.2. Silver permeation profiles

Fig. 2 shows silver permeation profiles of the three experiments. Twenty-four-hour silver flux penetration was $0.2 \text{ cm}^2 \text{ h}^{-1}$ (lag time: 8.2 h) for fresh skin, $0.3 \text{ ng cm}^2 \text{ h}^{-1}$ (lag time: 10.9 h) for cryopreserved skin, and $3.8 \text{ ng cm}^2 \text{ h}^{-1}$ (lag time: 6.3 h) for glycerol preserved skin.
Fig. 2 (a): Silver concentration of receiving phases in experiment with fresh, cryopreserved and glycerol preserved skin; 2 (b): silver concentration of receiving phases of experiment with fresh and cryopreserved skin (for fresh skin, two cases are highlighted: case 15 is the extreme value of silver concentration at 8 hour; case 20 is an outlier at 8 hour. Both the values have been included in statistical evaluation).

3.3.3. Silver skin content

At the end of the experiments, AgNPs present inside the donor phases were aggregated and deposed on the skin surface because of the elevated saline concentration. It means that a huge number of not-penetrated AgNPs remained on the surface even after the washing procedure. Silver was revealed in the epidermis of all the skin samples, with elevated standard deviation because of AgNPs deposition on the SC surface. Silver detected inside the epidermis of the three experiments was thus high and it was discarded for risk assessment evaluation. The silver amount found in fresh cryopreserved and glycerol preserved dermis is reported in Fig. 3. There was no significant difference between the silver content revealed in cryopreserved dermis of experiment 2 (median 3.08 µg g\(^{-1}\); 25th–75th percentiles 2.06–3.2 µg g\(^{-1}\); range 1.97–6.93 µg g\(^{-1}\)) and that found in glycerol preserved skin (experiment 3, median 3.53 µg g\(^{-1}\); 25th–75th percentiles 2.73–4.52 µg g\(^{-1}\); range 2.64–4.78 µg g\(^{-1}\)). The amount revealed in dermis of fresh skin was comparable to that found in the cryopreserved
skin (experiment 1, median 1.55 µg g⁻¹; 25th–75th percentiles 1.11–3.00 µg g⁻¹; range 1.05–3.47 µg g⁻¹). The Kruskal–Wallis test revealed no significant differences between silver amount revealed in the skin grafts of the three experiments (p = 0.075) even if it has been noted that glycerol preserved skin has higher content of silver, while fresh skin has lower content.

![Silver concentration in dermis after 24 hour application of AgNPs (<20 nm) dispersed in synthetic sweat. * Kruskal-Wallis test revealed no significant differences between silver amount revealed in the skin grafts of the three experiments (p = 0.075), (case 7: extreme case of silver concentration in cryopreserved dermis; the value was included in statistical evaluation).](image-url)
3.4. Discussion

AgNPs used in this study were purchased from NanoAmor Materials Inc.; therefore, the characterization was performed in order to verify the producer’s declaration. Silver concentration, nature of the polymer coating, and NP dimensions were all confirmed by the analysis, and TEM images are reported as the most representative data (Fig. 1).

At the beginning of the three experiments performed, the AgNPs were well dispersed in synthetic sweat and showed a homogeneous yellow coloration due to the excitation of surface plasmon vibrations on AgNPs surface [22]. The elevated content of chlorides and the acidic pH of the donor solutions enhanced aggregation of AgNPs that visibly precipitated on the skin surface after a few hours from the starting time. The aggregation process is time dependent and probably occurs also because of the presence of lipids and proteins in the SC [23–25]. Previous studies showed that the interaction of proteins and lipids with polyvinylpyrrolidone coated AgNPs led to time-dependent agglomeration of the particles in biological media [26,27]. The three in vitro experiments performed in this thesis document that silver can permeate fresh and cryopreserved human skin in similar way. This finding confirmed previous data [20,21] and is both of theoretical and methodological interest.

Taking into account that cell viability of cryopreserved skin was reduced by 36% compared to fresh skin (as assessed by the validated skin bank protocol)[27], our results indicate that differences in cell viability do not influence the percutaneous absorption. This finding suggests that silver permeation through the skin could be achieved through the mechanism of passive diffusion. From a methodological point of view according to OECD TG 428 (OECD 2004b, c) and EHC 235 (WHO 2006), nonviable but intact skin can be used to investigate percutaneous absorption. Starting from these previous considerations, and taking into account the peculiar properties of NPs, it was important to verify once more that the use of cryopreserved human skin for in vitro experiments could be a good approximation of reality. Our results demonstrate that the use of cryopreserved skin
allows simplifying the performance of in vitro experiments without misleading for evaluating silver permeation after skin exposure to AgNPs. Permeation profile of glycerol preserved skin is significantly different from both fresh and cryopreserved skin. It is known that glycerol-preserved dermis maintains intact tissue morphology and is comparable to fresh and cryopreserved dermis (same content of collagen fibers and elastic properties) even if it does not contain any viable cell. Otherwise, the SC of glycerol-preserved allografts is weaker than cryopreserved and fresh skin [27,30,31]. The experimental conditions used in this study weakened the glycerol-preserved epidermis which resulted in its easy removal from the dermis at the end of experiment. The process of de-epithelization is well known from the literature and results in detachment of epithelium from the basal membrane [30]. Therefore, the permeation profile of glycerol preserved skin must be interpreted as if AgNPs were deposited directly on the basal lamina. The amount of silver revealed inside the dermis used in the three experiments was not significantly different, but reflect the same trend observed in the receiving cells: fresh dermis has lower silver content whereas glycerol-preserved dermis has higher content. It is important to underline the clinical significance of these results because cryopreserved and glycerol-preserved skin allografts are commonly used in burn treatment, and wound dressing containing AgNPs are increasingly used for prevention of sepsis in burns and lesion treatment. Consequently, a glycerol preserved skin treated with this kind of product could lead to a relevant exposure to silver since absorption take place through a compromised SC. Systemic effects and also argyria-like symptoms could be generated after a prolonged exposure of damaged skin to silver nanoproducts as a case report of 2006 shows [32]. The elevated blood silver levels found in this case report are the evidence that silver released from products containing AgNPs can reach the dermis and enter the circulatory system. Glycerol preserved skin is comparable to necrotic skin from a morphological and structural point of view: the results shown in this thesis allow estimating silver permeation of a burn skin treated with AgNPs garments. Other studies report silver uptake after wearing silver nanoproducts demonstrating percutaneous absorption in vivo [33–35]. Silver can penetrate the skin as NPs or as ions released
from NPs: a previous study of Larese [10], performed with the same kind of AgNPs used in this thesis, reported the permeation of NPs through the SC of cryopreserved skin. Both silver ions and AgNPs are known to have relevant side effects on skin host cells depending on the dosage. Silver could be a cytotoxic agent on keratinocytes and fibroblasts at concentrations that are lethal for bacteria [36]. The main objective of burns treatment is reaching a controlled and selective release of silver ions in order to avoid side effects and delay of wound-healing process [37,38]. As this thesis demonstrates that silver can pass the skin barrier reaching the dermis, further studies are needed to relate in vitro and in vivo data, quantify silver skin uptake, and quantify the amount of silver passed as ion and/or as NP.

3.5. Conclusions

Permeation through glycerol preserved skin is significantly higher compared to both fresh and cryopreserved skin. This result can generate relevant clinical implications for burns treatment with products containing AgNPs as glycerol preserved skin has morphological and structural analogies with necrotic skin. No significant differences have been found between percutaneous absorption of silver in cryopreserved and fresh skin confirming the use of cryopreserved skin in in vitro experiments as a good model for skin permeation evaluation.
4. IN VITRO PERCUTANEOUS PENETRATION AND CHARACTERIZATION OF SILVER FROM SILVER CONTAINING TEXTILE

In the previous part of this thesis, the proper skin storage protocol for in vitro experiments was determined. Therefore, further aim of this investigation is to evaluate both release of silver from silver textiles and consequently the permeation of silver released through cryopreserved skin.

4.1. Background

Silver containing textiles and wound dressings are commonly used in sportswear and for the treatment of burned and infected skin e.g. in patients suffering from atopic eczema. The importance of silver as an antimicrobial agent has been known for centuries and its use has recently increased because of the advent of the nanoparticles formulations (Cho et al, 2005; Sadeghi et al, 2012). The versatility offered by nanoparticles on different substrates at a limited cost and the peculiarity silver has in avoiding bacterial resistance mechanisms have led to wide application of silver nanoparticles on different biomedical devices (Chen et al, 2008; Simchi et al, 2011). Silver nanoparticles have a large surface area, resulting in a larger amount of silver ions being released and potentially penetrating into and across the skin, particularly if the skin is not intact (Larese et al, 2009). This raises concerns that dermal exposure of larger areas of damaged skin may lead to systemic adverse health effects (Trop et al, 2006; Wang et al, 2009). In order to carry out a health risk assessment it is not only important to quantify and characterize the silver species which are released from a silver containing material but also to determine and characterize silver species which penetrate into the skin. Most commercially available products containing silver have no quantitative or qualitative data as to the presence of silver in the material and in particular details on its release when in contact with skin. Therefore, the characterization of silver species released from these materials under “an in-use scenario” is the first step for the risk assessment process. There is a paucity of
data on the silver percutaneous penetration of silver containing products (Some et al, 2011). Although percutaneous penetration of silver nanoparticles has long been under debate (Larese et al, 2009; Samberg et al, 2010), it still remains to be clarified whether they can penetrate the skin. Most studies have investigated the release of Ag from commercially available nanotextiles in water reporting that the silver released was either in a dissolved form, as very small nanoparticles (1-100 nm), or as aggregates of silver chloride particles (Benn et al, 2008; Benn et al, 2010; Lorenz et al, 2012; Pasricha et al, 2012).

Geranio et al have shown that there is a very low level of dissolved Ag for most textiles and that the majority of the particulate Ag released into washing liquids was present as particles larger than 450 nm and that only 5–15% of particles was smaller (Geranio et al, 2009). In contrast to what was reported by Geranio et al (2009), Farkas et al (2011) have reported that the Ag particulates, which are released into the water of a washing machine, are mostly present as nanoparticles in the range of approximately 10 nm. Impellitteri et al (2009) demonstrated that the chemical composition of washing solutions strongly affects the speciation of the silver released and that there is a relevant transformation of elemental Ag to AgCl in the presence of chlorides. Furthermore, Kulthong et al (2010) showed that silver release was likely to be dependent on the amount of silver deposed on the fibers, the fabric quality and the artificial sweat formulations including its pH.

The second step of this thesis was the assessment of quantitative and qualitative silver release from different silver containing materials for topical application. Among these, three biomedical materials with a relevant content of silver were selected for risk assessment evaluation. Furthermore, in vitro percutaneous absorption of silver released from the three materials (through human skin) and silver speciation in different skin layers was determined.
4.2. Experimental

4.2.1 Chemicals

All the chemicals used were of analytical grade. Urea, lactic acid, sodium chloride, sodium hydrogenphosphate, potassium dihydrogenphosphate, nitric acid (69% v/v) and hydrochloric acid (36.5-38% v/v), were purchased from Sigma Aldrich (Milan, Italy) and ammonium hydroxide (25%) from J.T. Baker (Milan Italy). Ultrapure water was produced with a Millipore purification pack system (MilliQ® water).

4.2.2 Silver containing textiles

Eight different silver containing materials were investigated. Material 1 is one of the most commonly used medical devices that contain silver nanoparticles (Acticoat™). High density polyethylene mesh with a core of rayon and polyester is coated by nanocrystalline silver (dunn and Edward-Jones, 2004). The manufacturer declares an antimicrobial effect that will last for 3 days, which is the maximum recommended application time. The material 2 was a wound dressing composed of nylon-spandex fibers plated with 20% (by mass) of metallic silver by an electroless deposition process. The antimicrobial barrier properties of this material have been evaluated by Krieger et al (2011) and Silver et al (2006). The material 3 contains 79% modal, 11% polyamide, 7% elastam and 3% silver plated by an electroless deposition process (according to the producer’s declarations).

Material 4, 5 and 6 were wound dressings of the same brand of material 2, but with a different content of silver. Material 7 and 8 were two different kinds of sports wear: a T-shirt and a polo shirt. Only in the case of materials 1, 2 and 3 it was possible to collect informations about their composition directly from the producer. Amongst investigated materials, only material 1 declares the presence of silver nanoparticles.
4.2.3 The silver release test

Three samples (9.6 cm$^2$) of each silver fabric were soaked in 11 mL of synthetic sweat (milliQ® water, 0.5% NaCl, 0.1% lactic acid, 0.1% urea, ammonium hydroxide to reach a pH of 4.5) for 24 hours. After 8 hours, 1.5 mL of the bathing solutions were sampled and immediately replaced with the same volume of fresh synthetic sweat. At 24 hours, 1.5 mL of the bathing solutions were sampled for ET-AAS analysis; after adding 1% (w/w) of nitric acid the final dilution was made in 2.8% (w/w) ammonium hydroxide immediately before analysis and the samples of material were drained and collected for SEM-EDX analysis.

Tab.1 Silver released in synthetic sweat from silver containing textiles.

<table>
<thead>
<tr>
<th>Release in synthetic sweat</th>
<th>SPORTS WEAR</th>
<th>WOUND DRESSINGS</th>
<th>GARMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag µg/L (8h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>35.5 ± 6.8</td>
<td>243.0 ± 29.2</td>
<td>941.7 ± 447.0</td>
</tr>
<tr>
<td></td>
<td>8 ± 3.3</td>
<td>187.3 ± 81.2</td>
<td>179.7 ± 8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.5 ± 3.3</td>
<td>183.0 ± 40.9</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>303.3 ± 55.0</td>
<td></td>
</tr>
<tr>
<td>Ag µg/L (24h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>30.9 ± 1.4</td>
<td>252.1 ± 25.6</td>
<td>1038.4 ± 661.3</td>
</tr>
<tr>
<td></td>
<td>8 ± 3.4</td>
<td>236.2 ± 10.3</td>
<td>209.5 ± 11.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.0 ± 1.4</td>
<td>209.3 ± 14.8</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>333.3 ± 59.0</td>
<td></td>
</tr>
</tbody>
</table>

For further studies, material 1, 2 and 3 were selected both for the high silver release and for the impact that their use was expected to have on consumers’ health.

4.2.3 The determination of silver in material 1, 2 and 3

Three samples (1 cm$^2$) of each material were dissolved in 5 mL of nitric acid 69% v/v assuring that the material was completely covered by the liquid. After 24 hours the resulting solutions were diluted to 50 ml with milliQ water and analysed for silver content by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES).
4.2.4 The in vitro test

The experiment was performed using human cryopreserved skin (as described in section 3, see also Castagnoli et al, 2003). The skin from back and arms was obtained from two donors (26 and 68 years). Each piece of skin was clamped between the donor and the receptor compartment; the exposed skin area was 1.77 cm² and the average skin thickness was 1 mm (percent relative error of 1%). Skin integrity was assessed by using electrical conductibility before and after the experiment as previously described, cells with a resistance $< 3.95 \pm 0.27 \, \text{k}\Omega \, \text{cm}^{-2}$ were considered to be damaged and rejected as suggested by Davies et al (2004).

Percutaneous penetration was investigated by using static diffusion cells according to the Franz method (Franz, 1975). Each donor chamber was filled with 1.5 mL of synthetic sweat (milliQ® water, 0.5% NaCl, 0.1% lactic acid, 0.1% urea, ammonium hydroxide to reach a pH of 4.5) in which a sample of silver material (1.77 cm²) was soaked. Six chambers were prepared for each material tested (2 donors, three samples per donor), while two donor chambers were filled with synthetic sweat as a blank. The total silver concentration of each donor phase was determined by ICP-MS.

The receptor compartment had a volume of 12.0 mL and was maintained at +32°C by the circulation of thermostated water in the jacket that surrounded the cell. The physiological solution used as the receptor fluid was prepared by dissolving 2.38 g of Na₂HPO₄, 0.19 g of KH₂PO₄ and 9 g of NaCl in 1 L of MilliQ® water (final pH = 7.4). The concentration of the salt in the receptor fluid was approximately the same as that present in blood. The solution in each cell was continuously stirred using a Teflon coated magnetic stirrer.

At selected intervals, 2, 4, 8, 16 and 24 hrs, 1.5 ml of the samples of the receptor solution were collected for the analysis of silver. Each receptor sample was immediately replaced with an equal volume of fresh physiological solution.
4.2.5 Skin sample preparation for analysis

At the end of the 24-hours penetration test, the skin was washed with a physiological solution to remove any excess of silver from the surface. From the three replicates of each donor, two of them were separated into dermis and epidermis by heat shock, immersing them in water at +60°C for 1 min. Subsequently, they were placed into a drying oven for 24 hours and put into glass tubes with 1 mL of HNO₃ 69% v/v for digestion. The resulting solutions were diluted with MilliQ® water for ICP-MS analysis. The third replicates of each donor were divided into two subsamples: the first was dried and digested as previously described for the analysis of total silver; the second was fixed with glutaraldehyde at 10%, washed with ethanol-water solutions with increasing concentrations of ethanol and then stored in ethanol at 98%, until SEM analysis.

4.2.6 The instrumental analysis

SEM-EDX analysis was performed using a Hitachi mod. TM-3000 - equipped with Oxford Instruments X-ray Microanalysis (Hitachi High-Technologies Europe GmbH, Krefeld, Germany). The total silver concentration in solutions resulting from the materials dissolution were conducted by means of Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) using a Spectroflame Modula E optical plasma interface (OPI) instrument (by SPECTRO, Germany). The samples were analyzed measuring against standard solutions for instrumental calibration (range 0-10 mg/L). The limit of detection (LOD) at the operative wavelength of 328.068 was 0.05 mg/l. The relative standard deviation (RSD %) of the measurements was always less than 5%.

The total silver concentration in the receptor fluid was determined by an ICP-MS 7500 CE (Agilent Technologies, Santa Clara, CA, USA) with an integrated autosampler. The selected ion mass was 107 u.m.a. The limit of silver detection was 0.05 µg/L (standard solutions: 0.1, 0.5, 1.0, 5.0, 10.0 µg/L) (RSD %, for the analysis was always <5%). The accuracy was determined analyzing NIST
certified reference material 1640-a (trace elements in natural water), the analyzed results fit in the range of the certified value (8.081 ± 0.046) µg/L.

The silver content in the skin and in the donor liquid was measured by an ET-AAS (Perkin Elmer, Waltham, MA, USA) mod. 4100 ZL with AS/71 autosampler and Transversely Heated Graphite Atomizer (THGA) Zeeman background correction (LOD: 0.5 µg/L; standard solutions range 5-50.0 µg/L).

The AFM analysis of silver particles on the surface of the material was performed using a Multimode AFM with Nanoscope IIIa controller (Bruker, Billerica, MA, USA) with a vertical engagement (JV) 125 µm scanner. Contact mode was used throughout the experiments, using silicon–nitride tips (NP-20, Bruker, nom. freq. 56 kHz, nom. spring constant of 0.32 N/m) and a scan resolution of 512 samples per line. The processing and analysis of images was done by NanoScope™ software (Digital Instruments, version V614r1). A previously developed protocol was used for textile fabric AFM imaging (Ražić, 2011). Samples of silver material (1 cm²) were fixed onto the sample holder (stainless steel, diameter 1.5 mm) immediately before testing. All measurements were performed under ambient conditions i.e. at room temperature and a relative air humidity of 50-60%. The sample surface areas investigated ranged from 25 µm x 25 µm to 2 µm x 2 µm, so as to ensure that the scan areas of 2 µm x 2 µm were representative of the features of the material of interest.

4.2.7. Data analysis

Data analysis was performed using the statistical software IBM SPSS Statistic 20 for Windows. The difference between independent data was assessed by means of the One Way ANOVA test. A p value <0.05 was considered as the limit of statistical significance.
4.3. Results

4.3.1 The content of silver in textiles
The amount of silver in the investigated materials is shown in Table 2. The digestion of the textiles with nitric acid as described in the Experimental section resulted in transparent and homogeneous solutions. Silver concentration in textiles reported in Table 2 are expressed as mg of silver per surface area and as percentage of silver in textile (weight/weight). It has to be highlighted that material 1 has the higher mass per surface area than materials 2 and 3. Thus, the expression of silver concentration per surface unit is more representative for the silver dose i.e. the amount of silver in the contact with the skin. The silver contents of the three materials (µg/cm²) were compared by means of One Way ANOVA test revealing significant differences (p<0.05). As showed in Table 2, the content of silver in the textiles follows this trend: material 1>material 3>material 2.

Tab.2 The concentration of silver in the textile and in donor chambers after 24 hour of soaking

<table>
<thead>
<tr>
<th>Ag concentration</th>
<th>Material 1 mean±SD</th>
<th>Material 2 mean±SD</th>
<th>Material 3 mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Textile [mg/cm²]</td>
<td>1.37±0.05</td>
<td>0.33±0.05</td>
<td>0.67±0.09</td>
</tr>
<tr>
<td>Textile [% w/w]</td>
<td>8.1± 1.3</td>
<td>7.1± 0.2</td>
<td>3.6± 0.1</td>
</tr>
<tr>
<td>Donor fluid [µg/cm²]</td>
<td>4.0± 1.6</td>
<td>0.6 ±0.1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Donor fluid [µg/ml]</td>
<td>4.7± 1.9</td>
<td>0.7 ±0.1</td>
<td>2.2±0.1</td>
</tr>
</tbody>
</table>
4.3.2 The characterization of silver particles in the investigated textiles before the release test

Material 1 is made of a silver nano-crystal grid enveloped by gauzes. The SEM analysis was performed only on the gauzes (Fig. 1). The analysis of particles on the grid surface was not possible due to its electron density. However, we analysed the grid with AFM which has a better resolution and additionally it is not influenced by the electron density of the grid (Fig. 2). Although the gauze was not expected to contain silver, some micrometric particles of silver were sporadically identified by SEM/EDX. AFM revealed tetrahedron-shaped nano-crystals with an average height of 16 nm. However, also crystals of 120 nm height and 300 nm wide were observed.

The SEM analysis of material 2 revealed a highly homogeneous nanostructured surface with silver particles ranging from 50 to 200 nm. The AFM analysis was in a good agreement with the size distribution of the silver particles detected by SEM. The high-resolution AFM topography on the fibers in material 2 is shown in Fig. 2B. The average height of the predominantly cubic-shaped nano-crystals was 36 nm and crystals of 330 nm high and 700 nm wide were also sporadically observed.

The SEM morphologic analysis of material 3 (Fig. 1C) revealed a mixture of silver coated and uncoated fibers. The silver coated fibers were brighter because of the electron density of silver and the coating was not homogeneous on the fiber surfaces. Silver aggregates with a wide dimensional range (300 nm-1 µm) were found also on the surface of the uncoated fibers. High-resolution AFM imaging revealed spheroid-shaped particles with a wide size distribution and an average height of 11 nm (Fig. 2C). Some spheroid aggregates reached a height of 130 nm and a width of 700 nm.
4.3.3 The characterization of silver particles in the textiles after the release test

After 24 hours of immersion in synthetic sweat, silver-silver chloride aggregates were detected by SEM-EDX on the gauzes attached to the silver grid of material 1, as shown in Figure 3A (diameter: 1 µm). The SEM analysis of the material 2 (Fig. 3B) detected the presence of silver-silver chloride clusters in a wide range of dimensions. Sodium was also noticed as a component on the surface of the clusters. Silver-silver chloride clusters were also detected on the surface of the uncoated fibres of material 3 (Fig. 3C).
Fig. 3 SEM analysis of nanosilver textiles after 24 hour of immersion in synthetic sweat, morphologic and electron image of: (A) Material 1 gauze fiber; (B) Material 2; (C) Material 3.

4.3.4 The percutaneous penetration

The amount of silver in the donor compartment is given in Table 1 as exposure dose (µg of silver per exposed skin area) and as concentration of silver in the donor solution (µg/mL). The silver concentration in the donor fluid was the highest for material 1 (4.7± 1.9 µg/ml). This finding is consistent with the highest amount of silver found after acidic digestion of material 1 (1.37±0.05 mg/cm²) in comparison with materials 2 and 3 (Table 1). The release of silver in donor compartment reflects the silver content in textiles: material 1>material 3>material 2. Thus, a higher content of silver in the material results in a higher release of silver into solution.
The silver concentration in the receptor fluid of all diffusion chambers sampled up to 12 hours was below the ICP-MS limit of detection. Silver was detected in the receptor fluid collected at 20 hours, although the concentrations were just below the ICP-MS limit of quantification (data not shown). At 24 hours, silver was detected in all receptor fluid samples (Table 3) ranging between 0.13 and 0.19 µg L⁻¹, with similar results for all investigated materials.

The amount of silver in epidermis and dermis was considerably higher than in the receptor fluid. Again, the amount of silver found in epidermis and dermis was the highest for material 1. There was a large difference in the silver amount between two donors; this difference was higher in the epidermis than in dermis.

### Tab.3 Silver concentration in the skin layers (median; 25ᵗʰ-75ᵗʰ percentiles) and in receptor compartments (mean± SD), after 24 hour of exposure

<table>
<thead>
<tr>
<th>Ag concentration</th>
<th>Material 1</th>
<th>Material 2</th>
<th>Material 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis µg/cm²</td>
<td>1.05; 0.02-3.56</td>
<td>0.26; 0.01-0.55</td>
<td>0.33; 0.01-0.67</td>
</tr>
<tr>
<td>Dermis µg/cm²</td>
<td>0.30; 0.01-0.59</td>
<td>0.03; 0.003-0.08</td>
<td>0.07; 0.01-0.15</td>
</tr>
<tr>
<td>Receptor fluid Ag µg/L</td>
<td>0.13±0.08</td>
<td>0.18±0.09</td>
<td>0.17±0.11</td>
</tr>
</tbody>
</table>

### 4.3.5 Characterization of silver particles in the skin

By using SEM/EDEX we identified the presence of electrondense aggregates of silver and silver chloride with a wide size distribution both in epidermis and dermis for all investigated materials.

Fig. 4 shows representative images obtained by SEM/EDX analysis in 3 samples. There was a considerable difference in the number of silver aggregates found in the skin of the two donors. The skin of donor 2 showed a larger number of hair than donor 1 (Fig.5). Consistently, a larger number of silver aggregates were detected in donor 2 than in donor 1.
Fig. 4 Left: SEM image of silver clusters and in the skin layers: (A) epidermis, material 1, (B) dermis, material 2, (C) epidermis, material 3. Right: EDX spectral identification

4.4. Discussion

4.4.1 The characterization of silver in investigated materials

The amount of silver in tested materials varied from 3.6 to 8.1% w/w. As determined by SEM/EDX and AFM, the material 1 (Acticoat™) showed a wide size distribution of tetrahedron-shaped nanocrystals. Consistently with our findings, the morphologic variability of silver aggregates on the surface of the Acticoat was also reported by Rigo et al (2012). Moreover, the manufacturer declared the possibility of an inhomogeneous coating of the fibers with nanocrystals.
Material 2 and 3 showed more regular and homogeneous coverage of silver particles (Fig 2). The AFM analysis was in a good agreement with the size distribution of the silver particles detected by SEM. It has to be noticed that only in the case of material 1 the presence of a nanostructured covering is declared.

After 24 hour exposure test, the investigated materials showed electrondense aggregates on the surface; EDX detected the presence of silver and chlorine as being the major components of these aggregates. This is consistent with previous studies showing that silver is released from textile mainly as a silver ion which subsequently reacts with chloride ions from the sweat forming silver chloride (von Götz et al, 2013). As silver chloride is poorly soluble its precipitation limits the amount of dissolved silver available for penetration.

The AFM and SEM analyses gave comparable results on the size of silver particles on the material. Combining these two surface characterization techniques allows for a complete characterization of the size and morphological properties of the nanomaterials which are relevant for silver release and, consequently, for the antimicrobial activity. While SEM-EDX provides information on the composition and dimensions of the silver particles and clusters on the x-y plane, AFM shows the three dimensional profile of the particles found on the fabric surface. Furthermore, the resolution of AFM (1 nm) is better than that of SEM (30 nm).

4.4.2 Percutaneous penetration of silver

The release of silver from materials, which determines exposure concentration, differed among three investigated materials and amounted to respectively 4.0, 0.6 and 1.8 to µg/cm² (respectively 4.7 to 0.7 and 2.1 µg /mL). The silver release in material 1 was comparable to that found in the study of Rigo et al performed in saline environment which amounted to 4.21 µg /g (w/w Ag/saline solution)²⁶. Brett at al has reported in a review that the typical bactericidal silver concentration is about 1 mg/L, while Greulich et al reported a minimum inhibitory concentration (MIC) against S.
*aureus* in a range of 2.5-5 mg/L depending on the inoculated cell number of bacteria (Brett, 2006; Greulich et al, 2012). Thus, the silver amount released by the three materials is in the bactericidal range.

Silver could reliably be quantified (i.e. the concentrations above the limit of quantification) only in the samples of receptor fluids collected after 24 hours of exposure suggesting low systemic absorption. However, the levels of silver in the epidermis and dermis were considerably higher than in the receptor fluid revealing that silver is able to penetrate across the stratum corneum which is the principle barrier of the skin. Low concentrations of silver in the receptor fluid might at least partly be explained by formation of aggregates in the epidermis and dermis which slows down their further penetration. Since one of the main aims of this thesis is to characterize the silver particles also in the deeper skin layers, we preferred the full thickness skin above dermatomed skin. Likely, if dermatomed skin was used the penetration of silver into receptor fluid might have been higher.

The large dimensions of the silver-silver chloride aggregates detected in the dermis suggest that silver chloride precipitation and silver particles aggregation occurred in the deeper layers of the skin as the aggregates of this size can not penetrate across the SC. Consistently the findings reported in this thesis, van der Zande et al (2012) found in an *in vivo* study in rats that silver containing particles, presumably composed of silver salts, can be formed from silver ions. The silver containing particles (larger than 20 nm) were found in liver, spleen, lungs and in the gastrointestinal contents after exposition to both silver nanoparticles (<20 nm) and silver nitrate. Moreover, George et al (2014) found silver aggregates (750 nm) in the reticular dermis after *in vivo* exposure of human skin to material 1. Thus, the *in vitro* method applied in this thesis seems to be a good model for evaluating penetration of inorganic particles through human skin.

In this thesis, we encountered a large difference in the amount of silver present in the epidermis of two donors. This was consistent for all investigated materials implying that these differences were dependent on the donor skin. One of the reasons might be higher abundance of hair in the donor skin showing higher levels of silver in the dermis. The importance of hair follicles as a penetration
route for solid particles (as liposomes and nanoparticles) has been reported in literature (Lane, 2011). Baroli et al (2007) observed iron nanoparticles deposited below the viable epidermis and within and proximal to the hair follicles. However, the nanoparticles did not permeate through the skin. Moreover, Graf et al (2009) found that gold particles with dimensions of 161± 13 nm were able to penetrate the superficial layers of the SC and hair follicles. Also in the study of Graf et al (2009), no deeper penetration was observed. It has to be noticed that in the present study, silver containing aggregates were localized not only in the vicinity of the hair but also in other areas (Fig. 5).

The risk of systemic effect is likely caused by the total silver amount. But if also nanosilver is responsible for toxic effects (e.g. local effects in the skin), for risk assessment evaluation it is necessary to collect information whether, the size and how many AgNPs are able to permeate the skin. In this thesis only the total amount of silver that penetrated into and across the skin could be determined. The AFM and SEM analysis provided qualitatively the presence of silver particles in the skin, however the relative composition of silver species is not known. Furthermore, at present there are no reference values for silver particles.

It must be emphasized that penetration through the skin layers becomes more relevant in the presence of damaged skin, as for example in the case of dermatitis or burns. A damaged skin barrier would not only increase the amount of silver that penetrate across the skin but will likely enable penetration of larger nanoparticles. At present, there are only a few in vivo studies on silver uptake after the application of nanosilver textiles on damaged human skin (Rigo et al, 2013; Moiemen et al, 2011; Vlachou et al, 2007).
4.5. Conclusions

To our best knowledge, this is the first time that both the extent of percutaneous penetration of silver and at the same time the characterization of the silver particles in different skin layers were performed. In this thesis was demonstrated that the use of silver containing textiles leads to the release of silver and its penetration across the skin, implying that silver is systemically available. By using AFM and SEM/EDX we have shown the presence of silver particles of nano and micro size on the materials. We also identified silver containing aggregates of micro size in the epidermis and dermis suggesting these aggregates are formed in the skin which is consistent with recent in vivo
studies. Formation of these aggregates likely slow down systemic absorption of silver. On the other side these aggregates may form a reservoir enabling prolonged release of silver ions which might lead to local effects.

In order to evaluate if these striking findings would be confirmed in \textit{in vivo} conditions, further experiments have been performed as described in section 5.
5. DEVELOPMENT OF THE PROTOCOL FOR THE DETERMINATION OF SILVER UPTAKE IN VIVO

The results of in vitro experiments reported in section 3 and 4 highlighted the needing to better understand if in in vivo conditions (under real scenarios) the presence of silver particles in the skin layers, after exposure to silver textiles, could be confirmed. In this section, the protocol for a case-control study with human volunteers has been determined as well as the analytical methods for silver determination in biological matrices (Stratum Corneum, urines).

5.1. Background

As previously debated, it is known that silver containing textiles may release silver ions or nanoparticles that could penetrate the skin, mainly if damaged. Atomic spectrometry leads at the moment to advances in the analysis of nanomaterials; while Ag appears to be the most popular element for investigations*. Hyphenated techniques for the on-line separation of nano Ag of different diameter are dominated by a symmetric flow field flow fraction (AF4) coupled to ICP-MS, for an elemental specific detection in aqueous matrices (Poda et al 2011). Various human exposure routes to nano Ag are of interest; e.g. via inhalation (Braakhuis et al, 2014) and food contact materials (Echegoyen and Nerin, 2013). For related risk assessment methods were successfully developed by ICP-MS*. The biodistribution and kinetics of nano-Ag in in vivo animal test systems were studied after intravenous injection. A number of target tissues like e.g. liver, spleen, kidney, but also organs of special interest like brain, aortic samples were analyzed by ICP-MS too (Lankveld et al, 2010). By indirect exposure, skin was a less relevant tissue in these cases (Poda et al, 2011) and the dermal uptake of e.g. Ag as well as possible health effects are insufficiently known (Gopee et al, 2009). To assess the risk due to dermal exposure to a (nano)Ag containing product the ability of Ag to penetrate into and across the skin should be known. Bioimaging of metals in tissue sections by laser ablation (LA)-ICP-MS seems to be a powerful
imaging (mapping) technique for depth profiling of elements (Becker et al, 2010). However, skin samples must be taken by surgery which should not be a method of choice for operational testing of engineered, inorganic nanomaterials in consumer and personal care applications. In addition, the skin sample pre-treatment prior LA-ICP-MS can be problematic too.

The Stratum Corneum (SC) can be removed sequentially by repeated application of adhesive tape on a site of interest. Thanks to this procedure, more information about the function of SC as the main barrier for skin penetration can be obtained. The amount of SC removed is a key parameter to establish the concentration profile of released chemicals after topical application (Dreher et al, 1998; Hostynek et al, 2001). With this approach, only a few micrometer of SC are removed in a nearly painless manner and no surgery is required for sampling of the outmost skin layer.

In this section, a new method consisting of skin layer sampling, sample pretreatment and analysis by ICP-MS is developed for the measurement of the Ag uptake after direct skin contact. Interestingly, the developed approach for skin depth profiling highlights the relation between the Ag amount and the protein amount per skin layer; it is known from literature the binding affinity of thiols and amines (contained in proteins) to silver ions*. Moreover, due to the possible biodistribution within the whole body after skin absorption, additionally indirect exposure matrices, which can be reached e.g. via the blood circulation, are of great interest (Krystek et al, 2013). Therefore, the ultra-trace concentrations of Ag in urine are studied too.

5.2. Experimental

5.2.1. Chemicals

Ultrapure water (H₂O) with a resistivity of 418 MΩ cm (at 25 °C) was obtained from a Milli-Q Plus system (Millipore, Amsterdam, TheNetherlands). Hydrochloric acid (HCl), 30%, ultra-pure and nitric acid (HNO₃), 65%, ultrapure, were purchased from Merck, Darmstadt, Germany. Ammonium hydroxide (NH₄OH) (28–30%), analytical grade, was purchased from Sigma Aldrich, Milan, Italy.
Calibration standard solutions of Ag and the solution of the internal standard related to the ICP-MS measurement (chosen element: rhodium (Rh)) were made of single element stocks solutions with a concentration of 1 mg/mL from Inorganic Ventures, Christiansburg, USA. For the on-line addition of the internal standard, a dilution of 2 µg/L Rh in 2 % HNO₃ was prepared.

5.2.2. Materials and samples

The “silver sleeves” used in this pilot study were made of material 3 previously described in section 4. Two different fittings were tested; skinny fit medical garment intended for use in the treatment of Atopic Dermatitis. Volunteers with healthy skin have worn the “silver sleeves” (Fig.1) on the forearm 8hrs a day for 5 consecutive days. Among these volunteers, three subjects have worn the silver sleeves on both the forearms: on the left one 8 hrs a day, on the right one 24 hrs a day for five consecutive days. The different timings of exposure were applied in order to evaluate if 8 hrs per day could be sufficient to evaluate silver permeation in SC.

Fig. 1 Silver sleeves: skinny fit.

Some tape strips were sampled from unexposed areas as skin blank control. Skin sampling took place after the first and fifth days of exposure. These experiments were carried out under the supervision of the Academic Medical Center (AMC), Coronel Institute of Occupational Health,
Amsterdam, The Netherlands. The human skin removal for the preparation of SC layers was taken by round adhesive tape discs (polyacrylate ester adhesive, 3.8 cm², D-Squame; CuDerm, Dallas, TX, USA). The tape discs were applied to the skin of the forearm on the sites of interest. Each tape disc was pressed for 10s with standardized force of 225 g cm² using a disc pressure applicator (CuDerm, Dallas, TX, USA) (Breternitz et al, 2007). From each skin site of interest the entire SC was collected by approximately 20–25 consecutive tapes. Each tape disc was gently removed with tweezers and stored in a 1.5 mL Safe-locktube (Eppendorf, Nijmegen, The Netherlands) at -20 °C until analytical pretreatment. The amount of SC proteins on the tape disc was determined by measuring the light absorption of the disc at about 850 nm (infrared radiation) using the D-Squame Scan 850 A Instrument (CuDerm, Dallas, TX, USA) (Voegeli et al, 2007). The concentration of Ag on most of the tape discs was determined after leaching and dissolution by ICP-MS. Furthermore, a selected number of tape discs were used for the studies by SEM-EDX.

The spot urine samples have been collected from 30 subjects before exposure and after five days of wearing of “silver sleeves”. The urines were stored at -20 °C until analytical pretreatment and quantification.

5.2.3. Instrumentation

Before leaching Ag, a series of tapes was analyzed with SEM-EDX (type TM-3000 – equipped with Oxford Instruments X-ray Microanalysis Hitachi High-Technologies Europe GmbH, Krefeld, Germany) in order to evaluate the presence of silver nanoparticles. For the pretreatment by leaching, an ultrasonic bath type Elmasonic S30H (from Elma, Singen, Germany) is used. The elemental detection and quantification, is carried out by ICP-MS. A quadrupole (Q)–ICPMS type7500CE,AgilentTechnologies,Santa Clara, CA, USA, with integrated autosampler and a high resolution (HR)-ICPMS type ELEMENT XR, Thermo Fisher Scientific, Bremen, Germany, were utilized. Similar sample introduction systems consist of a concentric nebulizer, a cyclone spray
chamber and nickel cones (all from the instrumental suppliers) were used. The operating conditions as well as the method and measuring parameters of the ICP-MS instruments are summarized in Table 1. Sample pretreatment and analysis were carried out in a clean-room facility class 10,000.

**Table 1. Operating conditions of ICP-MS instruments**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Settings at Q-ICPMS</th>
<th>Settings at HR-ICPMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF power [W]</td>
<td>1500</td>
<td>1250</td>
</tr>
<tr>
<td>Cool gas flow [L/min Ar]</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Nebuliser gas flow [L/min Ar]</td>
<td>0.78</td>
<td>1.04</td>
</tr>
<tr>
<td>Auxiliary gas flow [L/min Ar]</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Isotopes</td>
<td>$^{107}$Ag$^+$</td>
<td>$^{107}$Ag$^+$, $^{109}$Ag$^+$, $^{103}$Rh$^+$</td>
</tr>
<tr>
<td>Method</td>
<td>Acquisition mode: Spectrum</td>
<td>Acquisition mode: E-scan</td>
</tr>
<tr>
<td></td>
<td>multitune</td>
<td>Mass window: 150%</td>
</tr>
<tr>
<td></td>
<td>Sample time: 30 ms</td>
<td>Search window: 150%</td>
</tr>
<tr>
<td></td>
<td>Samples per peak: 10</td>
<td>Integration window: 80%</td>
</tr>
<tr>
<td></td>
<td>Detection mode: dual</td>
<td>Sample time: 50 ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Samples per peak: 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Detection mode: triple</td>
</tr>
<tr>
<td>Number of replicates</td>
<td>3</td>
<td>9</td>
</tr>
</tbody>
</table>

**5.2.4. Samples pretreatment of skin layers**

The stored tape discs were directly pretreated in the Safe-lock tubes with a volume of 1.5 mL. Three different leaching and dissolution media were tested; details see Table 2. After 15 min of
sonification, the extract was transferred into 15 mL polypropylene vial (Sarstedt, Etten-Leur, The Netherlands) and the extract was diluted with water to a final volume of 10 mL.

Table 2. Composition of leaching and dissolution media.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nitric acid</td>
<td>+ 0.5 mL water + 1 mL HNO₃ (60%) ultrapure</td>
</tr>
<tr>
<td>2</td>
<td>Aqua regia</td>
<td>+ 0.5 mL water + 0.25 mL HNO₃ (60%) ultrapure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ 0.75 mL HCl (30%) ultrapure</td>
</tr>
<tr>
<td>3</td>
<td>Ammonia</td>
<td>+ 0.5 mL NH₄OH (28-30%) + 1 mL water</td>
</tr>
</tbody>
</table>

5.2.5. Samples pretreatment of urine

Urine sub-samples of 2 mL were transferred into 15 mL polypropylene vials prior adding 0.5 mL HNO₃ (60%) ultrapure and 1.5 mL HCl (30%) ultrapure. After 5 min shaking the samples were diluted with water to a final volume of 10 mL.

5.2.6. Quantifications by ICPMS and quality control aspects

Ag concentrations in extracts were determined by ICP-MS; the two available ICP-MS instruments were used interchangeably for the purposes of this study. As long as the measured Ag isotopes (107 Ag and 109 Ag) are interference-free, both instruments can be used as substitutes. All calibration curves were prepared in the same matrix as the related extracts were. By Q-ICP-MS, external calibration curves of 107 Ag were performed between 0 and 50 mg/L for skin extracts in ammonium hydroxide and in nitric acid. By HR-ICP-MS, external calibration curves of 107Ag were performed in the low-resolution mode (between 0 and 25 µg/L for skin extracts in nitric acid and between 0 and 75 µg/L for skin extracts in aqua regia); while 109 Ag was used for control.
However, for the measurement of Ag with adequate detection power in urine samples, a matrix match approach in aqua regia for the optimized calibration range (between 0 and 2.5 µg/L) was used. On-line addition and correction with an internal standard (103 Rh in the low-resolution mode) was applied too. For the analysis of both matrices (tape discs with SC and urine), the chemical blanks were controlled and the reported results are corrected for them. For quality control aspects of the instrumental performance, the standard reference materials 1640a (Trace Elements in Natural Water) and 1643e (Trace Elements in Water) from the National Institute of Standards & Technology (Gaithersburg, MD, USA) was controlled in each series. The analyzed results fulfill to the certified values of silver (8.081 ± 0.046) µg/L Ag respectively (1.09 ± 0.11) µg/L Ag.

5.3. Results

5.3.1. Imaging of silver particles in the Stratum Corneum on tape discs

SEM-EDX characterization of the textile composing the silver sleeves is reported in section 4. In Fig. 1 a photo of silver sleeves and of placebo sleeves is reported. For the identification of Ag particles on a tape disc after contact with treated skin, SEM-EDX was used too. The resulting image is given in Fig. 2. It is shown that mainly particles with a size of approximately 1 µm were formed. As investigated by EDX, these particles consist of Ag.
Fig. 2. Presence of silver on tape discs (layer 4 shown) after contact to exposed skin; image by SEM-EDX.
5.3.2. Comparison of nitric acid and aqua regia as leaching media for silver in the Stratum Corneum on tape discs

The most relevant aspect for method optimization deals with leaching and dissolution of Ag from the tapes containing SC prior quantification by ICP-MS. The leaching of Ag from tape discs has been investigated by nitric acid versus aqua regia and it has been applied for the time dependent silver permeation through 25 layers of SC. No interferences were found and the exposure to a placebo resulted in non-detectable values of silver. All results after wearing the “silver sleeves” for 8 hrs. per day on one respectively five days are summarized in Fig. 3. It has been demonstrated that the method was sufficiently sensitive to construct a curve of absorption of Ag versus the SC depth needed to deduct the percutaneous penetration parameters even after 8 hrs. of exposure. By comparison of two sleeve fittings, it has been shown that the skinny fit with a rather permanent skin contact leads to higher concentrations than the loose fit. The sleeve, even as skinny fit, cannot provide the same contact to the forearm skin in each point for geometric reason. Comparing the leaching profiles for the two people reported in this study (see Fig. 3A and B), it is possible to hypothesize that the variability in concentration could be mostly due to a different exposure to the textile of the single spots stripped than to a difference between the methods. The comparison between nitric acid and aqua regia shows comparable SC depth profiles (see Fig. 3A versus B) but at different concentration levels. This leads to additional information about the abundance of various Ag species (e.g. AgCl) with different solubility in the uppermost layers of the SC. The amount of Ag in the skin is dependent on exposure duration and the SC depth which is in accordance with the diffusion laws. The highest level of Ag has been found in the uppermost layers of the SC. Furthermore, repetitive exposure (i.e. exposure on five consecutive days) leads to higher amounts of Ag implying accumulation of Ag in the SC (see Fig. 3). With the developed procedures, the limit of detection (LOD) is 2 ng Ag per tape disc and the limit of quantification (LOQ) is 6 ng Ag per tape disc. The upper limit of applicability is depending on the used leaching medium. The
determination of methodological performance characteristic is restricted as long as independent
duplicate samples are necessary for the determination of e.g. the repeatability and the
reproducibility. Cutting a sampled tape disc for obtaining two independent samples is avoided due
to possible variation by inhomogeneous distribution of Ag on a tape disc. The same applies to
sampling at two independent skin positions. As the best approach, the repeatability was calculated
between couples of tape discs which were sampled directly after each other at the same skin site and
at nearly steady state concentration levels; see e.g. Fig. 3 (tape discs with numbers above no. 20).
Based on this approach, the repeatability for the leaching medium nitric acid is approximately 30% which is also related to lower concentration ranges of up to 7 times the LOD. For aqua regia, the
repeatability is approximately 20% for concentration ranges which are mainly above 10 times the
LOD.
Fig. 3 Concentration of silver in the SC layers obtained after wearing silver containing sleeves for 1 and 5 days.
5.3.3. Comparison of the “silver to protein” ratios on SC layers and testing of ammonia as leaching medium

Since the amount of SC which is collected by a tapedisc is variable, the concentration of Ag (in mg) has been normalized for the protein amount (in mg) on the strip as measured by IR spectrometry (D-Squame Scan 850A/ IR radiation at 850 nm). As an example the normalized Ag concentrations versus SC depth is presented in Fig. 4. For a limited number of samples, next to nitric acid and aqua regia, ammonia was tested as third leaching medium. The use of nitric acid and ammonia gave comparable results; see Fig. 4.

Fig. 4 Silver concentrations normalized for protein amount in the Stratum Corneum layers after leaching with different media.

5.3.4. Results of the systemic uptake by ultra-trace analysis of silver in urine
Based on the matrix composition (urine) and the chemical blanks, the LOD, which was determined under repeatability circumstances, amounted to 0.010 µg Ag/L urine. The concentrations of Ag in urine before and after exposure, that were determined in 30 urine samples, are low and ranged from <0.010 to 0.012 µg Ag/L urine in the samples before the exposure and < 0.010 to 0.23 µg Ag/L urine after five days of exposure. These results are within the concentration range reported in the literature for general population as studied in Sweden and Germany (Rodushkin et al., 2001a,b; 2004).

5.3.5. **Comparison between different time of exposure**

To evaluate if 8 hrs per day could be sufficient for the evaluation of silver absorption profile, three volunteers worn on the both forearms two silver sleeves with different timings. As shown in Fig. 5 the absorption profiles after 8 hrs or 24 hrs were comparable. Since 8hrs per day provide a more reliable scenario for risk assessment evaluation, this finding shows that the analytical method used in this thesis is enough sensitive for this specific case.

![Graph](image)

**Fig.5 Silver concentration found in volunteer 3 (pilot 3) after 8hrs and 24 hrs per day of exposure to silver garment**
5.4. Conclusions

In this section, a reliable and feasible ICP-MS method for profiling Ag in the outermost layers of SC is applied after *in vivo* dermal exposure. The method allowed for the measurement of the Ag concentrations at different depths of the SC enabling the deduction of the percutaneous penetration kinetics. The detection power of the method was sufficient to measure the ultra-trace concentrations of Ag in urine before and after dermal exposure. The developed methods were successfully applied within a clinical study for the assessment of dermal uptake of Ag from (nano) Ag containing textiles. This further investigation is reported in section 6. In this clinical study next to healthy volunteers also patients with a very common skin disease (Atopic Dermatitis) are investigated. As (nano) Ag is increasingly being used in medical garments, smart textiles, and toys, this method will facilitate a better control and monitoring of Ag release and absorption during dermal exposure enabling appropriate health risk assessment.
6. IN VIVO COMPARISON OF SILVER PERMEATION THROUGH HUMAN HEALTHY AND ATOPIC SKIN AFTER EXPOSURE TO A SILVER TEXTILE

Starting from the first in vitro experiments of section 3 until the preliminary in vivo experiments, of section 5, the findings reported in this thesis justified a further in vivo study. Briefly, once demonstrated that silver released from nano-silver containing textiles is able to permeate the skin and that silver particles are present in exposed skin, it is important to verify if this preliminary results are confirmed. In the final part of this thesis, the previously optimized protocol, described in section 5, is therefore applied to a larger population. Since one of the main targets of silver textiles is atopic skin, AD patients together with healthy controls were subjects of this investigation.

6.1. Background

The role of silver as topical agent to reduce the colonization of bacteria is well known and is strictly related to the release of silver ions. Silver has mainly been used for medical purposes e.g. in wound care, medicating clothing for health care personnel and in garments designed to combat super infections in patients with atopic dermatitis (AD). The treatment of wounds and burns with silver evolved from topical application of the silver nitrate solution to silver sulfadiazine cream. In the last decades the use of nanosilver as source of silver ions is strongly increased in various formulations (Ansari et al. 2011; Martinez-Castanon et al. 2008; Moiemen et al., 2011). Otherwise the use of silver in the form of thin coverings applied to textiles and polymers is increasing as well. As silver containing textiles are in direct contact over large body surface areas for prolonged periods this has raised concerns over possible adverse health effects, also realizing that these products are worn by individuals who might be increasingly susceptible e.g. children and individuals with skin diseases. Since even thin coverings of metallic silver could have a nanostructured surface (Oriňáková et al. 2013), the characterization of these silver materials at the nanometric scale is needed. Often, the presence of AgNPs in commercially available products is not stated by the manufacturer. Indeed,
for substances in textiles no registration is required in many EU countries, since textiles in the EU are treated as articles (von Götz et al., 2013). Substances in articles also do not need to be listed on a product label or be evaluated according to their potential for consumer exposure (von Götz et al., 2013). Dermal exposure to silver might potentially lead either to local effects in the skin like irritation or systemic adverse effects after its absorption in the body. The likelihood of such effects is dependent on the amount of silver which penetrates into and across the skin.

In section 4 the presence of AgNPs in the material was shown, subsequently in section 5 our pilot study highlights the possibility of AgNPs penetration in healthy skin after dermal exposure.

AgNPs devices nowadays are increasingly used to treat skin diseases whose symptoms are often associated with bacteria proliferation, such as Atopic Dermatitis (Jain et al. 2009; Keck and Schwabe, 2009; Kim et al. 2008). The European Scientific Committee on Cosmetics and Non-Food Products (SCCNP) nanotechnology report declares that the data on skin penetration of nanoparticles through diseased skin are limited. An impaired SC could not perform an efficient barrier to nanoparticles penetration increasing the risk of systemic uptake. In this section, thanks to a larger in vivo study we investigated the silver species permeated and retained within the SC of healthy and AD volunteers after the application of a nanosilver containing garment. AD patients have been selected from two reasons. Firstly, AD patients, in particular children are frequent users of silver containing garments due to often bacterial superinfections. Secondly, one of the main features of AD is reduced skin barrier thus they represent a model for damaged skin barrier. In addition to percutaneous penetration we investigated for possible proinflammatory effect associated with dermal exposure to silver and measured the levels of inflammatory mediators IL-1 and IL-1RA in the skin after exposure to silver. Therefore, a further purpose of this study was to compare the differences in the type and amount of silver particles penetrated between atopic and healthy skin.
6.2. Materials and Methods

6.2.1 Investigational material

For this study two sleeves (see section 4 and 5 for characterization and composition) marked with “L” (left arm) and “R” (right arm) were used in 5 different sizes to avoid that the sleeves are not too tight or too loose. One sleeve contained silver, the other one was a placebo i.e. silver free. A fiber coated with pure silver is knitted in such a way that silver yarn lies exclusively on the inside of the textiles (Fig. 1). The area of the sleeve was on average 600 cm².

The sleeves were worn for 8 hours during the night on 5 consecutive days. The exposed skin sites, sampling schedule and investigated parameters in the stratum corneum (SC) are shown in Fig. 1.

![Fig. 1 Investigated skin sites and sampling schedule](image-url)
6.2.2. Study population

30 Caucasian subjects (fifteen healthy subjects and fifteen subjects with AD), with a mean age of respectively 41.9 and 26.5 years participated in the study. The Medical Ethics Committee of the Academic Medical Center, University of Amsterdam, approved the experimental protocol. The study was conducted according to the Declaration of Helsinki Principles and informed consent was obtained from the participants before the start of the study. Participants did not use anti-inflammatory, anti-histamines and antibiotic medicine during, and 4 weeks prior the study period. Furthermore, volunteers with systemic inflammatory, immunological and malignant diseases except AD were excluded. No sunbathe during, and 4 weeks prior the study period was permitted. Also pregnant and lactating woman were excluded.

6.2.3. Calculation of the exposure concentration from the silver release test

As described in section 3, the concentration of Ag released into sweat solution after 8 hrs amounted to 300.3 µg/L which equals the release of 0.12 µg Ag / cm² of surface area. We assumed that in the in vivo dermal exposure all released silver will be transferred into the sweat layer on the skin. The volume of sweat has been estimated from literature data of sweat rate of 4.03 mg/cm³ (Eishi et al., 2002). Assuming density of sweat of 1 g/ cm³, the calculated total silver concentration (i.e. exposure concentration), was 29.8 µg/ cm³ (29.8 ppm). (0.12 / 4.03 x 10⁻³).
6.2.4. **Tape stripping of the stratum corneum**

The stratum corneum (SC) layers were sequentially removed with circular adhesive tapes ($3.8 \text{ cm}^2$, standard D-Squame, Monaderm, Monaco, France). The tapes were consecutively attached to the volunteer’s underarm and pressed for 10 seconds with a constant pressure as previously described (section 5). A total of 30 subjects were stripped for total silver and cytokines amount; among these the strips of 11 volunteers (7 healthy and 4 AD) were analyzed also by means of SEM-EDX and AFM.

6.2.5. **Determination of silver in tape strips**

Among the three extraction media previously tested (see section 5), nitric acid was selected. Briefly, to each tape strip 1 mL MilliQ water and 0.5 mL of ultrapure HNO$_3$ (69 %) was added. Samples were sonicated for 15 minutes and diluted to a final volume of 6.5 mL with MilliQ water. The total silver concentration in the tape strips was determined by an ICP-MS 7500 CE (Agilent Technologies, Santa Clara, CA, USA). The limit of detection of the method, LoD was 0.05 $\mu$g/L, RSD < 5 %).

6.2.6. **Determination of silver in urine**

Urine samples were collected before and after ending of the 5-days exposure. As only spot urine has been collected, the Ag concentrations were normalized with creatinine levels. The total silver concentration in urine was determined by a high resolution inductively coupled plasma mass spectrometry, HR-ICP-MS (Thermo Fisher Sientific, Bremen, Germany) (methodological limit of detection, LoD = 0.015 $\mu$g/L). For the quantification, an external calibration with internal standard correction by the element rhodium (Rh) was applied. For full method description see section 5.
6.2.7. Analysis of cytokines in tape strips

The cytokines, interleukin IL-1α, its receptor antagonist, IL-1RA were determined in the SC tapes after the 5th day collected from the exposed and placebo forearm. To determine the amount of soluble protein and cytokines, the tape strips were extracted in phosphate-buffered saline using ultrasound sonification (de Jongh et al., 2006). The concentration of soluble protein was determined with a Pierce Micro BCA protein assay kit (Thermo Scientific, The Netherlands) and cytokine concentrations were determined using ELISA assay (Bio-Source International, CA, USA) as described elsewhere (de Jongh et al., 2006).

6.2.8. Data analysis

To estimate the penetration parameters, approach based on Fick’s second law of diffusion, which applies to the non-steady state condition (Crank, 1975; Pirot et al., 1997), was used. In this method, two parameters, K and D/L^2, are determined by best-fit regression of the concentration of silver as a function of relative SC depth (x/L) (Fig. 3) to the following equation:

Equation 1:

\[ c(x) = K \cdot c_{veh} \left( 1 - \frac{x}{L} \right) - \sum_{n=1}^{\infty} \frac{2}{n\pi} K \cdot c_{veh} \sin \left( \frac{n\pi x}{L} \right) \exp \left( \frac{-Dn^2\pi^2t}{L^2} \right) \]

where \( c_{veh} \) is the applied silver concentration (i.e. the concentration of silver in the sweat layer covering the skin surface) (29.8 µg/cm³), \( c \) is the silver concentration (mg/cm³) at SC depth x (cm), K is the SC/water partition coefficient, L is the total thickness of the SC (cm), D is the apparent diffusion coefficient of silver through the pseudo-homogeneous SC (cm²/h), D/L² (h⁻¹) is the diffusion rate constant, and t is the exposure duration (h). In deriving equation, K and D/L² are assumed to be constant throughout the SC. The apparent permeability coefficient (\( K_p \), cm/h), which
is commonly used for the risk assessment was calculated from the relationship \( K_p = K \cdot \frac{D}{L} \). Dermal kinetics can be derived by this method only after a single exposure (Day 2). After day 5, only a cumulative amount of silver in the SC has been deducted from the area under the curve (AUC). For curve fitting and statistical calculations Prism 5 (Graph Pad Software Inc., San Diego, CA) was used. Student’s t-test (or Mann-Whitney test in case of non-Gaussian distribution of data) was used to test the differences between AD and healthy subjects. For cytokines, Wilcoxon matched pairs test was used to compare the values of the silver containing and placebo sleeves. P-value < 0.05 was considered significant. In the case of deviation from Gaussian distribution of data, median and interquartile ranges have been shown.

**6.2.9. SEM-EDX analysis of tape discs**

On average, 20 strips were sampled from the forearms of seven healthy subjects and four AD patients in order to investigate silver particle penetration in the SC after silver sleeve’s exposure. The strips were fixed rigidly onto a circular metallic sample holder by means of an adhesive tape (silver free). All the analyses were run at 15 kV. A prior analysis at 50x was performed to have an overview of the whole sample stub. SEM analysis has been done at magnification of 2000x to identify the presence of silver aggregates for 500 fields (corresponding to an area of about 4 mm\(^2\)). Any aggregate was then characterized at magnifications up to 8000x and the EDX spectrum was recorded. The starting field of analysis was randomly chosen at the upper left corner of the sample stub, and then the resting fields were selected at grid points formed by evenly spaced horizontal and vertical lines, with the distance between fields being sufficient to prevent field overlap.
6.2.10. **AFM analysis of SC on tape discs**

SC tapes (r=3.8 cm\(^2\)) were stored until analysis in the cryo vials at 4°C. Before imaging, strips were taken out with previously cleaned tweezers. An area of approximately 0.7 x 0.7 cm\(^2\) was cut with cleaned scissors as shown in the schematic drawing:

![Schematic drawing of area for AFM imaging](image)

The AFM imaging was performed using a Multimode AFM with Nanoscope IIIa controller (Bruker, Billerica, MA, USA) with a vertical engagement (JV) 125-µm scanner. The tapping mode was applied using silicon tips (RTESP, Bruker, resonance frequency 289–335 kHz, spring constant in the range of 20–80 N/m). The linear scanning rate was optimized between 1.0 and 2.0 Hz with scan resolution of 512 samples per line, except for large scans (15 µm × 15 µm) were resolution was 256 samples per line. The processing and analysis of images was carried out using NanoScope\(^{TM}\) software (Digital Instruments, Version V614r1). All images presented are raw data except for the third order two-dimensional flattening. For each SC sample we have recorded at least 3 randomly selected large areas. The large area images were used to select the areas of interest that were imaged at high resolution. The high-resolution images were analyzed for presence of Ag NPs and the objects suspected to be silver particles were measured using section analysis (height profiles along indicated lines). Scanned areas were 225 µm\(^2\), 25 µm\(^2\) and 4 µm\(^2\) corresponding to 15 x 15 µm, 5 x 5 µm and 2 x 2 µm scan size, respectively.
6.3. Results

6.3.1. Percutaneous penetration of silver as determined by tape stripping of the Stratum Corneum

Fig. 3 shows a typical concentration profile of silver across the SC obtained after the first 8 hours of exposure. The fitted curve represents the solution to the Fick’s second law of diffusion. The curve fitting could not be performed for 3 controls and 3 AD patients because of excessive variability of data points. The apparent permeability coefficient, \( K_p \) has been determined as described in Data analysis section. From the \( K_p \), exposed skin surface and exposure duration the steady state flux has been calculated as follows: \( J \text{ (mg cm}^{-2} \text{ h}^{-1}) = K_p \cdot C_{\text{veh}}. \) Steady state dermal fluxes of silver calculated as described above are shown in Table 1.

Figure 3. Concentration decay of silver as a function of normalized position \((x/L)\) in the SC after the first 8-\( h\) exposure to silver sleeve.
There was no significant difference in the fluxes between control subjects and subjects with AD (Table 1). By using the area under the concentration vs. depth curve (AUC) the amount of silver present in the entire stratum corneum has been estimated (Table 1). As seen from Table 1 the amount of silver in the SC was higher after Day 5 as compared to Day 2 which indicates accumulation of silver in the SC during repeated exposure. However, also for the absorbed amounts of silver in the SC there were no significant differences between healthy individuals and AD patients (Table 1).

Table 1. Dermal flux and absorbed amount of silver in the stratum corneum (SC) in control subjects (Ctrl) and subjects with AD (AD) after the 1st and 5th 8-h exposure. Results are expressed as median (25% and 75% quartiles).

<table>
<thead>
<tr>
<th>Exposure concentration</th>
<th>Ctrl</th>
<th>AD</th>
<th>Ctrl + AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>36.6 µg/cm³</td>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 24</td>
</tr>
<tr>
<td>Steady state flux mg/cm²/h *10⁻⁷</td>
<td>2.3 (1.2-3.8)</td>
<td>2.0 (0.8-4.1)</td>
<td>2.1 (1.1-3.8)</td>
</tr>
<tr>
<td>(1st exposure) Ag (ng) absorbed in the SC</td>
<td>57.4 (26.1-100.9)</td>
<td>73.9 (25.7-115.0)</td>
<td>62.7 (26.1-108.6)</td>
</tr>
<tr>
<td>(5th exposure) Ag (ng) absorbed in the SC</td>
<td>105.0 (57.3-156.6)</td>
<td>119.8 (65.7-262.1)</td>
<td>116.3 (59.0-186.0)</td>
</tr>
</tbody>
</table>

6.3.2. Silver concentrations in urine

The concentrations of silver in urine sampled before and after 5 days of exposure are shown in Table 2. There was a large interindividual variation in the concentrations of silver, even after normalizing with creatinine and there was no significant increase of silver in urine after 5 x 8 hours
of dermal exposure either in healthy controls or AD patients (as tested by a Wilcoxon matched paired test).

**Table 2. Silver concentrations in urine of control subjects (Ctrl) and subjects with AD (AD) before (Day 0) and after 5 days of exposure to a silver sleeve. Results are expressed as median (interquartile ranges).**

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>AD</th>
<th>Ctrl + AD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µg Ag/L of urine</td>
<td>0.014</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>(0.008 – 0.04)</td>
<td>(0.008 – 0.012)</td>
<td>(0.008 – 0.012)</td>
<td></td>
</tr>
<tr>
<td>µg Ag/mol creatinine</td>
<td>0.96</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>(0.57 – 2.3)</td>
<td>(1.1 – 4.0)</td>
<td>(0.7 – 3.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Day 5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>µg Ag/L of urine</td>
<td>0.020</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>(0.011 - 0.028)</td>
<td>(0.008 - 0.23)</td>
<td>(0.008 - 0.23)</td>
<td></td>
</tr>
<tr>
<td>µg Ag/mol creatinine</td>
<td>2.04</td>
<td>1.3</td>
<td>1.9</td>
</tr>
<tr>
<td>(0.38 - 4.2)</td>
<td>(0.39 - 16.4)</td>
<td>(0.9 – 3.3)</td>
<td></td>
</tr>
</tbody>
</table>

**6.3.3. IL-1 Cytokines in healthy and AD volunteers**

The results of IL-1 cytokines in the SC collected on day 5 after the last 8-h exposure are shown in Fig. 4. There were no significant differences in the levels of investigated IL-1 cytokines between silver- and placebo treated arm in both healthy controls (CTRL) and AD patients, which indicates absence of any pro-inflammatory effect of silver in the garment. The values of IL-1RA were higher in AD patients compared to controls, independently on the exposure (i.e. silver containing sleeve or placebo).
**6.3.4. Silver particles in the SC tape strips**

Table 4 summarizes the information regarding SEM-EDX analysis of inorganic aggregates found in healthy and AD subjects exposed to the silver garment. As shown in Table 4, the silver particles were detected in all SC samples collected from the silver exposed forearm in both healthy and AD subjects. The presence of silver aggregates was shown even in the 25\textsuperscript{th} strip. Silver aggregates were located both in the intercellular space (Fig. 7B) and on the corneocyte surface (Fig. 7A, 7C). As assessed by the SEM-EDX analysis, silver aggregates varied in the morphology with a dimensional range of 150-2000 nm with irregular shapes (Fig. 7C, 7D).
Fig. 7 SEM-EDX image of SC tape strips after topical application of silver garment. A: micrometric aggregates revealed. Onto corneocytes surface in the 3° strip; B: micrometric aggregates in the intercellular space; C: submicrometric aggregates onto corneocytes and in the intercellular space; D: micrometric aggregates onto corneocytes surface. EDX confirmed the presence of silver as main component of the aggregates

Most of silver aggregates showed in the EDX spectra the presence of silver as major component although some aggregates showed next to silver also chlorine, calcium, potassium, sulphur and sodium (Tab.1). Silver aggregates were easily distinguished from other inorganic crystals by SEM-EDX analysis (Fig.8).
Fig. 8. SEM-EDX image of tape strips. A: 5° strip of SC with inorganic particles with different morphology, B: EDX spectrum of silver aggregate; C: EDX spectrum of an inorganic crystal (probably a sulfate) composed of potassium, sodium and sulphur.

The electrondensity of silver resulted in brighter particles respect to other inorganic salts (Fig. 8A); moreover the shape of silver aggregates was very irregular often revealing the single components borders. In the control samples (placebo exposed skin) no silver was detected. In addition to silver aggregates SEM-EDX revealed the presence of KCl particles (0.8-2.0 µm) both on the corneocyte surfaces and in the intercellular space. However they differed in morphology from silver aggregates as they showed more regular and compact shapes (Fig.9).

Fig. 9 SEM-EDX image of tape strips. A: 5° strip of SC with inorganic particles with different morphology than silver aggregates, B: EDX spectrum of particles revealed a compositon of potassium, sodium and sulphur.

No differences were found between healthy and AD subjects regarding the morphology and number of the silver aggregates revealed.
Tab.4 SEM-EDX results of SC samples analysis in healthy and AD subjects.

<table>
<thead>
<tr>
<th>ID volunteer</th>
<th>Skin typology (H/ AD)</th>
<th>Ag aggregates 1-10 layers (Y/N)</th>
<th>Ag aggregates 11-25 layers (Y/N)</th>
<th>Location of Ag aggregates</th>
<th>Presence of other inorganic particles</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>AD</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface</td>
<td>Few particles (about 1 µm) containing titanium were found in the intercellular space of tape n. 10</td>
<td>Silver aggregates were the most abundant form, but also few aggregates with Ag-Cl-K-Na were found</td>
</tr>
<tr>
<td>26</td>
<td>AD</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface, few in the intercellular space</td>
<td>Few particles (about 1 µm) containing titanium were found in the intercellular space of tape n. 15</td>
<td>Silver aggregates were the most abundant form, but also few aggregates with Ag-Cl-K-Na were found</td>
</tr>
<tr>
<td>28</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface, few in the intercellular space</td>
<td>Submicrometrical particles of KCl were found on the corneocytes surface and in the intercellular space (tape n. 1)</td>
<td>Silver aggregates were the most abundant form, but also few aggregates with Ag-Cl-K-Na were found</td>
</tr>
<tr>
<td>29</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface</td>
<td>Submicrometrical particles containing K-Cl-Ca-Al-Ti were found on the corneocytes surface and in the intercellular space (tape n. 1)</td>
<td>The aggregates in this volunteer were more abundant than in other subjects. Silver aggregates were the most abundant, but also few aggregates with Ag-Ca-Al were found</td>
</tr>
<tr>
<td>Pilot 1</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface, few in the intercellular space</td>
<td></td>
<td>Silver aggregates were the most abundant form, but also few aggregates with Ag and Cl were found</td>
</tr>
<tr>
<td>Pilot 2</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface, few in the intercellular space</td>
<td></td>
<td>Only silver aggregates were found.</td>
</tr>
<tr>
<td>Pilot 3</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface, few in the intercellular space</td>
<td></td>
<td>Silver aggregates were the most abundant form, but also few aggregates with Ag and Cl were found.</td>
</tr>
</tbody>
</table>
As the elemental composition cannot be determined by AFM, the identification of silver particles was based on the particle shape (sharp edges, pyramidal or oblong shape, clusters) and comparison between placebo and silver exposed samples. In addition, the recorded phase images provide a clear distinction between “organic” and “inorganic” particles. Sometimes, large objects were encountered but the edges were not sharp. In such cases we changed the imaging parameters by applying a larger force while imaging. This way we succeeded in peeling-off the organic layer (presumably lipid) uncovering a sharp objects. Table 5 summarize the most striking results of AFM analysis performed on tape strips of healthy and AD subjects exposed to the silver garment.

**Tab.2 Silver particles and nanoparticles revealed on SC tapes revealed by AFM analysis**

<table>
<thead>
<tr>
<th>ID volunteer</th>
<th>Skin typology (H/AD)</th>
<th>AgNPs 1-10 layers (Y/N)</th>
<th>AgNPs 11-25 layers (Y/N)</th>
<th>Location of AgNPs</th>
<th>Presence of aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>AD</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface</td>
<td>Aggregates were found</td>
</tr>
<tr>
<td>25</td>
<td>AD</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface</td>
<td>Large aggregates were found</td>
</tr>
<tr>
<td>26</td>
<td>AD</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface</td>
<td>Agglomerates and aggregates were found</td>
</tr>
<tr>
<td>29</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface</td>
<td>Agglomerates were found</td>
</tr>
<tr>
<td>31</td>
<td>H</td>
<td>Y</td>
<td>Y</td>
<td>Mostly onto keratinocytes surface</td>
<td>Few agglomerates</td>
</tr>
</tbody>
</table>

According to AFM analysis, inorganic nanoparticles (30-100 nm), with a morphology specific for AgNPs were detected mostly on the cells, fewer in the space between the cells (Tab. 5). Consistently with the SEM-EDX analysis, also AFM revealed presence of aggregates and agglomerates (Fig.10).
Fig. 10. AFM imaging of SC strips. Phase and Amplitude Images and depth profile of silver aggregates

In some images, it can be seen that AgNPs are covered by a lipid layer suggesting penetration across the lipid bilayers (Fig.11).
Fig. 11. AFM imaging of SC strips. Silver particles appear covered by lipids

Consistently with SEM-EDX analysis, no differences were revealed between healthy and AD subjects regarding the number and typology of nanoparticles and aggregates or agglomerates found.
6.4. Discussion

6.4.1. Total silver permeation through healthy and AD skin

Wearing of silver containing sleeves resulted in low but measurable amounts of silver in the SC even after the first 8 hrs of exposure revealing that silver is able to penetrate into and across the SC. The concentration-depth profiles of silver obtained from the silver concentrations in different layers of the SC after the first 8 hrs exposure period enabled the determination of the steady state dermal flux which amounted to respectively 2.0 x 10^{-7} and 2.3 x 10^{-7} mg/cm^2/h in the AD patients and healthy controls. There were no significant differences in dermal fluxes between healthy subjects and AD patients although it has to be noted that the skin sites investigated for the percutaneous penetration were not visually affected by disease and the patients had only mild symptoms.

Literature data on \textit{in vivo} silver absorption after dermal exposure are scarce and to our best knowledge this is the first study in which dermal flux has been determined after repeated, controlled exposure. Several studies reported systemic uptake of silver by measuring increase of silver plasma and urine levels in patients with burns treated with silver dressings, however the quantitative relationship between exposure to silver in these studies and the silver levels in biological samples is difficult to establish (Moiemen et al., 2011).

Several studies showed that silver is released from fabrics mainly in ionic form as Ag\(^+\) although dependently on the physico chemical properties of used silver and method applied to treat the fibers, also metallic silver particles of nano or micro size can be released (Stefaniak et al., 2014; von Götz et al., 2013). Silver ions readily react with different constituents present in the sweat on the skin surface and dependently on the solubility of these compounds silver can be present in dissolved form or as particulate. Silver ions, its soluble forms, but theoretically also particles of several nm size could penetrate into the skin. Von Götz et al. (2013) investigated in an experimental setting the release of silver into artificial sweat from various textiles with claimed antimicrobial properties and found particles smaller than 450 nm in detectable amounts (23–74 µg of silver/g of textile/L of sweat). The concentration of soluble Ag species which are able to penetrate the stratum corneum
varied in that study from 43-45 µg/g/L. Stefaniak et al. (2014) found that the relative amount of ionic silver is also dependent on its concentration in sweat solution. At higher concentrations the silver rapidly reacted with sweat chloride ions to form poorly soluble silver chloride particles. In contrast, at lower silver ion concentrations measured in the use scenario experiment, silver was primarily in the ionic fraction. Clearly, the prediction of the concentration of bioavailable silver is the biggest challenge in determining the percutaneous penetration of silver and assesses the health risk for a certain exposure scenario. In this thesis for the calculation of kinetic parameters we assumed that all silver which is released from the garment is available for penetration and further that silver present in the SC is available for systemic absorption.

The question arises whether dermal absorption under real life exposure scenarios can lead to adverse health effects. For this purpose we calculated the hypothetical uptake of silver in a baby who wears a comparable silver containing garment for 8 hours/day. There is currently no reference dose for dermal exposure but US Environmental Protection Agency (EPA) has established a chronic oral Reference Dose (RfD) for silver ingestion of 5 µg per kg of body weight per day based on a review of 70 cases of argyria that were associated with oral and other uses of silver compounds (EPA, IRIS). Argyria is a medically benign but permanent bluish-gray discoloration of the skin. To calculate the uptake associated with dermal exposure the following equation has been used:

$$\text{Dermal uptake (mg/kg/day)} = \frac{J\ (mg/cm^2/h) \cdot \text{Exposure area (cm}^2) \cdot \text{Exposure time (h)}}{\text{Body weight (kg)}}$$

The skin surface area of a child from three to six months of age is estimated to be 0.38 m² (EPA, Exposure factor handbook, 2011) with average body weight of 7.4 kg. Contribution of trunk, arms and legs to the total skin surface is 70 % and corresponds to 0.266 m² (EPA, 2011). The experimentally determined flux of 2.1 mg/cm²/h has been used for the calculation (Table 1). Assuming that a child (average body weight of 7.4 kg) is exposed to silver coated textile covering
only trunk, legs and arms (with skin surface area of a 0.266 m$^2$) during 8 hrs and that all textile is in close contact with the skin, the average dermal uptake can be calculated using the equation:

\[
\text{Dermal uptake (mg/kg/day)} = \frac{2.1 \times 10^{-7} \text{ mg/cm}^2/\text{h}.2660 \text{ cm}^2.8 \text{h}}{7.4 \text{kg}} = 6.01 \times 10^{-4} \text{ mg/kg/day}
\]

This equals to the uptake of 0.6 µg/kg of body weight/day. This value is below the RfD value of 5 µg per kg of body weight per day. For comparison, the current norm for drinking water in the USA is 0.1 mg/L (EPA, 2012). Assuming daily drinking water consumption for the 10 kg child to be 1 L/day, the uptake via water is approximately 0.01 mg/day/kg which is higher than dermal uptake 0.6 µg/kg of body weight/day calculated above. It has to be noted that the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. In general, silver garments are used only in periods with disease exacerbations and furthermore AD clears up during infancy or childhood in majority of children (Garmhausen et al., 2013) so the exposure to silver will be intermittent and over a limited period during a lifetime. Low systemic uptake as calculated from the experimentally determined dermal flux is consistent with low amounts of silver detected in urines. The concentrations of silver in urine before and after exposure were within the concentration range (Table 2) reported in the literature for general population which is caused by environmental exposure to silver e.g. via drinking water and food (Rodushkin et al., 2001a,b; 2004) (Fung et al., 1996). Clinical studies in patients treated with silver medical products for severe burns have shown that elevated silver levels were detectable in urine within 24 hrs, thus the exposure time of 5 days applied in this thesis would have been long enough to detect increase of silver in urine (Wan et al., 1991). Boosalis et al. (1987) found a value of approximately 1100 µg/L for the mean urinary silver excretion after treatment for severe burns with
silver sulfadiazine. This value is so much higher than the average levels detected in this thesis of 0.02 µg/L. Exposure to silver did not lead to increased expression of IL-1 cytokines in the SC. These cytokines are highly abundant in the SC and are released after the damage of the skin barrier or inflammation. IL-1α when released, mediates local inflammation, which is prevented by IL-1RA. Though, in placebo treated skin IL-1RA was higher in AD patients as compared to healthy controls which can be explained by increased inflammatory status in atopic dermatitis (Kezic et al., 2013).

6.4.2. Silver particle penetration in healthy and AD Stratum Corneum

In a restricted number of subjects, next to percutaneous penetration, we characterized the silver particles in the SC. The presence of AgNPs and their aggregates in different matrices was detected using two techniques: AFM and SEM-EDX. The main advantage of AFM is high resolution (1-2 nm), but on the other side absolute identification and elemental analysis is not possible. For that reason we did additionally SEM-EDX analysis. Both techniques used in this thesis don’t need any preparation of the samples before the analysis; therefore the possibility of artifacts is strongly reduced. Moreover, SEM-EDX and AFM analysis were performed by two distinct laboratories with different instrumentations showing consistent results.

The garment characterization, fully described in section 4, revealed the presence of silver particles on the textile surface of nano and micro size. After dermal exposure, the presence of silver containing particles of different size and composition was shown across the whole SC in both atopic and healthy skin (Fig.7, Fig.8); while on the other side these particles were not present in the placebo samples. Interestingly, most of the Ag-particles on the strip 1 (i.e. approximately 2nd-3rd layer of the SC) appeared between the cells, whereas the preferred localization on the deeper strips was rather on cell surfaces. In some cases, the EDX spectra revealed also sulphur and chlorine as components of silver aggregates found in SC. The presence of chlorine in the aggregates could be
ascribed to the reaction of silver ions released from the garment with chlorine which is as one of the main components of biological media. The formation of silver chloride after immersion of nanosilver textiles in biological media was previously reported (Impellitteri et al. 2009; Stefaniak et al. 2014). Stefaniak suggested that silver chloride binding on nanoparticles surface is more reliable than silver chloride precipitation. The presence of sulphur in silver aggregates might reflect silver binding to cysteine residues of skin proteins as, for example, keratin (van der Zande et al. 2012). Our findings are consistent with previous studies which reported the presence of silver salts (AgS and AgSe) in different organs after oral exposure to AgAc and AgNO₃ (Liu et al. 2010; Loeschner, 2011). Furthermore, the formation of AgS and AgSe in the skin was also reported in argyria patients (Massi and Santucci, 1998).

As AFM can not provide the elemental composition of the particles, the first selection was made according to the morphology of the particle and in comparison with the placebo samples and further SEM-EDX analysis. SEM resolution was sufficient to highlight morphology differences between (larger) silver particles and other inorganic salts found in the SC for which EDX confirmed that these particles indeed do not contain silver (Fig.9).

To date, there are few in vivo studies concerning the penetration of AgNPs in human healthy skin after topical application (George et al. 2014; Prow et al. 2011; Zhu et al.; 2015) and only some papers reported the penetration of AgNPs in burned skin (Rigo et al. 2013; Trop et al. 2006; Vlachou et al. 2009; Wang et al. 2009). Consistently with our results, George et al. (2014) showed the presence of silver clusters up to 750 nm in size below the epidermis after the 5-day dermal exposure of 16 healthy subjects to nanocrystalline silver dressing. Prow et al. (2011) reported by using reflectance confocal microscopy the presence of AgNPs aggregates in furrows and hair follicles after treatment with an AgNPs spray. In a recent study Zhu et al., (2015) showed by using a sensitive surface-enhanced Raman scattering that the penetration depth of coated AgNPs (70 ± 20 nm) can exceed the stratum corneum. As in that study AgNPs were coated, it confirms that next to Ag ions also AgNPs can penetrate through the SC, although according to the authors it was not
clear whether the presence of AgNP is caused by the penetration of AgNPs through the SC or by the measurements inside the hair follicle. Some authors state that the skin penetration of nanoparticles larger than several nm through the intercellular space is not possible due to the small dimensions of the lipid bilayers which are the principal penetration route for most of chemicals (Watkinson et al., 2013).

Nevertheless, in this thesis SEM/EDX shown the presence of silver clusters on the cell body and furthermore AFM images revealed AgNPs and a lipidic layer on the silver aggregates surface (fig.7). Even if silver nanoparticles penetration has been demonstrated, this process is thought to be occasional; therefore the formation of such large silver aggregates shown in this study and in literature could be more likely due to silver ions precipitation.

The aggregation of silver in the SC might explain the low penetration of silver that we previously found both in in vitro and in vivo conditions. This is consistent with previous studies which report low serum and urinary levels of silver after topical application of a nanosilver textile (George et al. 2014). Interestingly, George found that silver nanoparticles released by a nanosilver textile (10-40 nm) could diffuse through the epidermis, but there was no increase in serum silver levels in the subjects exposed to the nanosilver garment.

On the other side, silver particles found in SC layers can be seen as reservoir for silver ions that could prolong the antibacterial effect in the skin. As the aggregates are of μm size, they will not be able to penetrate further and will eventually be removed from the SC by desquamation. In that way, the formation of aggregates can be seen as a detoxification pathway as less silver will reach the systemic circulation. On the other side, the risk of local effects (e.g. pro-inflammatory effect) of silver in the skin will theoretically be increased. However, the analysis of IL-1 cytokines (reported in paragraph 6.4.1.) revealed the absence of the inflammatory pathway in the SC.

Previously, the permeation of AgNPs through the SC was reported by Larese (2009) in in vitro conditions. Larese showed significant differences between intact and damaged skin permeation of
AgNPs dispersed in synthetic sweat. This justifies the hypothesis of higher penetration of AgNPs through atopic skin since it is known that the SC of AD patients has a reduced barrier function. In this thesis, at the first sight no clear differences between atopic and healthy skin penetration of AgNPs have been revealed; otherwise it has to be noted that the number of subjects considered was limited and the number of the clusters at certain depths has not been assessed in a systematic manner. However, there was no striking difference between the atopics and non-atopics concerning the number and size of the clusters and their abundance at different depths. In addition the patients involved in the study had either no active disease or only mild symptoms.

Even if the penetration of AgNPs through the skin was shown in different models and under a wide range of scenarios (Larese et al. 2009; Samberg; Tian et al. 2007, Zhang and Monteiro-Riviere 2013), there are no data about the fate of silver particles in the SC or in the uppermost layers of viable dermis. This should be investigated in further studies.

6.5. Conclusions

The in vivo study reported in this last section contains several uncertainties which should be considered by the interpretation of the results. Several assumptions have been made to calculate the exposure concentration. It has been assumed that the total amount of silver found in the release test is available for dermal absorption. However, as addressed previously released silver ions in contact with sweat forms complexes and salts which limits the amount of dissolved silver available for penetration. Furthermore silver can be present as particulate (von Götz et al., 2013) that might have different penetration kinetics dependently on the particle size. As, in this thesis a complete speciation of silver released has not been done we assumed the worst case scenario, i.e. the total amount of silver which is released from the garment into sweat is available for penetration. Another assumption made in the present study is that silver present in the SC is available for systemic absorption. However, silver ions once in the SC can react with different functional groups of the present proteins and free amino acids which might reduce the amount of silver available for the
penetration and systemic uptake. The value for the volume of sweat on the skin surface needed for the calculation of the exposure concentration has been taken from the literature (Eishi et al., 2002). Possible changes in the sweat volume due to absorption into the garment or occlusion have not been taken into account. In conclusion, the in vivo data based on the percutaneous penetration and excretion of silver in urine revealed that dermal absorption of silver after wearing a garment containing 13% silver is low and not likely to lead to systemic effects. Furthermore, dermal exposure to silver from the investigated garment does not lead to altered expression of inflammatory IL-1 cytokines in the skin indicating absence of local pro-inflammatory effect.
7. Final conclusions and future perspectives

One of the most important results of this thesis, from a methodological point of view, is the good agreement between the findings derived from the in vitro and the in vivo studies of silver permeation through human skin. Since the use of in vivo studies is limited, both for practical and ethical reasons, it is important to verify the trustworthiness of in vitro methods and avoid misinterpretations.

We have demonstrated that silver released in synthetic sweat from the tested materials reaches the antibacterial concentration needed for the therapeutical use. There is the needing of a sensitive technique for the speciation of silver released. Indeed, to date it is hard to evaluate if silver is released in the form of nanoparticles or ions without introducing artifacts. It is even more difficult to understand if silver nanoparticles found in solution are migrated from the material or due to precipitation phenomena of ions released.

We have highlighted the importance of the characterization at the nanometric scale of silver textiles in case of risk assessment evaluation. Indeed, we found the presence of silver nanoparticles in the three tested materials although in only one case there was the statement of nanoparticles containing material.

Even if the in vivo study conducted in this thesis had some limitations, the data obtained under this scenario revealed that dermal absorption of silver after wearing the garment is low and not likely to lead to systemic toxicity. In the short term exposure the retention of silver did not result in any local pro-inflammatory effect. Another important finding is the presence of silver micro-aggregates in the skin after exposure to the nanosilver garment. It is important to investigate the destiny of these aggregates in order to understand if, in case of prolonged exposure, they could be reservoir species for further ion release or if they could be related to the developing of Argyria. These findings strengthen the need to evaluate both the systemic effects after a prolonged exposure in subjects with damaged skin and the fate of the silver species retained in time.
References


EPA Integrated Risk Information System (IRIS) (http://www.epa.gov/iris/subst/0099.htm)


EPA 2012 Edition of the drinking water: Standards and Health Advisories, EPA 822-S-12-001, Office of Water U.S. Environmental Protection Agency Washington, DC.


Labouta, H.I., Schneider, M., 2013. Interaction of inorganic nanoparticles with the skin barrier: current status and critical review, Nanomedicine. 9, 39-54.


