Silver nanoparticles: an overview of scientific toxicity and safety data and introduction of a new dressing, Venus Ag

KEY WORDS
- Nanoparticles of silver
- Safety
- Silver dressings
- Toxicity

Nanotechnology has opened a new area of scientific research. This field deals with materials within the dimensions of 1–100nm and a plethora of new technologies have emerged. In wound care, silver nanoparticles are used to aid wound healing as an antimicrobial agent, but also as an anti-inflammatory agent. The properties of silver nanoparticles differ from that of the material on a larger scale and their production can be controlled to give varied properties and characteristics that have different uses. These resultant properties are very important and differences in characterisation can alter their biological and physical attributes. All wound dressings have to undergo rigorous scrutiny around toxicity and safety when regulatory review is undertaken, yet some users still have concerns over long-term effects of silver nanoparticles *in vivo*. This review will address some of these concerns and reviews the current health and safety data associated with introduction of new products containing silver nanoparticles using Venus Ag dressings (SFM LTD, UK) as an example.

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Nanoparticles are clusters of atoms in the size range of 1–100 nm (Williams, 2008, Wong, 2010; Ge et al, 2014) and show unique physical, chemical and biological properties compared with their macro-scaled counterparts due to their high surface-to-volume ratio (Li et al, 2001). Nanomaterials are classified according to their dimensions, morphology, state and chemical composition (Gleiter, 2000), and they can be further divided into four sectors based on zero, one, two or three dimensions. For example, nanorods, quantum dots, nanowires and nanotubes (Saleh, 2020).

Silver nanoparticles (AgNPs) are the most widely exploited and have been incorporated into a range of wound care applications including composite fibres, textiles and wound dressings. (Caro et al, 2016; Balanga et al, 2020; Tremiliosi et al, 2020). There are several common methods of producing silver nanoparticles, these include mechanical milling, laser ablation (Zhao et al, 2006), sputtering, vapour deposition (Dunn and Edwards-Jones, 2004) and other chemical processes (Pal et al, 2007; Almatroudi, 2020). The various AgNPs produced are usually characterised using varying analytical methods such as Surface-Enhanced Raman scattering (Elechiguerra et al, 2005), high thermal and electrical conductivity, catalytic activity (Ribeiro et al, 2014) and non-linear optical behaviour. The physical, biochemical and antimicrobial properties vary depending upon the size (Sotiriou et al, 2010), shape, surface charge (El Badawy et al, 2011), coating (Yang et al, 2012), and solubility (Wu et al, 2008) and these differences confer different optical, magnetic and catalytic properties. In antimicrobial assays, Pal et al (2007), showed that truncated triangular AgNPs displayed the strongest antimicrobial action, compared with spherical and rod-shaped nanoparticles and the silver ions from $AgNO_3$.

AgNPs are incorporated into wound dressings, primarily for their antimicrobial properties, but they can also have modulatory effects (through inhibition of matrix metalloproteases MMPs) on wound healing (You et al, 2017). The level of AgNPs within the dressing has to be optimised so that there is sufficient to excert antimicrobial activity against the microbial cell yet, simultaneously, be safe for use against the eucaryotic cell (the host cell) within

Box 1. Mechanism of action of silver ions

- In bacterial cells, Ag^{+} inactivates respiratory and intracellular enzymes by reacting with sulphydral groups (Chappell and Grenville, 1954). Consequently, processes such as cell respiration, ion transport, (Marambio-Jones and Van Hoek, 2010), ATP production and DNA replication (Feng et al, 2000) are affected
- Silver ions interchelate with DNA, binding specifically with GC groups (Rosenkranz and Rosenkranz, 1972; Modak and Fox, 1973; Feng et al, 2000), so disrupts replication and reproduction
- Interaction with sulphur-containing membrane proteins cause disruption in the membrane structure. This can lead to increased membrane permeability and allow more silver to enter the cells (either ionic or nanoparticle form) and could possibly cause cell lysis in some cases. AgNPs have a large surface area-to-volume ratio and provide good contact with the bacterial cell membrane, allowing attachment and subsequent penetration of the particle. Once inside, sustained release of the Ag^{+} ions enhances bactericidal activity (Bryaskova et al, 2011).

the wound bed. This review will address some of the concerns around AgNPs and review the current health and safety data associated with them.

ANTIMICROBIAL EFFECT OF AGNPS ON MICROBIAL CELLS

Different oxidation states of silver exist, Ag^{+}, Ag^{++} and, Ag^{+++}. However, singly charged silver, Ag^{+}, is considered the most biologically active with its availability dependent upon the solubility of the salt used (Richards et al, 1991). Ag^{++} and Ag^{+++} show some antimicrobial activity but are more likely to form insoluble complexes and be rapidly inactivated by the proteins, phosphates, sulphates and chlorides frequently found in tissues and wound exudate (Khansa et al, 2019). AgNPs and subsequently Ag^{+} has a broad spectrum of activity and acts on multiple sites within the bacterial cell (*Box 1*) (Feng et al, 2000; Dibrov et al, 2002), and inhibits the growth of bacteria, viruses and yeasts at concentrations between 8–80 parts per million (ppm) (Liao et al, 2019). AgNPs interact with structural proteins on the surface of extracellular viruses inhibiting their entry to cells at an early stage, by either damaging proteins or preventing viral attachment (Galdiero et al, 2011: Jeremiah et al, 2020).

The addition of AgNPs to dressings allows a continuous slow release of silver ions from the nanoparticles, which can extend the use of dressings over several days. This sustained release of silver ions allows levels to be maintained a therapeutic concentration of >30ppm (Khansa et al, 2019). AgNPs have now been incorporated into a variety of different carrier dressings from fibres to hydrogels (Singh et al, 2021) and their broad-spectrum activity against a wide range of wound pathogens, including multidrug (antibiotic) resistant (MDR) strains, make their use an excellent alternative to other antiseptics and antibiotics.

ANTI-INFLAMMATORY EFFECT OF AGNPS

The anti-inflammatory effect of AgNPs has been extensively assessed using a variety of *in vitro* (cell lines e.g. fibroblasts and keratinocytes) and *in vivo* (murine, guinea pig, rabbit and pig) models and show a positive effect on wound healing (Nadworthy et al, 2008; Hebeish et al, 2014; Parnsamut et al, 2015; Hartmann et al, 2016; You et al, 2017; Nešporová et al, 2020).AgNP's can bind to metallothioneins, cysteine rich proteins found in the host cell, and this contributes to tissue repair. (Lansdown, 2006). Dressings such as Acticoat inhibit or sequester matrix metalloproteinases (MMP) and modulate the immune system by their removal from the wound bed (Wright et al, 2002; Walker et al, 2007; Nešporová et al, 2020). Using a porcine model, healing, inflammatory response, restoration of the epithelium and blood vessel and collagen formation were compared up to 15 days post-wounding using Acticoat and a polyurethane film control dressing. No difference was found in the rate of healing, however, the epithelium of the Acticoat-treated wounds more closely resembled normal epithelium and contained a higher proportion of mature blood vessels, and collagen deposition. This showed that Acticoat has a beneficial effect on healing compared to the controls (Hartmann et al, 2016).

A 1% w/w AgNP cream suppressed the expression of interleukin-2 and tumour necrosis factor α (TNFα), in a mouse model (Bhol et al, 2004) and a topical AgNP dressing suppressed inflammatory cytokines and induced apoptosis in inflammatory cells in mice with allergic contact dermatitis (Bhol and Schechter, 2005). In patients, toxic epidermal necrolysis and Stevens-Johnson Syndrome showed marked improvement when treated with a topical nanocrystalline silver dressing (Acticoat) (Asz et al, 2006; Dalli et al, 2007).

The inflammatory response associated with the topical delivery of AgNPs was further investigated

by Tian et al using a mouse model. The authors analysed the expression patterns of IL-6, TGF-β1, IL-10, VEGF, and IFN-γ using a quantitative real-time RT-PCR and confirmed modulation of the cytokine profile by AgNPs. They also demonstrated reduced scar appearance in the presence of AgNP and supported the beneficial application of AgNPs in wound care (Tian et al, 2007). You et al (2017) showed that at a concentration of 10ppm, AgNPs promoted the migration of fibroblasts, and expressed higher levels of the marker α-smooth muscle actin (α-SMA), indicating the capability of AgNPs to transform fibroblasts into myofibroblasts and to speed the wound healing process. They concluded that AgNPs of certain size and concentration could represent a valuable tool to maintain a reasonable activation of macrophages thus modulating the local inflammatory response (You et al, 2017).

Therefore, *in vitro* studies imply the use of AgNP dressings could have a bi-functional effect, depending upon the AgNP incorporated into the dressing: toxic to microbes yet modulatory to the immune system.

TOXICITY OF SILVER NANOPARTICLES

Silver has been used topically for over 50 years in burns and in recent years in chronic wounds, and the reported cases of silver toxicity are limited. Reports of localised argyria (a result of silver toxicity that turns skin, eyes, internal organs, nails, and gums a blue-gray colour) following application of silver sulphadiazine have been reported (Payne et al, 1992; Tomi et al, 2004; Trop et al, 2006) but since the controlled usage of silver in dressings these reports are limited (McCague et al, 2016). When used topically, silver ions are absorbed across the skin and enter the systemic circulation as a protein complex to be eliminated by the liver and kidneys. If levels of silver are too high, then systemic toxicity may manifest, with diarrhoea, stomach irritation, decreased respiration and damage to the liver and kidneys (Lansdown, 2006). A study undertaken by Vlachou and colleagues (2007), on 30 burn patients, showed an increase in silver blood levels when treated with an AgNP dressing. The median of 56.8μg/L correlated with the size of the burn and length of exposure to silver and levels returned to to normal (1–10μg/L) within six months post treatment. No long-term effects were noted (Vlachou et al, 2007).

Most studies of AgNP's toxicity to human cells are based on *in vitro* cellular experiments using silver ions and relatively short-term animal experiments, so interpretation of any results with real-life needs caution (Asghari et al, 2012; 2016: Atiyeh et al, 2007; Poon and Burd, 2004; Skebo et al, 2007). It is therefore important that *in vitro* assays are interpreted carefully, as results of effects on single parameters may not reflect the interaction in a complex wound environment that has complex architecture in the epidermis and dermis (Lansdown and Williams, 2004).

Dissolution of AgNPs into silver ions is the main factor leading to toxicity in test systems and this has been repeatedly shown, indicating the toxicity arises from ionic silver rather than the intrinsic property of AgNPs (Trop et al, 2006; Aranout et al, 2012). AgNPs have reduced activity when ion formation is removed (Xiu et al, 2012). When both AgNPs and ionic silver were tested for antibacterial efficacy under strict anaerobic conditions, it was shown that ionic silver was more effective than AgNPs, supporting the hypothesis that the primary toxic action of nanoparticles is from ion release (Xiu et al, 2012) Prevention of ion formation by the addition of the anti-oxidant, N-acetylcysteine, to AgNPs was shown to inhibit antimicrobial activity (Kim et al, 2007; 2009), suggesting that bioactivity arises from the ionic silver released by nanoparticles in an oxygenated environment. Further studies on dissolution of AgNPs in ultrapure water suggested that toxicity to bacteria increased with a higher dissolution rate and this was proportional to the specific surface area of the nanoparticle (Helmlinger et al, 2016). The rate and extent of dissolution *in vitro* is determined by the chemical composition of the media; the temperature; pH; the concentration of nanoparticles in solution; the size of the nanoparticle and the coating of the nanoparticle (Liu and Hurt, 2010; Ma et al, 2011). Whether this also happens *in vivo* is not known and NIOSH (the National Institute for Occupational Safety and Health) raised concerns about the significant lack of technical knowledge pertaining to the mode of action and long-term effects of AgNPs *in vivo*

(NIOSH, 2021). It has been suggested that AgNPs might act as a "Trojan horse", bypassing typical barriers based on size and then releasing high levels of silver ions at the site of potential damage (Park et al, 2010; Lubick, 2011).

AgNPs are 1–100nm in size (over 100X larger than a silver ion, approxima 115pm equivalent to 0.115nm), therefore concern over the size of the nanoparticle and ability to overcome the body's normal protective barrier must be based on the unique properties of AgNPs compared to the normal ionic silver species (Nasterlack, 2008; Schulte et al, 2008).

In 2009, Tang et al showed, in rats, that AgNPs were able to enter the bloodstream and cross the blood-brain barrier, where they induced damage to the barrier membrane and subsequently caused astrocyte swelling and neurodegeneration in the brain. This paper is regularly cited in the case for AgNPs' toxicity. However, several issues were raised on the design of the study, namely: the AgNPs were administered into rat models by subcutaneous injection rather than allowing normal levels of elution from a wound dressing. The quantity injected into each rat was 62.8mg/kg — over 12,500 times the maximum dose recommended by the FDA (5µg/kg/day). Therefore, it was unsurprising that such a large dose caused damage. A study into accumulation of silver in the liver, spleen and kidneys was undertaken on rats and it was shown that a dose of <10mg/kg AgNPs was safe for biomedical application and there were no observed side-effects. However, a higher dose >20mg/kg was toxic (Tiwari et al, 2011).

In human neutrophils, AgNPs were shown to induce apoptosis and act as an inhibitor of protein synthesis (Poirier et al, 2014). An interaction with mitochondria and induction of the apoptosis pathway via the production of reactive oxygen species, which leads to cell death had previously been demonstrated (Hsin et al, 2008). Reports of cytotoxicity to several cell types, including human peripheral blood mononuclear cells, human alveolar epithelial cell line, human alveolar macrophage cell line, neuroendocrine cells, rat liver cell line and mouse germline cells, continues to confound the problem (Shin et al, 2007; Greulich et al, 2011: Pratsinis et al, 2013). It is suggested that AgNPs are ionized in the cells, leading to activation of ion channels and changes in the permeability of the cell membrane to both potassium and sodium studies cite organ toxicity after administration of AgNPs; with the liver, lungs and brain affected but whether this is following dissolution and release of silver ions is not elucidated (Ahamed et al, 2010). Hepatoxicity may be due to thiol-rich proteins in the liver, such as glutathione, which may act as a reservoir for silver (Knetsch and Koole, 2011).

Translocation of AgNPs from wound dressings into deeper tissue, brain and other organs is unlikely from lower extremities (as in chronic wounds) as low-pressure venous flow and gravity to reduce movement towards the barrier is small (Tiwari et al, 2011).

Using a transmission electron microscope, it was shown that AgNPs can enter the stratum corneum and upper epidermis, tending to accumulate near the hair follicles. Using a static model, it was shown that AgNPs (below 30nm diameter) passively penetrated the skin (Tinkle et al, 2003). Using Polyvinylpyrrolidone (PVP)-capped AgNPs, Larese et al (2009) demonstrated that there was a 5-fold increase of AgNPs transversing abraded skin compared with intact skin. A recent study using ICP-MS detected silver nanoparticles transferring across porcine membranes, demonstrated the potential for toxicity if accumulation occurred within local or adjacent tissues (Zanoni et al, 2021). However, the concentration of silver ions in dressings, and the subsequent dose eluted from the dressings required to achieve any organ toxicity is still not clear. Therefore, it is important to establish the degree of migration of nanoparticles from a dermal wound, the ability of the body to remove nanoparticles translocated from the wound site, and the resultant levels of silver ions before reaching firm conclusions.

Further studies of dermal toxicity are of paramount importance going forward if AgNPs are to be used and, whatever *in vivo* method is chosen, it should be representative of realistic doses and modes of entry into the body or wound environment (Singh et al, 2021).

Long-term application of topical antimicrobial silver or AgNPs, either in ointments or dressings, should be frequently monitored by clinicians reviewing its administration.

EVIDENCE FOR THE SAFETY OF SILVER NANOPARTICLES IN DRESSINGS

Wound exudate can bind excess silver ions and form bio-inactive salts, which act as a protective mechanism against metal toxicity (Lansdown, 2005). Whereas this may be a major feature for silver ions, it is not known whether this is applicable to fully inactivate AgNPs to a safe level. In physiological salt concentrations, AgNPs show a tendency to form large aggregates (Lok et al, 2007), which reduces the problem of small size particles translocating through the skin and this slows down the penetration into viable skin layers (Bianco et al, 2016). It has also been shown that the AgNPs from dressings remain in the wound locality and do not penetrate deeper because AgNPs primarily act on bacterial cells near the surface (Knetsch and Koole, 2011). Alteration of the structure of the AgNPs in the wound dressing may reduce toxicity and PVP-capped AgNPs have a slightly negative ζ (-10mV) charge, which generates an electrostatic repulsion from the surface charge on the cell membrane of bacteria. Uncapped AgNPs had a surface charge of –28mV and as the electrostatic potential was less than the capped AgNPs, there was a greater attraction between them and the bacterial cells with increased antimicrobial activity (El Badawy et al, 2011).

When introducing any medical device to the market, it is important to validate any possible negative effects against positive benefits of the device. Compliance with an International standard ISO 10993, compiled by a panel of international experts from scientific and industrial communities, must be met in order to achieve acceptance by the regulatory bodies for medical device. (ISO 10993, 2018).

INTRODUCING VENUS AG

Venus Ag™ (SFM-LTD, Coventry, UK) is a new dressing, which is a soft conformable, non-woven fabric made from sodium carboxymethyl cellulose (CMC) and strengthening cellulose fibre(s). The structure remains intact, thus minimising pain when removing the dressing. There is low lateral wicking to protect the periwound area and the dressing has low shrinkage. It has a high fluid absorbency and supports autolytic debridement and entrapment of bacteria and cell debris within the dressing. The indications for use are second-degree burns, lower leg ulcers, pressure ulcers and diabetic foot ulcers, surgical wounds and wounds that are prone to bleeding.

The silver nanotechnology is effective against biofilm and a broad spectrum of pathogens such as MRSA. More importantly, *in vitro*, Venus Ag has a low cytotoxicity profile compared to other commercially available silver dressings (data on file, SFM Ltd).

Safety studies on a new dressing, Venus Ag, have been undertaken and the dressing has recently received CE (conformité européenne) marking from Europe and is approved for use in humans, having undergone extensive testing with reassuring results.

In safety testing, Venus Ag dressings were shown to be non-toxic, and have no detrimental effect on wound healing when tested in a rat and porcine model (data on file, SFM Ltd). Silver ions are released into the dressing from fibres impregnated with spherical PVP-capped AgNPs, approximately 5–30nm (mean 14nm) in diameter, imparting an antimicrobial action in the dressing when wound exudate encounters the dressing. Venus Ag contains carboxymethyl cellulose fibres, a component used in clinical wound care in a variety of dressings for more than 30 years, including in Aquacel Ag. Using these attributes, the new dressing removes exudate and kills organisms within the dressing. The AgNPs do not elute from the dressing, thus reducing any toxicity issues. There are different formulations and weights of dressings produced (120, 160 and 200gsm in rope and flat form).

Estimating the amount of available AgNPs eluted within the dressing was undertaken by taking samples of Venus Ag 200gsm weight fabric incubated for 72 hours. The liquid was analysed using a UV spectrophotometer to estimate the concentration of AgNPs released from the dressing into the liquid. The estimated quantity of silver was shown to be 1.6µg/day for a 70kg adult, far below the WHO recommended limit of 5µg/kg body weight/day (data on file, SFM Ltd).

As the Venus Ag dressing is intended for prolonged contact with breached or compromised skin, an assessment of cytotoxicity, skin irritation, skin sensitisation, acute systemic toxicity, sub-acute/sub-chronic toxicity, implantation and material-mediated pyrogenicity was carried out using appropriate *in vitro* and *in vivo* models accepted to

comply with ISO 10993 testing. All results of these tests were passed for the criteria set by the standard (ISO 10993,2018) and deemed safe for use with low skin and cell toxicity.

One concern of the manufacturer's was that if the dressing is worn for long periods, then AgNPs may become embedded in the tissue and continue to release silver ions after the 4-week treatment period, therefore, exposing the wearer to silver for a longer time period. Due to this reasoning, the Venus Ag dressing was tested for genotoxicity, carcinogenicity and chronic toxicity to be compliant for an additional ISO 10993 category (part 3).

With regards to the assays for cytotoxicity (using appropriate cell lines), skin sensitisation (using guinea pigs) and skin irritation (using New Zealand white rabbits), acute systemic toxicity (injected into white mice), material-mediated pyrogenicity (rabbit pyrogen test), and sub-acute/sub-chronic toxicity effects (using a rat model), Venus Ag at three different concentrations of AgNPs demonstrated reassuring results, passing all criteria set. Wound-healing studies in pigs and rats also provided evidence of a good healing performance, without signs of toxicity (data on file: SFM ltd).

Other detailed biological, chemical and physical analysis was undertaken on the dressing and a comprehensive risk analysis was independently conducted for Venus Ag and all risks were identified and mitigated. The reports on biocompatibility tests according to ISO 10993 concluded that Venus Ag is safe.

Currently, detailed patient studies are being undertaken to evaluate their effectiveness.

CONCLUSION

It is agreed that the concentration of released silver ions is a significant contributor to the toxicity of AgNPs, even if there is disagreement on other factors (Matzke et al, 2014). It appears that there are particle-specific effects, but it is unknown whether these directly contribute to the toxicity. The smaller the particle, the greater the surface area-to-volume ratio and therefore the greater potential of silver ions being released. The shape of the AgNP is important, particularly with reference to the surface area-to-volume ratio in terms of ionic release potential. Assuming silver ion concentration is the key driver for AgNP toxicity, then it may be possible to estimate toxicity by estimating the concentration of silver ions released from the AgNPs. More data and studies are needed to confirm the *in vivo* effects of Ag^{+}.

The concerns generated by *in vitro* studies are not replicated in *in vivo* studies or during clinical use (Samberg et al, 2010). There are numerous reports of using silver products in wound care and AgNPs have been used for over 20 years without any major adverse event (Wound International, 2012). All nanoparticle use needs to be monitored but it appears that, for AgNPs in wound care, their benefits outweigh the risk.

Conflict of interest

The work was commissioned by SFM Ltd. the manufacturer of Venus Ag. The author has no conflict of interest to declare.

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