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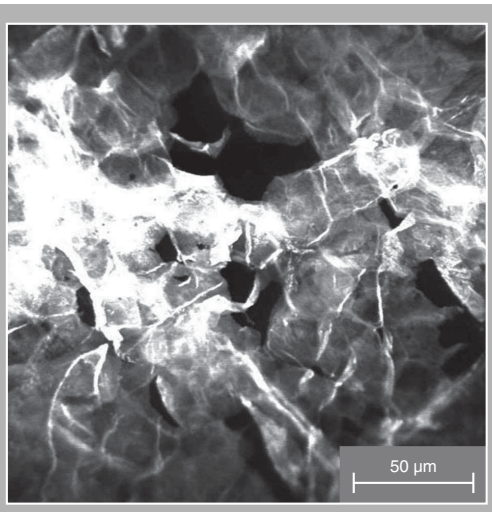
Abstract: A high number of treatments in dermatology are based on the penetration of topically applied drugs through the skin barrier. This process is predominantly inefficient, on account of the strong protection properties of the upper skin layer – the *stratum corneum*. If the skin barrier is damaged, the penetration efficiency of topically applied drugs increases. Therefore, different methods have been developed to influence the barrier properties of the skin.
Recently, it could be demonstrated that a cold tissue tolerable plasma (TTP) produced by a plasma-jet can strongly enhance drug delivery through the skin. These investigations were performed by using a solution of fluorescent dye as a model drug. In the present study, these investigations were carried out using fluorescent silica particles at different sizes. The aim of the study was to investigate whether or not there is a limitation in size for topically applied substances to pass through the skin barrier after plasma treatment.



Typical distribution of the 56 nm particles on the non-plasma treated skin

Stimulation of the penetration of particles into the skin by plasma tissue interaction

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1. Introduction

The *stratum corneum* is the uppermost layer of the human skin representing the barrier to the environment. The barrier function of the skin includes prevention of water loss and protection against the penetration of external substances and microorganisms [1–3].

Various dermatological diseases are treated with topically applied drugs providing that the substances penetrate through the *stratum corneum* into living tissue [4–7]. However, in intact skin, this process can be inefficient for various topically applied substances [8–10]. Hence, special formulations have been developed to stimulate the penetration through the skin barrier [11-13]. Liposomes

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Skin area	Type of treatment	Applied formulation
Area A	No plasma treatment	Application of 56 nm particles (1 mg/cm^2)
Area B	Plasma treatment	Application of 56 nm particles (1 mg/cm^2) after plasma treatment
Area C	No plasma treatment	Application of 689 nm particles (1 mg/cm^2)
Area D	Plasma treatment	Application of 689 nm particles (1 mg/cm^2) after plasma treatment

Table 1 Schema of the four different treatments on each pig ear

or nanoemulsions, e.g., are increasingly applied as drug carriers [14–16]. Additionally, mechanical methods, such as electrophoresis [17] or microneedles [18,19] are sometimes utilized to modify skin barrier properties in order to increase penetration. Recently, it was reported that also the application of cold tissue tolerable plasma (TTP) was able to increase the penetration of topically applied model drugs [20,21].

The aim of the present study was the investigation of the influence of plasma treatment on the penetration process of silica particles of two different sizes (56 and 689 nm). Next to the relevance concerning drug delivery, the investigations are also important in regard to safety aspects of the plasma-jet technique. Biogenic hazards, such as bacteria and fungi, typically have a size of 1 – 10 and 3 – 200 μm [22,23], respectively. The question addressed in the present work was whether the penetration of such relatively large structures through the skin barrier is facilitated during plasma treatment.

2. Materials and methods

2.1. Tissue samples

The investigations were performed on porcine ear skin, which represents a suitable model for human tissue [24]. The unscalded tissue samples had been freshly obtained from the slaughterhouse. The Veterinary Medical Council, Berlin-Treptow, had granted approval for the experiments. Pig ear as a tissue model was chosen affording the removal of an unlimited amount of biopsies for histological analysis.

2.2. Plasma treatment

In the present study, the plasma-jet "kinpen09®" was used, which was developed at the Institute of Plasma Physics, Greifswald [25] and manufactured at neoplas GmbH [26].

The plasma-jet consists of a small size and lightweight plasma generation unit, which allows fast and almost arbitrary 3D movements. Recently, it was reported that during the application of cold tissue tolerable plasma (TTP), a temperature between 35 up to 45°C was produced in the plasma tissue interaction zone [20]. The device "kinpen09®" has successfully passed CE-certification (electromagnetic compatibility) and fulfils therewith the standards for electrical safety.

Argon gas was used as a discharge medium in the plasma-jet. A detailed description of the plasma-jet has already been published [20,27].

2.3. Applied formulations

Two types of silica particles were investigated in this study. Both samples were prepared by modified Stoeber syntheses [28,29]. The inner core of the particles was covalently labeled with fluoresceine isothiocyanate (FITC) [30], whereas the outer shell of at least 3 nm thickness was free of dye. Fluoresceine is a strongly fluorescing food dye with an excitation wavelength of approximately 488 nm. Transmission electron microscopy (TEM) images were taken using a Zeiss EM 10 CR, in order to determine the size and the size distribution of the particles. The final diameter of the large particles was 689±26 nm and that of the small particles 56±4 nm. The particles were transferred into water by repeated (at least three times) centrifugation/redispersion before the experiments with skin started. The particles were applied to pig skin in the form of aqueous dispersions. The concentration of the large particles was 62.4±0.8 g/L and that of the small particles was 5.4±0.2 g/L.

The small particles were developed and produced at the Physical Chemistry Laboratory, Freie Universität Berlin, Germany. The large particles are a gift from Dannis't Hart, Debye Institute, Department of Physics and Astronomy, University of Utrecht, The Netherlands.

2.4. Laser scanning microscopy

The distribution of the particles on the skin surface and in the *stratum corneum*, *stratum granulosum*, *stratum spinosum*, and *stratum basale* was analyzed by laser scanning microscopy ("Stratum", Optilas Ltd., Melbourne, Australia). The laser scanning microscope consists of a base station containing the excitation laser (argon laser, $\lambda = 488$ nm) and the spectrometer in the control unit [31]. The base station is connected by optical fibers to the hand

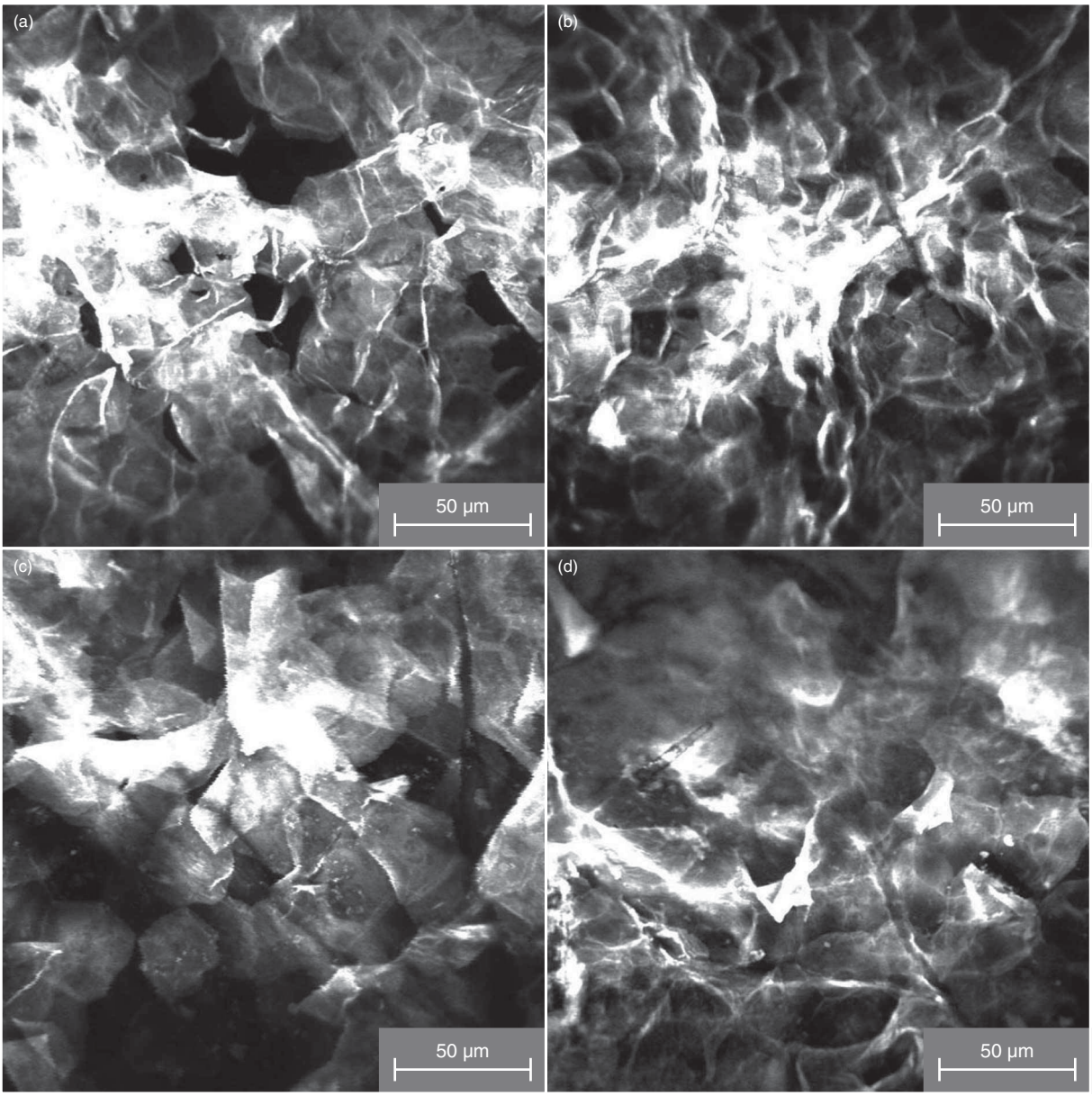

Figure 1 Detection of 56 and 689 nm diameter silica particles on the skin surface of the non-plasma treated and plasma treated skin. (a): 56 nm – untreated skin, (b): 689 nm – untreated skin, (c): 56 nm – plasma treated skin, and (d): 689 nm – plasma treated skin

piece, where the optical imaging system and the focus control unit are positioned. The maximal penetration depth of the radiation into the tissue was approximately 150 μm, which implies that the skin could be analyzed up to the upper papillary structure [32–37].

2.5. Study protocol

The pig ears were washed under running cold water and subsequently gently dried with paper towels. Four different skin areas (A – D) at 2×2 cm^2 were marked on each pig ear, which were treated in accordance with the procedures presented in Table 1. The position of the four marked skin areas rotated during the investigation of the six pig ears.

The velocity of the plasma stream with a diameter of 2 mm on the skin surface was 10 mm/sec. After treatment, immediately 1 mg/cm^2 of the nanoparticle-containing formulations were applied to the skin samples. Following a penetration time of 15 min, the distribution of the nanoparticles on the surface of the samples and in the deeper layers of the skin was analyzed by laser scanning microscopy.

Skin area	Type of treatment	Cell layers where the particles were detected
Area A	No plasma treatment (particles of 56 nm diameter)	Upper cell layers of the *stratum corneum* (Fig. 1a)
Area B	Plasma treatment (particles of 56 nm diameter)	All cell layers of epidermis: *stratum corneum* (Fig. 1c), *stratum granulosum* (Fig. 2a), *stratum spinosum* (Fig. 2b), and *stratum basale* (Fig. 2c)
Area C	No plasma treatment (particles of 689 nm diameter)	Upper cell layers of the *stratum corneum* (Fig. 1b)
Area D	Plasma treatment (particles of 689 nm diameter)	Upper cell layers of the *stratum corneum* (Fig. 1d)

Table 2 Location of particles in the tested skin areas

3. Results

The untreated skin showed no fluorescence signals, neither on the skin surface nor in deeper skin layers. After application of both types of particles onto the skin, the particles could be detected as bright areas on the skin surface in the laser scanning microscopy (LSM) images of all skin areas A – D, due to their strong fluorescence properties. In the case of the skin areas A and C (no plasma treatment), no penetration of both types of particles into deeper parts of the *stratum corneum* and into the *stratum granulosum* could be detected. Typical laser scanning microscopic images of the distribution of the fluorescensing particles on the skin surface with and without plasma treatment are presented in Fig. 1.

In Figs. 1a and 1b, the typical distribution of the 56 nm particles (area A) and the 689 nm particles (area C) on the non-plasma treated skin are shown. The skin, which was not treated with plasma, shows a regular undamaged structure of the first layers of corneocytes. This homogeneous structure is slightly disturbed in the case of plasma treated skin, which is also typical for dry skin. Typical LSM images are shown in Fig. 1c (area B) for the 56 nm particles and in Fig. 1d for the 689 nm particles (area D).

Only in the case where the particles with a diameter of 56 nm were applied after plasma treatment (area B), the particles could also be detected in deeper skin layers. The particles were found in the *stratum granulosum* (Fig. 2a), *stratum spinosum* (Fig. 2b), and *stratum basale* (Fig. 2c), up to a depth of 150 μm, which corresponds to the detection limit of the laser scanning microscope. In any case, the larger particles with a diameter of 689 nm do not pass through the *stratum corneum*. The results are summarized in Table 2. Identical results were found in all six investigated skin samples.

4. Discussion

Penetration of topically applied substances through intact skin barrier is an inefficient process, which is due to the strong protection properties of the *stratum corneum* [1]. Only 0.1% of the topically applied drugs in cosmetic products pass through the skin barrier [38]. Therefore, different clinical and mechanical procedures have been developed to increase the drug penetration [39–42]. The effect of these methods is always based on damage to the skin barrier [43]. On the one hand, damage of the skin barrier is advantageous for the penetration of topically applied substances through living cells [44]. On the other hand, the reduced barrier function can increase the risk of infections caused by bacteria and fungi [23]. Therefore, in the case of damage to the skin barrier, a disinfection of the skin should always be applied [45].

It has been reported that a plasma-jet, which produces a cold "electrical plasma" of approximately 45°C can be applied to biological material including human tissue [46]. The tissue tolerable plasma has a high efficiency of disinfection [46]. Additionally, it could be demonstrated that during the plasma skin interaction, the barrier properties of the *stratum corneum* were changed, allowing topically applied substances to penetrate highly efficiently into the living tissue [46]. The experiments were performed using an aqueous solution containing a fluorescent dye.

In the present study, it was investigated whether this efficient penetration could also be observed for nanoparticles. Recently, it was demonstrated that particles are efficient carrier systems for topically applied substances into the hair follicles [19]. Additionally, in the present study, investigations were performed as to whether particles of variable sizes, which are almost of a similar order of magnitude in size as bacteria and fungi, could penetrate into the living tissue after plasma treatment.

The results of the present study clearly demonstrate that particles of 56 nm can penetrate through the skin barrier after plasma treatment. The larger particles with a diameter of 689 nm did not pass through the *stratum corneum*, even when the velocity of the plasma impinging on the skin surface was extremely reduced to 1 mm/s. Thus, it can be expected that bacteria and fungi, which have a size of 1 – 10 and 3 – 200 μm, respectively, do not pass the skin barrier during the plasma tissue interaction. This is in contrast to the application of mechanical systems used for barrier interruption, such as microneedles, which produce pores in the skin with a diameter of several

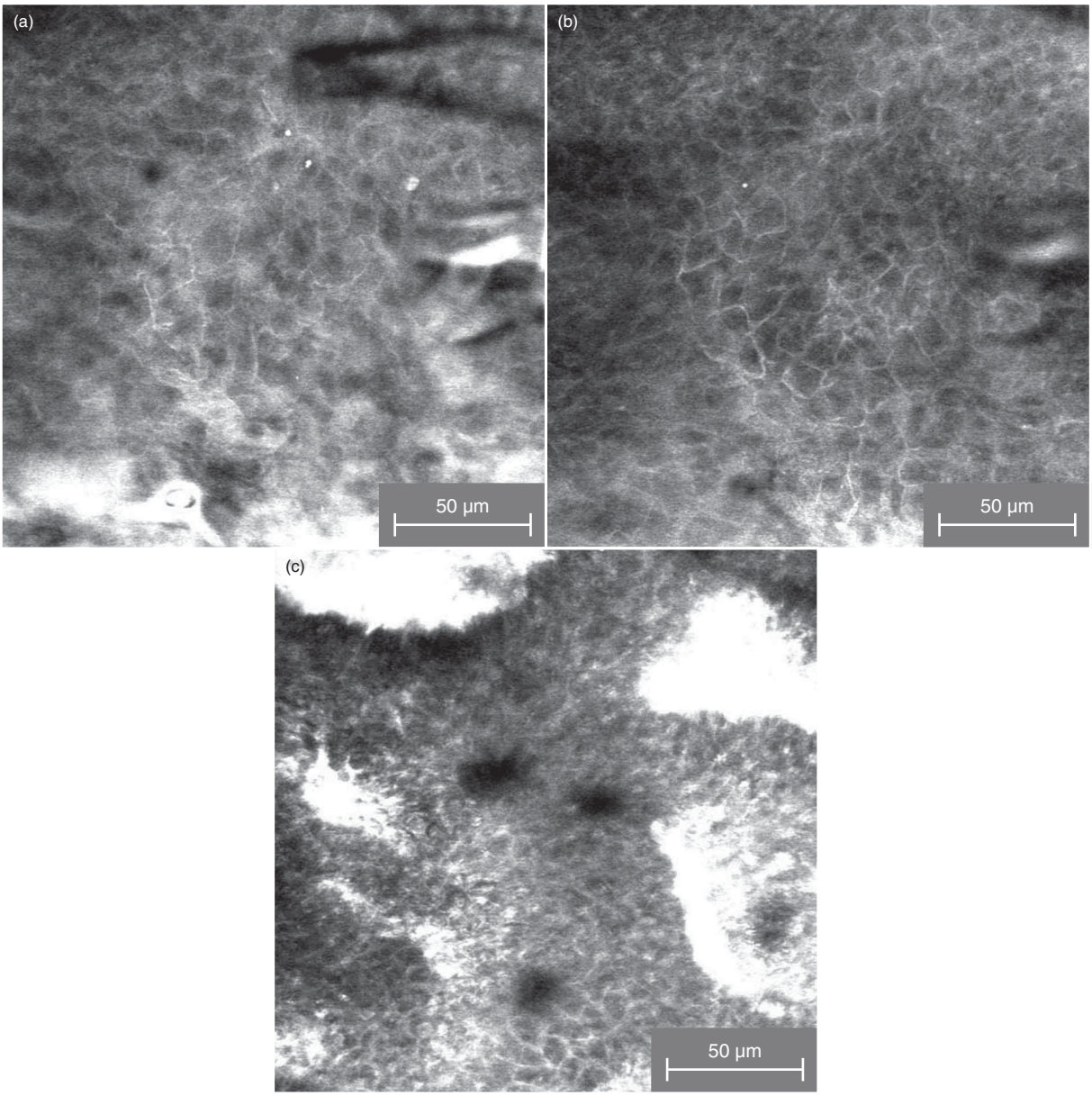

Figure 2 Detection of 56 nm diameter silica particles applied after plasma treatment in the *stratum granulosum* (a), the *stratum spinosum* (b), and the *stratum basale* (c)

10 μm [18,47]. Consequently, the tissue tolerable plasma shows two safety aspects regarding the impact of bacteria and fungi. Firstly, as the plasma has disinfection properties, during laser tissue interaction, the skin surface becomes disinfected [48]. Secondly, it could be shown in the present study that even larger nanoparticles, which are significantly smaller than bacteria and fungi, do not pass the skin barrier during the plasma tissue interaction. As a result, side effects of the plasma application, such as infection can be excluded in contrast to other physical and chemical methods, used for barrier interruption.

Regarding penetration of topically applied substances by the plasma tissue interaction, it was shown in the present study that particles at a size of ≤ 56 nm could pass efficiently through the skin barrier. The high fluorescence signal detected in all cell layers of the epidermis demonstrates the high efficiency of this process.

Therefore, the maximum diameter, which allows particles to pass through the skin barrier during plasma tissue interaction, is supposed to be larger than 56 nm and smaller than 689 nm. The exact determination of these critical values requires further investigations. The results

of this study were obtained under *in vitro* conditions. Consequently, barrier recovery after plasma tissue interaction did not occur. Under *in vivo* conditions, the recovery of the barrier can occur rapidly. However, it must be verified, whether the topically applied substances, which penetrate through the skin barrier *in vitro*, can be applied *in vivo* after plasma treatment, or whether they should be applied prior to the treatment, if the skin barrier recovers very quickly. An application prior to plasma treatment may have the disadvantage that the topically applied drugs interact with the plasma, thus changing their properties.

In conclusion, it must be stated that the plasma tissue interaction is not only efficient in disinfection of the skin, but also changes the barrier properties. This effect opens up new aspects in drug delivery. In this case, drug delivery and skin disinfection occur simultaneously. Particles at a size of ≤ 56 nm, are able to pass the skin barrier efficiently, larger particles at a size ≥ 689 nm will remain outside the skin barrier during plasma tissue interaction. This effect is important for the safety aspects of plasma application, because these results suggest that components, such as bacteria and fungi do not penetrate through the skin barrier when plasma is applied. Furthermore, future investigations will clarify whether drugs, which are supposed to penetrate through the skin barrier *in vivo*, need to be applied before or after plasma treatment of the tissue.

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