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Note

Combined multiphoton imaging-pixel analysis for semiquantitation of skin penetration of gold nanoparticles

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ABSTRACT

Interaction of nanoparticles with the skin barrier is a recent area of research that draws a lot of attention from the researchers. However, monitoring nanoparticles in or through the skin is mainly based on qualitative microscopical techniques. Yet, a quantitative approach is required for a better basic understanding. In response, a combined "multiphoton-pixel analysis" method was developed in this study for semiquantitation of gold nanoparticles penetration into different skin layers. The developed approach provides a useful tool for future studies focusing on skin penetration of nanoparticles for the aim of health risk assessment or for the design of topical and transdermal drug delivery systems.

Studying the skin penetration of nanoparticles is of great pharmaceutical importance for the design of potential topical and transdermal nanocarriers. Understanding the behavior of nanoparticles and their interaction with different skin layers would ultimately lead to the design of the "ideal" carrier (Schneider et al., 2009). Hence exploration of the relevant parameters of the nanoparticles, e.g. size, shape, surface chemistry, influencing skin penetration of nanoparticles is necessary. Furthermore, the amount of penetrating particles is crucial. Quantitative estimation of skin penetration of nanoparticles is however problematic due to detection limits of the available techniques with regard to the scarce concentrations of nanoparticles present in the skin in typical permeation–penetration experiments. The uncertainty whether penetration really occurs and the expected reduced diffusion rates of nanoparticles (Schneider et al., 2009) are the key reasons for that. Therefore, monitoring skin penetration of nanoparticles has been based mainly on qualitative visualization by microscopy techniques. These include electron (Samberg et al., 2010), fluorescence (Mortensen et al., 2009), confocal and multiphoton (Stracke et al., 2006), Raman (Patlolla et al., 2010) and nuclear (Kiss et al., 2008) microscopy. Other analytical methods including inductively-coupled plasma mass (Huang et al., 2010) and atomic absorption (Wu et al., 2009) spectroscopy do not detect the particles themselves. A quantitative approach based on nanoparticles' detection would allow a better understanding of skin penetration of nanoparticles and provide a sound scientific basis for diverse biomedical applications and health risk assessment.

We have established a method by multiphoton microscopy for investigating skin penetration of gold nanoparticles (AuNP) based on gold luminescence (Labouta et al., unpublished). The objective of this study was to explore this method for semiquantification of AuNP penetrating into the stratum corneum (SC) and deeper skin layers (DSL). Pixel frequency was chosen as an indicator for the amount of AuNP avoiding the limitations of intensity measurement introduced previously (Kuo et al., 2009).

Thiol-coated AuNP dispersion (diameter = 6.00 ± 0.81 nm) were applied on human skin *in vitro* at c_{AuNP} = 437 μg/ml in a vertical Franz diffusion set-up at 32 °C for 24 h under occlusive conditions. Skin was then longitudinally cryo-sectioned, 10 μm thickness, at −20 °C and subjected to two-photon excitation fluorescence microscopy (ZEISS_LSM_510_META system, Carl Zeiss, Jena, Germany). On sectioning, skin piece was not placed parallel to the cutting blade to avoid dislocation of the particles from outside into DSL or vice versa, but in a perpendicular position limiting sectioning artifacts (Fig. 1). Furthermore, imaging was done within the tissue and not on the surface of the cut. Longitudinal skin sections were used over full-thickness skin to reduce loss of laser power going deeper inside the skin. A wavelength of 800 nm and energies of 0.485 and 0.647 mW in the focal plane were used for both excitation of AuNP and scanning the skin, respectively. Signal due to gold luminescence and a light transmission image of the skin were simultaneously collected with no signal interference among tracks.

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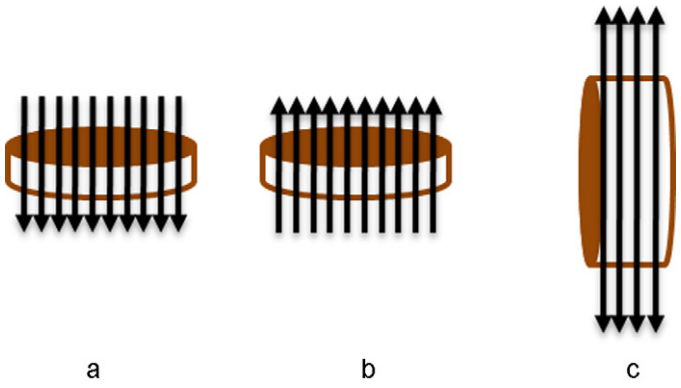

Fig. 1. A schematic presentation showing different possibilities for longitudinal cryosectioning of a skin punch. The arrows indicate the direction of cutting. Placing the skin punch parallel to the cutting blade (a, b) results in possible dislocation of the particles on the surface into the DSL (a) or vice versa (b). On the other hand, placing the skin punch in a perpendicular position (c) (adopted cryosectioning method) avoids particle dislocation limiting sectioning artifacts.

Images of the longitudinal skin sections were then analyzed using ZEISS-LSM software, also feasible by any image analysis software. Semiquantitative data for the distribution of AuNP in different skin layers were extracted as follows: z-stacks, with optical layers 1 μm thickness each, were adopted for analysis. The starting position ($n = 1$) was defined as the first optical layer with detected signals in the AuNP track. Similarly, the end position ($n = x$) was the last optical layer showing AuNP. Each optical scan was composed of 512×512 pixel^2 and 71.4×71.4 μm^2. For each layer, the intensity was first thresholded in order to remove the contribution of the background such as second harmonic generation from collagen (Sugata et al., 2011). The pixels due to gold luminescence were determined in the SC and in the DSL for this optical layer. Summing up these values for all the optical layers of the z-stack resulted in Σpixel frequency (ΣPixel) due to AuNP in the SC and in the DSL of this z-stack. Fig. 2 illustrates the method used for semiquantitation.

Finally, ΣPixel of AuNP was normalized to determine the weighed number of particles (N_w) as follows: Considering an emission spectrum for AuNP of $\lambda = \sim 530$–640 nm (Farrer et al., 2005), the mean theoretical lateral resolution (r_{xy}) was calculated from Eq. (1) (Jonkman and Stelzer, 2002) as ~0.341 μm, i.e. the area of a single diffraction-limited AuNP (A_{particle}) = ~0.365 μm^2.

$$r_{xy} = \frac{0.7\lambda}{\text{NA}} \tag{1}$$

where NA is the objective numerical aperture. Knowing the area of one pixel (A_{pixel}), 0.139×0.139 μm^2 in this study, N_w, the weighed number of nanoparticles, is calculated from:

$$N_w = \frac{\sum \text{Pixel} \times A_{\text{pixel}}}{A_{\text{particle}}} \tag{2}$$

This method of analysis has the advantage of measuring the number of events not their intensity. Therefore, the gain settings of multiphoton imaging could be freely adjusted for each measurement individually according to the energy of the laser required to excite AuNP at different depths in the examined skin specimens reducing the limitations for typical comparative measurements. Only the objective (water immersion objective, 63× magnification, NA = 1.2) and the image size should be kept the same throughout the measurements. This represents a great advantage over methods based on intensity measurement (Kuo et al., 2009), where the gain settings have to be fixed for all experiments. This would rather limit the imaging capability of nanoparticles in deep positions of some imaged skin specimens resulting possibly in inaccurate results. This limitation was discussed by Gratieri et al. (2010) when measuring the intensity of quantum dots as an indicator of their concentration in different skin layers using multiphoton microscopy. Our method overrides previous attempts involving manual counting of the number of fluorescent spots per field in confocal images

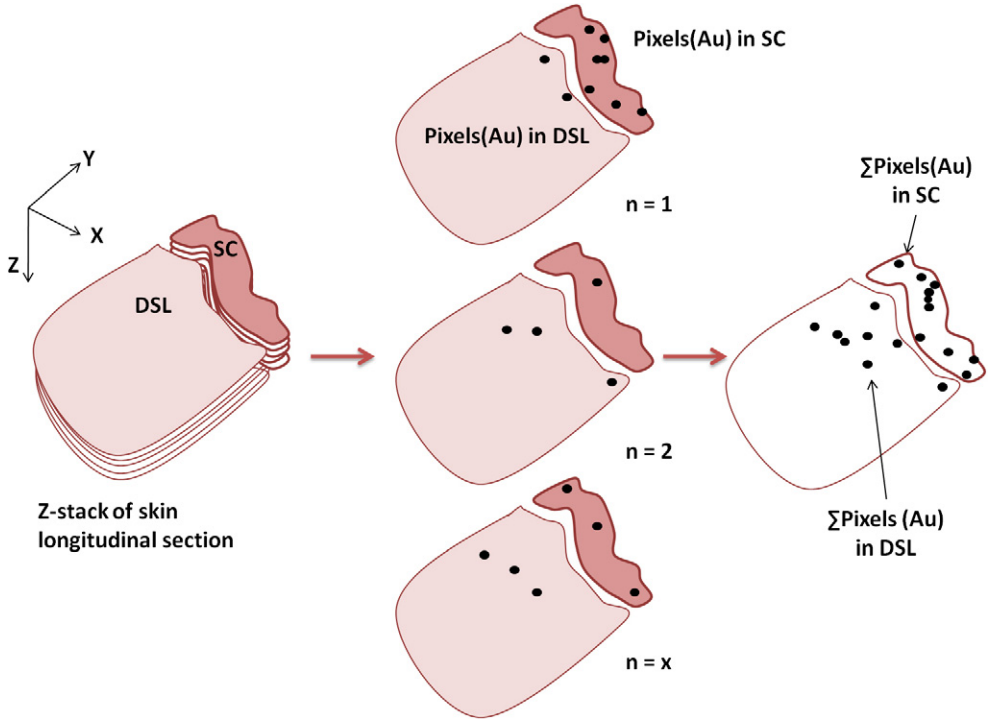

Fig. 2. A schematic presentation of the experimental approach used for semiquantitation of the penetration of AuNP in a z-stack of a longitudinal skin section imaged by multiphoton microscopy.

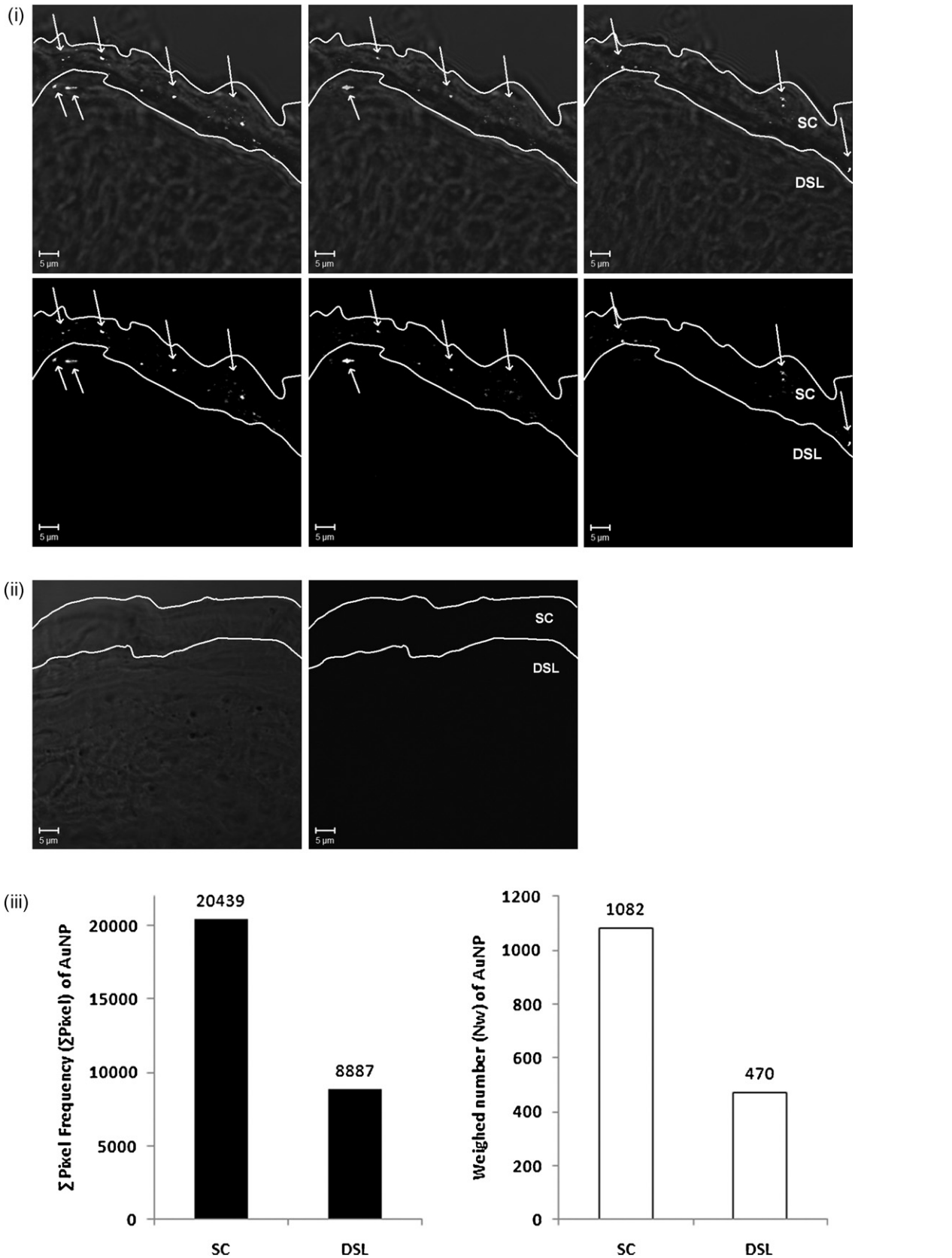

Fig. 3. (i) Representative overlaid multiphoton/transmission images (upper panel) and the correspondent AuNP track (lower panel), each showing AuNP (indicated as white spots) at different optical layers of a z-stack of a longitudinal skin section, in which different amounts of AuNP in the SC and DSL after 24 h of skin exposure were detected in each layer. A single layer is not descriptive for the overall penetration pattern. This is in comparison to (ii) an only vehicle-treated control skin specimen; the left image is an overlaid multiphoton/transmission image and the right image is the gold track only. (iii) Skin penetration of AuNP into the SC and DSL, expressed as Σpixel frequency of AuNP (ΣPixel) in all optical layers of the z-stack was determined and the respective weighed number of particles (N_w) were then calculated, as shown above, showing depth-profile for AuNP concentrating more in the SC rather than in DSL. Note that pixel values due to AuNP nanoparticles were recorded following thresholding of background intensity. Therefore, zero pixels were recorded for control skin specimens (not displayed).

(Mahe et al., 2009). Apart from human errors, adoption of the latter approach results in overlooking the area of the fluorescent spots if larger than the resolution limit.

Moreover, a previous attempt to analyze fluorescence images (Upadhyay, 2006) used only one image field of the examined skin for each experimental condition for analysis. However, as shown in Fig. 3(i), one optical layer is not always descriptive for the overall penetration profile of nanoparticles. On the other hand, calculation of ΣPixel and N_w of AuNP in the SC and DSL in optical z-stacks of the examined longitudinal skin sections showed a depth-profile for AuNP concentrating more in the SC rather than in DSL (Fig. 3(iii)).

In conclusion, a combined multiphoton imaging-pixel analysis approach was developed for semiquantitation of AuNP population in different skin locations in terms of pixels, from which the weighed number of particles could be calculated. These values could be used to determine the amount of AuNP penetrating into the SC and DSL in the same skin penetration experiment and correlate data among different experiments. The experimental approach described herein provides a valuable tool to advance our understanding of the interaction of nanoparticles with biological barriers, e.g. skin, and help identify various factors that enhance or limit their penetration. For future work, this method should be validated by the aid of analytical techniques such as inductively-coupled plasma mass and atomic absorption spectroscopy.

Conflict of interest

The authors state no conflict of interest.

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