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DISSERTATION

The Penetration, Efficacy and Potential of Antiseptic Nanoparticle-Emulsions

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Abstract (English)

Despite established prevention strategies such as standardized skin antisepsis with alcohol rubs, the consequences of nosocomial infections continue to take serious social and economic tolls. Recent discoveries concluded that new targets must be considered for the development of modern antiseptic agents and procedures. Studies have shown that a large number of microorganisms are located within and in the immediate vicinity of the hair follicle shaft, suggesting that the hair follicles act as bacterial reservoirs. Recent investigations have demonstrated that hair follicles also play an important role in skin penetration. In particular, nano-sized liposomes were found to be efficient carriers for drug delivery into the hair follicle. Based on these results, this summary provides an overview of subsequent in vitro and in vivo experimental studies assessing the antiseptic effectiveness of nano-sized liposomes with the bound disinfectant polihexanide. The in vitro study examined the follicular penetration of the curcumin-labeled particle-associated polihexanide into porcine ear skin using laser scanning microscopy. The follicular penetration depth was compared to that of the same curcumin-labeled nanoparticles without bound antiseptic. The images acquired in the transmission and the fluorescent modi were superimposed in order to visualize the distribution of the fluorescent dye inside the hair follicles. While quantitative and qualitative results confirmed the efficient penetration of both solutions into the hair follicles, the average penetration depth of the particles with bound polihexanide was significantly higher than that of particles without bound antiseptic. In the in vivo investigation on the volunteers, bacterial growth was monitored after antisepsis over a period of 2.5 h using swabbing of the skin and bacterial counts. The antiseptic potential of the particle-associated polihexanide was compared to that of a conventional aqueous polihexanide solution. Though not statistically significant in this case, results suggested that the use of a particle-bound antiseptic could accomplish a better and longer lasting antisepsis than in non-particular form. An additional review article was published to provide an overview on the subject of targeted delivery of antiseptics to hair follicles and to evaluate their clinical potential. This overview addressed, in particular, the emerging methods of particle-associated disinfectants and tissue-tolerable plasma (TTP).

Abstract (Deutsch)

Die Folgen von nosokomialen Infektionen führen trotz etablierter Präventionsstrategien wie die standardisierte Hautantiseptik mit Alkohollösungen weiterhin zu schwerwiegenden sozialen und wirtschaftlichen Schäden. Neuere Untersuchungen haben gezeigt, dass künftig neue Ziele für die Entwicklung von modernen Desinfektionsmitteln und Verfahren berücksichtigt werden müssen. Forschungsarbeiten haben offenbart, dass sich eine große Anzahl von Mikroorganismen innerhalb und in unmittelbarer Nähe der Haarfollikel Kanäle befinden. Diese Ergebnisse legen nahe, dass die Haarfollikel ein bakterielles Reservoir darstellen. Andere Arbeiten haben gezeigt, dass Haarfollikel eine wichtige Rolle beim Eindringen von Substanzen in die Haut spielen. Nanopartikel, und insbesondere Liposome, haben sich als effiziente Träger für Substanzlieferungen in die Haarfollikel erwiesen. Basierend auf diesen Entdeckungen wurden in der folgenden Publikationsarbeit zwei aufeinander aufbauende experimentelle in vitro und in vivo Studien durchgeführt, um die antiseptische Wirksamkeit von Liposomen mit angeheftetem Antiseptikum zu untersuchen.

Ein weiterer Artikel wurde veröffentlicht, um einen Überblick über das Thema der zielgerichteten Desinfektion zu geben und deren klinischen Anwendungsmöglichkeiten zu beurteilen. Dieser befasst sich insbesondere mit zwei neu aufstrebenden Technologien: partikuläre Desinfektionsmittel und Gewebefreundliches Plasma (tissue-tolerable-Plasma, TTP).

1. Introduction

The natural skin flora plays an important role in the development of surgical site infections (SSIs). Exogenous factors are estimated to be the underlying cause in only 10% of cases. [1] Nosocomial infections such as SSIs cause high additional costs and are associated with high mortality. The efficacy of skin antisepsis as a prevention tool has been repeatedly demonstrated in clinical studies comparing SSI rates after the use of skin antiseptic formulations. [2] The combination of alcohol with antimicrobial compounds such as octenidine-dihydrochloride has been established as state-of-the-art. [3] However, recent investigations in dermatology and skin physiology indicate the need for further engineering of antiseptic agents and procedures in order to improve their efficacy. In particular, as the prevention of many nosocomial infections relies heavily on efficient skin antisepsis, new targets need to be taken into consideration.

Lange-Asschenfeld et al. examined the quantitative and qualitative distribution of human resident flora within the corneal layers of human skin using a tape- and cyanoacrylat-stripping method. Approximately 25% of the bacterial colonies appeared to be located within the hair follicles, with high densities of microorganisms found deep in the hair follicle shafts. These findings identified the hair follicle as a highly significant target region for antiseptics. [4] Due to the anatomical structure of the hair follicle, it was postulated that a conventional liquid disinfectant could not penetrate sufficiently into the follicle structure to result in adequate microbial reduction. [5] Two of five trials showed that by sealing off the skin pores with an acrylat (Integuseal) foil immediately after skin desinfection, possibly blocking bacterial mobilisation from within, SSI rates could be significantly reduced. [6] This indicates that surviving microorganisms may re-emerge from non-sealed follicles, recolonizing the skin during long surgical procedures. Other studies examining the distribution of bacterial flora on human skin have supported the view that the hair follicle acts as an important reservoir for topically applied substances. The storage capacity of the human hair follicles was shown to be comparable to that of the stratum corneum on several parts of the body. [5] Another study demonstrated that substances applied to the skin were stored 10 times longer in the hair follicle infundibulum when compared to the storage time within the stratum corneum. [7] These observations suggest that the hair follicle structure could provide a bacterial growth reservoir and a potential storage location for topically applied substances. Taking these findings into consideration, the next generation of

antiseptics should penetrate into deeper skin regions, and especially into hair follicles in order to provide an improved and possibly prolonged skin antisepsis.
In recent years, nanoparticles have been used extensively in vitro and in vivo for drug delivery research, and they are already being used in a number of medical fields such as oncology and cardiovascular medicine. By artificially loading compounds in the aqueous spaces within the lipid membrane, liposomes can function as carriers for the delivery of substances to particular anatomical structures. [8] Nano-sized liposomes have been shown to display enhanced follicular penetration and prolonged storage properties, making them particularly efficient carriers for drug delivery into the hair follicles. [9]

Combining these findings, an investigation assessing the potential of antiseptic delivery to the hair follicles using nano-sized liposomes was conducted. The first part of the study was devoted to visualizing and measuring the *in vitro* penetration capability of a nanoparticle-emulsion containing the antiseptic polihexanide (PHMB) into hair follicles of porcine ear skin. Penetration depths of particle-associated solutions with and without PHMB were monitored using laser scanning microscopy. In the *in vivo* investigation, the disinfectant potential of the test solution was compared to that of PHMB in its conventional liquid form on the human skin. The degree of bacterial contamination was determined using microbiological cultures and bacterial colony counts. A literature search was also conducted to summarize the current state of research regarding skin antisepsis. This overview addresses, in particular, two emerging methods of follicle-targeted skin antisepsis.

This article thesis is submitted to obtain a doctoral degree in medicine. The following text is a summary and discussion of the experimental research work, examining three separate articles on the subject of targeted delivery of antiseptics to the hair follicle and on novel methods of skin antisepsis. The research articles discussed are bound at the end of this document.

2. Materials and methods

2.1 Particles and testing solutions

2.1.1 Antiseptic

Polyhexamethylene biguanide hydrochloride (PHMB, polihexanide) is a polymeric cationic antimicrobial agent used in commercially available antiseptic solutions approved for topical application to the human skin. It is a fast-acting broad-spectrum biocide acting against a wide range of both Gram-positive and Gram-negative bacteria. (Cosmocil PG®, Arch Chemicals Inc., Norwalk**,** Connecticut, United states)

2.1.2 Particles

Liposomes were obtained from a Lipofundin® MCT²-20% solution (B. Braun, Melsungen, Germany). Lipofundin® is suitable for peripheral venous administration, being generally used for the substitution of caloric fat components, insuring the intake of essential fatty acids in parenteral nutrition. Lipofundin® MCT 20% as an oil-in-water (o/w) emulsion contains 200g/L of a 1:1 mixture of medium chain triglycerides (MCT) and long chain triglycerides (LCT; soybean oil), glycerol (2.5%), sodium oleate (0.03%) and d,l-a-tocopherol, and is stabilized with egg lecithin. The carrier particles selected for this investigation had an average size of 295.1 ± 8.2nm (z-average) and a polydispersity index (PI) of 0.081 ± 0.08. (B. Braun, photon correlation spectroscopy, PSC).

2.1.3 Final testing solutions

The annexure of PHMB to the oil droplets was achieved by mixing 25mL Lipofundin MCT 20% with 25mL 0.1% polihexanide. The resulting oil-in-water (o/w) emulsion with bound PHMB was composed of 0.05% polihexanide in Lipofundin MCT solution with 0.6% egg lecithin. An aqueous 0.05% PHMB solution was prepared by combining 10mL 0.1% PHMB with 10mL sterile water.

2.1.4 Tracer substance

Penetration into the porcine hair follicles was monitored using the fluorescent tracer substance curcumin (Merck®), a naturally occurring dye known to be non-toxic to normal tissues, which is widely used in various experimental studies. The loading of curcumin inside the liposomes and the binding of PHMB to the outer surface were based on the instructions of Reeves et al. [10] 3.8mg curcumin was diluted in 10mL

unalloyed ethanol so as to obtain 1mM curcumin in ethanol. 100mL Lipofundin MCT-20% was combined with 1mL of a 1mM curcumin in ethanol solution. The obtained reactive mixture was incubated overnight in the dark at 37°C. The solution was then dialyzed against deionized water for a week with a daily change of the dialyzing fluid in order to remove the ethanol from the solution and monitor a secondary precipitation of the curcumin. Since no precipitation was observed, it could be assumed that all of the remaining curcumin within the final solutions was localized in the lipid phase of the emulgated fat droplets. The same procedure was repeated to obtain a fluorescent 0.05% polihexanide in Lipofundin MCT solution with 0.6% egg lecithin.

2.1.5 70% Iso (Propan-2-ol (Isopropyl alcohol))

70% isopropyl alcohol was used as a positive quality control in the *in vivo* investigation. The 99.9% isopropyl alcohol product from MERCK® (Darmstadt, Germany) was diluted with distilled water to obtain 70% propan-2-ol (70% Iso).

2.2 Skin preparation and application protocols

2.2.1 *In vitro* investigation on a tissue model

The investigation was conducted in vitro on the ear skin of 6 month old German domestic pigs. Studies concerning percutaneous penetration of topically applied substances have shown that porcine ear skin is a suitable model for human skin. Porcine ear skin was found to be superior to excised human skin for the analysis of follicular penetration, since, unlike human skin, pig ear tissue does not contract after excision, provided the cartilage is not removed. Pig ears from freshly slaughtered pigs were obtained from a local butcher and used experimentally within 24h after slaughter. Prior to the investigation, the hairs on the pig ears were abscised, the skin washed with cold water and gently dried with paper towels. 2 x 2cm testing areas were demarcated with a silicone barrier (Windows Colours®) on each pig ear. Approval for this study had previously been obtained from the Veterinary Board of Control, Berlin, Treptow-Köpenick. 20$\mu l/cm^2$ of the nanoparticle-emulsion with bound polihexanide was applied to the silicone-demarcated areas using a pipetting system. The dispersion was then spread equally and rubbed onto the skin by means of an electric massage appliance for 3 min according to the protocol of Lademann et al. [9] The same procedure was repeated for the control dispersion without bound polihexanide.

2.2.2 *In vivo* investigation on human skin

The *in vivo* investigation was performed on 12 healthy male Caucasian volunteers aged from 20 to 50 years with normal body mass index (BMI). The Ethics Board of the Charité had previously approved the study. (EA1/080/09) Each volunteer signed an informed consent prior to the experiment. All volunteers were prohibited from using medicated shampoos or systemic antibiotics previous to the study and did not wash their forearms with anything other than cold water for at least 12h prior to the experiment. Each palmar forearm was divided into 6 squares, resulting in a total of 12 squares per volunteer. The testing solutions were applied to the skin of the volunteers using a pipetting system. A sterile glass spatula was used to spread the test substances evenly on the skin surface and to massage the solutions into the skin.

2.3 Biopsies and Laser Scanning Microscopy (LSM)

In the *in vitro* experiment, solutions were left to penetrate 1 hour on the pig ear skin. Punch biopsies of 5mm in diameter were then collected and immediately shock frozen. In order to ascertain the penetration depth of the particles, vertical cryohistological sections of approximately 7µm were prepared using a special-purpose skin sectioning device (SLEE Kryostat MTE®, SLEE Technik GmbH, Mainz, Germany), and embedded (embedding medium: Killiks—cryostat embedding medium, Bio Optica, Milano, Italy) onto microscope slides (R. Langenbrinck®, Emmendingen, Germany). The sections were analyzed using a laser scanning microscope (LSM 410 invert, Zeiss®, Jena, Germany). The microscope was operated in transmission and fluorescence mode for each selected follicle. Since porcine ear skin emits natural autofluorescence within 520–560nm, the spectral range chosen for the measurements was set between 590 and 680nm. The penetration depth was measured using a digital image analysis and a special software program (confocal laser scanning microscope, software LSM 410 invert, Zeiss, Jena, Germany). The transmission mode allowed the analysis of the skin relief and follicle structure while the fluorescent mode enabled the visualization of the fluorescent dye. Both images were superimposed and the maximal penetration depth of the curcumin signal was measured for each follicle. A total of n = 14 follicles containing curcumin-loaded particles (control dispersion) and n = 12 follicles containing curcumin-loaded particles with polihexanide (test dispersion) were analyzed.

2.4 Bacteriological sampling of the skin areas of the volunteers

One of each of the correspinding areas was sampled for microorganisms at t^1= 30min, t^2 = 90min, and t^3= 150min respectively. Sampling was performed using a sterile microbiological swab and transport System (EM-TE-Vertrieb®, Hamburg, Germany). The swab was conducted alternately from right to left and left to right for twenty strokes, and the process was then repeated from top to bottom. The swabbing also included a rotatory movement and light pressure was applied.

2.5 Immersion in TSHC solution

In order to prevent any residual microbial activity after sampling, the inferior third of the swab was cut off and submerged into 1mL of a neutralizing solution containing 3% tween 80.3% saponin, 0.1% histidin and 0.1% cysein (TSHC) in tryptic soy broth. The effective neutralization was previously tested in accordance with DIN EN 1040 (Institut für Hygiene und Umweltmedizin, Greifswald, Germany). The 1mL neutralization solution containing the inferior part of the swab was then immediately shaken for 30s using a vortex (Vortex-2-Genie Scientific Industry, New York, USA) on level 6 and set aside. The process was repeated with a fresh solution for all samples.

2.6 Inoculation and incubation of agar plates

Inoculation started after all areas corresponding to the according sampling time were swabbed. Each solution was shaken briefly and 100µl was then applied to an agar plate using a pipetting system (Eppendorf®, Wesseling-Berzdorf, Germany). A multiple streaking method was employed using a sterile inoculating loop moving in a crisscross pattern and repeating the process twice after a 60° angle to obtain a maximum of isolated colonies. Columbia Blood Agar Base “Sheep blood +” (5-7%, OXOID™, Cambridge®, UK) is a highly nutritious, general-purpose medium for the isolation and cultivation of non-fastidious and fastidious microorganisms. Its components are a special peptone 23.0g/l, starch 1.0g/l, sodium chloride 5.0g/l and agar 10.0g/l. The pH was maintained at 7.3 ± 0.2 at 25°C. The processed agar plates were immediately incubated at 36 ± 2°C for 48 h. Since many pathogens naturally require carbon dioxide on primary isolation, the atmosphere was set to contain approximately 5 ± 2% CO^2.

2.7 Quantification of bacterial presence

After 48h of incubation, the agar plates were photographed. The numbers of colonies on each plate were determined by bacteriological count. A colony was defined as a circular cluster of microorganisms growing on the surface of the medium. If two or more distinct circular clusters were superposed they were counted as separate colonies. The culture dishes with a colony count above 500 were considered contaminated and not taken into consideration ("cut-off").

2.8 Graphical and statistical evaluation

Graphical and statistical analysis was performed using the software programs Excel® (Microsoft, Washington, USA) and SPSS® (IBM Cooperation, New York, USA). For SPSS calculations, the data was logarithmized and, after verifying the Gaussian distribution of the data, a paired T-Test was used to compare the bacterial growth of the solutions isolated for each sampling time. In this case the T-Test was chosen over ANOVA, as results would have been similar while the additional information concerning the other fields would have exceeded the scope of the study. Indeed, comparative analysis between both the Iso70% and the native skin fields and the testing solutions was not the focus of the study, as these were primarily used as positive and negative control fields.

2.9 Bias management

2.9.1 Randomization

The distribution of the testing areas was determined by using the general-purpose computer algebra system Maple® (Version 13, Waterloo, Canada) resulting in a random arrangement of 12 numbers, each corresponding to a testing field. This process was repeated to create a unique topical application map for each volunteer.

2.9.2 Blinding

A double-blind procedure was used to avoid bias during the investigations and placebo effects. The volunteers were oblivious to the substance applied onto each area. The areas on the arms of the volunteers were marked with letters, which were also used to mark the agar plates. The labels on the agar plates were removed during incubation, and replaced by a code unknown to the person performing the experiment. After completion of the bacteriological counts, the results were decoded using a pre-set key.

3. Results

3.1 In vitro investigation on porcine ear skin

Distribution of the fluorescent dye, and thus of the bound PHMB within the porcine ear skin biopsies, was visualized by superposing images acquired in transmission and fluorescent mode. Both longitudinal and cross sections were analyzed. In the visualized cross sections, the infundibulum of the hair root was highly fluorescent when compared to the surrounding skin layers. The fluorescent dye outlined the entire convexity of the visualized part of the hair canal. Longitudinal sections showed a fluorescent signal extending deep into the hair follicle, outlining the longitudinal shape of the follicular wall and that of the remaining lower hair shaft. The deeper skin layers around the hair follicle shaft appeared to lack fluorescent enhancement. Both the cross and the longitudinal sections showed very little fluorescent dye on the skin surface and deeper skin layers, when compared to the infundibulum and the hair root. Similar results were found for all biopsies.

Measurements of the penetration depths showed that both dispersions penetrated efficiently into hair follicles. The direct comparison of the penetration depth showed that particles without polihexanide (control) had an average penetration depth of 651± 81µm, while the penetration depth of the test dispersion corresponded to an average of 920± 47µm. The penetration depth of the test dispersion was significantly deeper ($p<0.05$), indicating that the emulsified particles of the test dispersion penetrated considerably deeper into the hair follicles of porcine ear skin than the control without polihexanide.

3.2 In vivo investigation on human skin

3.2.1 Average bacterial growth curves

Bacterial growth on the examined skin areas was determined by counting the bacterial colony forming units (CFU) obtained after incubation. Results were used to calculate the average CFU found on the different testing fields for the sampling times t^1 = 30, t^2 = 90, and t^3 = 150min. Overall, the results of the colony counts were highly heterogeneous. Untreated skin (negative control, C) areas displayed the highest average number of CFU throughout the experiment. Areas treated with 70% Isopropyl alcohol (70%Iso) showed the lowest average number of CFU throughout the trial period. Areas treated with the liposomal o/w-emulsion with bound polihexanide (PHMB

+ L) presented similar results. The largest gap was found at t^3 = 150min, where the average number of CFU found on PHMB + L was superior to 70%Iso by 8 CFU.
PHMB + L showed a relatively stable average number of CFU over time with an overall average variation of only 1 colony forming unit. The highest bacterial growth evolution over time was found for PHMB-treated skin areas (PHMB) with an average increase of 34 additional CFU, whereas the average colony number for untreated skin C and 70%Iso decreased over time. Tendency curves calculated for each solution showed an exponential growth only for PHMB. Bacterial growth on areas with topically PHMB + L and 70% Iso showed a linear tendency curve. The tendency curve calculated for the colony numbers registered on the untreated areas C was polygonal.

3.2.2 Statistical analysis

Statistical analysis was conducted with a two-paired T-Tests performed for all three sampling times t^1 = 30, t^2 = 90, and t^3 = 150min. Significance was set at $p < 0.05$.

Negative and positive control fields: number of colonies found on native skin (C, negative control) and areas treated with 70%Iso (positive control) remained significantly different throughout the sampling times t^1 ($p = 0.000$), t^2 ($p = 0.012$) and t^3 ($p = 0.001$).

70% Isopropyl alcohol and testing solutions: differences in the number of colonies found on areas treated with 70% Iso, when compared to areas treated with PHMB + L at t^1 ($p = 0.301$), t^2 ($p = 0.142$), and t^3 ($p = 0.196$), remained insignificant throughout the experiment. The number of CFU on areas treated with 70% Iso was significantly lower than that found on areas treated with PHMB after 30min ($p = 0.019$) and 90min ($p = 0.037$). However, at sampling time t^3 = 150min, the difference between the two reverted back to being insignificant ($p = 0.138$). This particular comparison showed the highest standard deviation (1.07) and the highest mean standard deviation value (0.31).

Native skin and testing solutions: the amount of CFU found on the native skin (C, control field) remained significantly higher than that found on areas treated with PHMB + L throughout the sampling times t^1 ($p = 0.000$), t^2 ($p = 0.033$), and t^3 ($p = 0.006$). Conversely, the number of colonies found on the native skin (control field) and areas treated with PHMB remained significantly different only for sampling time t^1 ($p = 0.019$). After 90min ($p = 0.097$) and 150min ($p = 0.083$), the native skin and skin areas treated with PHMB produced statistically similar numbers of CFU.

Testing solutions: There were no statistically significant differences between PHMB and PHMB + L after 30min ($p = 0.245$), 90min ($p = 0.172$) and 150min ($p = 0.450$).

3.3 Review (tissue tolerable plasma, TTP)

Recent developments in the area of plasma desinfection have opened new possibilities for skin antisepsis. Physical plasma contains a range of highly energetic aggregates composed of ions, free electrons, radicals, photons in the visible and infrared range, neutral and excited atoms and molecules, UV-radiation and electric fields. [11] Various plasma types such as hydrogen peroxide plasma are widely used for a range of procedures, such as the decontamination of medical devices or in-vitro food sterilization. The required high temperatures, ranging at several hundreds of Celsius, have thus far restricted the use of these methods. However, the recent development of low temperature plasma, also referred to as tissue-tolerable plasma (TTP), operating at temperatures of 30 °C - 50 °C, now allows this method to be used for the decontamination of living tissue.

TTP targets the upper cell layers, which absorb the produced energy leading to antisepsis and debridement of the surface. The proliferating layers below are exposed to only a residual amount of energy and a tolerable, non-harmful level of heat that has a stimulating effect on wound healing. [12] The resulting stimulation of blood flow, immunological processes and cell proliferation make TTP suitable for the treatment of chronic wounds. Studies examining TTP-treatment in combination with polihexanide or octenidine-dihydrochloride have demonstrated the positive influences of TTP on wound healing, and have confirmed its low cytotoxicity and antiseptic efficiency.

The microbicidal efficacy of TTP was shown to be comparable to the efficacy of the standard antiseptic octenidine-dihydrochloride and appears to rely on the production of large amounts of free radicals. These free radicals are found on the skin surface, but also specifically within the hair follicle structures after TTP application, suggesting the penetration of plasma into the hair follicles. In addition to its antiseptic efficacy, it could also be demonstrated that the penetration of particles and substances into the hair follicles can be stimulated by TTP-treatment of the skin. [13, 14]

4. Discussion

The thorough distribution of fluorescent dye within the infundibulum, hair canal and hair root, in most cases outlining the total convexity of the hair canal including the lower hair shaft, indicates an effective and deep penetration of the emulsions into hair follicle structures. It can be assumed that PHMB was transported into hair follicles along with the lipid particles when bound to their surface. Since the skin surface and corneal layer around the hair follicle shaft appeared to lack fluorescent enhancement, the results indicated that hair follicles are the preferred location for the distribution of the applied particles. Measurements of penetration depths of both emulsions indicated an effective penetration in both cases. Interestingly, PHMB seemes to enhance follicular penetration, as the average penetration depth of the dye-labelled oil-in-water (o/w) emulsion with bound PHMB was significantly higher ($p<0.05$) than that of the o/w-emulsion without PHMB. This phenomenon is probably due to the increase in size after the annexure of the PHMB to the oil droplets. As the emulsified lipid particles used in the investigation had an average size of 295.1 ± 8.2 nm, the annexure of PHMB probabily approached their dimension to the ideal particle size of 500 nm, which has been shown to increase follicular penetration. [9] The *in vitro* penetration behaviour of PHMB without liposomes was not examined. However, it can be assumed that the penetration depth would have been further reduced, since the size of PHMB alone would have been too small. It has been stipulated that penetration of particles of a particular size may be enhanced by the rigid hair shaft, which acts as a geared pump, moving applied nanoparticles down the hair follicle and resulting in a significantly deeper penetration into the skin. [7] In this case, it can be assumed that the liposomal carrier pulled the bound antiseptic into the hair follicle shaft, allowing the antiseptic PHMB to reach these otherwise inaccessible bacterial reservoirs. [4]

Graphical analysis from the *in vivo* investigation on human skin showed a continuously low average bacterial count on the positive control area, while the negative control areas displayed the highest average bacterial presence throughout the experiment. Differences between the average bacterial counts of both fields could be statistically confirmed for the entire trial. These results are in accordance to the anticipated bacterial presence on the control fields. The polygonal growth curve describing the average growth on the native skin was probabily due to a natural heterogeneity of

bacterial presence dependent on the location of the testing field on the forearm. The o/w-emulsion with bound PHMB resulted in a low average bacterial presence without significant difference from areas treated with isopropyl alcohol, showing it to be a highly effective antisepsis for at least 150min. However, the bacterial presence of areas treated with PHMB without a carrier steadily increased over time, presenting a significant difference to the positive control area for the first two sampling times. This suggests an antiseptic superiority of the o/w-emulsion after just 30min. This seems to be confirmed when comparing the average number of colonies found on native skin and skin areas treated with PHMB: these show no statistically significant difference for the last two sampling times, indicating a recontamination with increased bacterial growth after 90min.

While statistical analysis provides significant results when comparing the average bacterial growth over time for selected samples, no significant difference could be shown by directly comparing the antiseptic behaviour of PHMB to that of the o/w-emulsion with PHMB for any of the sampling times. It was possible to evaluate the tendencies indirectly when comparing the bacterial presence on areas treated with the test solutions to the positive and negative control areas. The tendencies observed indicate a superiority of the o/w-emulsion both in regard to the primary antiseptic potential and to the antiseptic efficiency over a time period of 150min.

This tendency suggests that the o/w- emulsion with bound PHMB provides an improved, but most of all prolonged, desinfection when compared to PHMB in its conventional liquid form. Results from the in vitro and in vivo studies confirmed that the rapid recontamination that takes place after the use of a standard skin antiseptic originates from within the hair follicle structure - a process that the o/w- emulsion was designed to prevent. [4] It seems that by attaching PHMB to follicle penetrating liposomes and thus blocking the endogenous recontamination pathway, the effective disinfecting time of PHMB was extended. These findings underline the potential of particle-associated delivery systems such as particle-associated antiseptics for specific, targeted drug application and delivery to the hair follicle.

The experience gained from this trial allows for an improved experimental protocol for future studies investigating the antiseptic potential of nano-bound antiseptics. The relatively small number of subjects may account for the failure to reach significant results. A follow-up study with a considerably larger number of participants should be

conducted in order to confirm and quantify the antiseptic superiority of the o/w-emulsion with PHMB. This is confirmed by the high standard deviation and high mean standard deviation values, in particular for PHMB at the last sampling time. The need for a larger trial group is also illustrated by inter- and intra-individual differences among the volunteers. The individual bacterial growth curves displayed a high heterogeneity. This phenomenon is likely due to differences in hair follicle distribution, follicle density, total number of hair follicles and bacterial growth conditions such as humidity, temperature and pH (power of hydrogen). These variables can be assumed to vary from one volunteer to the next, despite precautionary measures. Randomisation of the testing fields may not have been sufficient to prevent these factors from affecting the results.

Intra-individual differences also have to be considered. Results showed that the bacterial presence was highest in areas proximal to the inner elbow and lowest towards the wrist. Through randomisation, the testing fields were located on different areas for each volunteer. This is also why the analysis of the average growth curve was considered more meaningful than the comparison of the individual growth curves.

Aside from a larger trial group, future studies should therefore consider the inclusion of baseline samples to provide information on the initial bacterial presence and distribution on the forearms. Follicular mapping to establish the pre-treatment follicle density should also be considered in this case. [4]

An extension of the trial period must be considered for future studies. Since that the storage capacity of the hair follicles is comparable to the storage capacity of the stratum corneum on several parts of the body, it was stipulated that the human hair follicles are potential reservoirs for topically applied substances. [5] Investigations have shown a prolonged storage in the hair follicles for a time period of up to 10 days in the case of the nanoparticle associated dye, while the non-particulate form could be detected for only up to 4 days. [9] These studies confirm that the hair follicle structures also present a promising reservoir for applied substance. An extension of the trial period would allow for the evaluation of the long-term antiseptic efficacy of particle-associated antiseptics.

In order to avoid potential field contamination by neighbouring substances, follow-up studies should consider a repelling border for the demarcation of testing fields. This

could possibly be achieved with (non-toxic) window paint or another liquid repelling material that is non-absorbant, so as to avoid the loss of fluid through spreading or soaking, thus retaining the same concentration on all testing fields. Alternatively, further studies should be conducted with a safety margin between the different application areas.

Liposomal o/w-emulsions with bound antiseptics present an interesting opportunity for modern prevention methods. The application is fast and simple, not unlike the already widely used prevention method with alcohol-based hand disinfectants. Both the simplicity of application and the low manufacturing costs make particle-associated antiseptics suitable to widespread implementation and daily individual use. Due to the storage capacity of the hair follicles, nanoparticle carriers for antiseptics may be able to reduce the required frequency of application. This property addresses the often low adherence to the widely used hand rub method that requires frequent repetitions and often causes adverse side effects, such as dry skin and rashes.

As explained in the bound review on modern methods for antisepsis, TTP is another emerging technology enabling the antisepsis of the hair follicle reservoirs. As in the case of the liposome associated antiseptic solution, the penetration of plasma into the hair follicles presents an important technological advantage over conventional antiseptic methods. In addition to its antimicrobial efficiency, TTP has been shown to promote wound healing in chronic wounds, especially when combined with antiseptic agents. Recent findings have shown that TTP-treatment of the skin stimulates and enhance the penetration of particles and substances into the hair follicles. [13, 14] Therefore, these findings may also be linked to the enhanced penetration of the antiseptic agent through TTP-treatment. Future studies should address the possibility of combining TTP with antiseptic agents, or merging both TTP- and nanotechnology. This method could be further improved by administering prolonged released antiseptics, allowing the release of the active substance over time. Combined with the storage properties of the hair follicle structure, this could result in dramatically longer effective desinfection periods, which are needed, for instance, in long operative procedures.

TTP is currently restricted to specialized institutions, there is however hope that further development may soon make this an effective prevention method suitable for widespread implementation.

As both TTP and particle-associated antiseptics represent novel technologies and only a limited number of data is available to date, further investigations need to be conducted before a standardized use of either method can be considered. Thus far, results suggest that intact liposomes do not penetrate deeper than the corneal layer after topical application, but further investigations should focus on the penetration depths of liposomes with bound antiseptics so as to exclude an uptake of antiseptic agent into the organism. Though the cumulative dose of PHMB applied to the subjects during the in vivo experiment was very low, so that the possible intake of PHMB can be considered harmless, a crossing of the skin barrier needs to be evaluated and potential health risks assessed before considering daily use. Likewise, although it is not expected that the application of TTP on skin or wounds is associated with long-term risks, further risk assessment needs to be conducted to evaluate the exact penetration depths and long-term effects.

Conclusion

Recent studies have demonstrated that the hair follicle is both an efficient long-time reservoir for topically applied substances and a focal point for bacterial growth, shielding microorganisms from harmful exogenous factors such as conventional antisepsis with liquid rubs. As such, the hair follicle should be viewed as a strategic target for efficient long-term antisepsis. It was shown that nanoparticles such as liposomes penetrate efficiently into and can be stored within the hair follicle structure. Based on this approach, *in vitro* and *in vivo* investigations were conducted to determine the efficacy of an oil-in-water (o/w) dispersion, consisting of liposomes and the bound antiseptic polihexanide (PHMB), for long-term antisepsis. Examination of biopsies showed an effective penetration of both the o/w-dispersion with and without bound PHMB into the hair follicles of porcine ear skin. Likely due to the superior size of the particles, the penetration depth of the o/w-emulsion with associated PHMB was significantly superior. A second study analysed the *in vivo* efficacy of the o/w-emulsion with associated PHMB compared to that of a standard PHMB solution on human skin. Though statistically significant results could not be obtained by directly comparing the two test solutions, tendencies suggested an effective antisepsis on the skin surface treated with the o/w-emulsion with associated PHMB for at least 150min, whereas the areas treated with the standard antiseptic showed an increase of bacterial colonies over time within 30min after application.

Combining these results, and those of previous investigations, it is likely that the effective follicular penetration of the o/w-emulsion with associated PHMB is responsible for the longer and improved antiseptic effect. The faster recontamination on skin treated with standard PHMB is likely due to a spreading of bacteria from the hair follicle. The nanoparticle-emulsion ensures a longer decontamination period by transporting the bound PHMB deep into the follicle, disinfecting the hair follicle shaft and infundibulum. Though further investigations are needed, these results appear to confirm the role of the hair follicle as a bacterial reservoir and suggest that the human hair follicle represents an endogenous pathway for rapid skin recontamination following desinfection. Nano-sized particles such as liposomes are therefore likely to play an important role in the research and synthesis of new antiseptic formulations in the near future.

As shown in this summary, tissue-tolerable plasma (TTP) is another promising pathway for skin antisepsis currently under development. Results suggest that TTP may also effectively reach the hair follicle reservoir, thus preventing an endogenous recontamination. Interestingly, TTP-treatment was also shown to stimulate the penetration of particles into the skin. High costs, special training, and the need for high level technical support currently restrict TTP use to specialized institutions. However, the antimicrobial properties and low cytotoxicity of TTP are advantages that make this method a promising concept for improved antisepsis and treatment of chronic wounds in the near future.

As the hair follicle structure emerges as a new target, this study underlines the need for further engineering of antiseptics in order to improve their efficiency. The results indicate that conventionally applied commercially available skin antiseptics do not sufficiently target the microbial population within hair follicles. Both liposomes combined with PHMB and TTP are novel methods that target the hair follicle structure. They are not expected to be associated with long-term risks. However, further risk assessments must take place before either method may be implemented. TTP and the delivery of antiseptics using nano-sized liposomes are novel technologies with a strong potential. Future possibilities such as liposome-associated antisepsis with an extended-release antiseptic will have to be explored. In particular, the combined application of TTP and nano-sized antiseptic liposome emulsions presents a promising opportunity to expand the possibilities of modern antisepsis.

References

1. Kramer A, K.G., Oldhafer K et al, *Prävention von SSI in der Chirurgie. In: Kramer A, Assadian O, Exner M et al (Hrsg) Krankenhaus- und Praxishygiene.* . 2011 München: Elsevier Urban & Fischer.
2. Dumville, J.C., et al., *Preoperative skin antiseptics for preventing surgi-cal wound infections after clean surgery.* status and date: New search for studies and content updated (conclusions changed), published in, 2013(3).
3. Dettenkofer, M., et al., *Skin disinfection with octenidine dihydrochloride for central venous catheter site care: a double‐blind, randomized, controlled trial.* Clinical Microbiology and Infection, 2010. **16**(6): p. 600-606.
4. Lange-Asschenfeldt, B., et al., *Distribution of bacteria in the epidermal layers and hair follicles of the human skin.* Skin Pharmacol Physiol, 2011. **24**(6): p. 305-11.
5. Otberg, N., et al., *Variations of Hair Follicle Size and Distribution in Different Body Sites.* J Investig Dermatol, 2004. **122**(1): p. 14-19.
6. Dohmen, P.M., et al., *A retrospective non-randomized study on the impact of INTEGUSEAL, a preoperative microbial skin sealant, on the rate of surgical site infections after cardiac surgery.* International Journal of Infectious Diseases, 2011. **15**(6): p. e395-e400.
7. Patzelt, A., et al., *Hair follicles, their disorders and their opportunities.* Drug Discovery Today: Disease Mechanisms, 2008. **5**(2): p. e173-e181.
8. Medina, C., et al., *Nanoparticles: pharmacological and toxicological significance.* British journal of pharmacology, 2007. **150**(5): p. 552-558.
9. Lademann, J., et al., *Nanoparticles-An efficient carrier for drug delivery into the hair follicles.* European Journal of Pharmaceutics and Biopharmaceutics, 2007. **66**(2): p. 159-164.
10. Reeves, A.E., *Curcumin-Combretastatin Nanocells as Breast Cancer Cytotoxic and Antiangiogenic Agent*. 2008, GEISINGER CLINIC DANVILLE PA.
11. Fridman G, F.G., Gutsol A, Shekhter AB, Vasilets VN, Fridman A. , *Applied Plasma Medicine.* Plasma Proc Polymers, 2008. **5 (6)**: p. 503-33.
12. Kramer A, A.O., Below H, Bender C, Hammann A, Hübner NO, Koban I, Kocher T, Lademann J, Matthes R, Weltmann KD, *Perspektiven der Plasmamedizin durch Anwendung von gewebekompatiblen Atmosphärendruckplasmen (Tissue Tolerable Plasma, TTP).* Vakuum Forsch Prax 2010. **22(2)**: p. 33-38.
13. Lademann, O., et al., *Drug delivery through the skin barrier enhanced by treatment with tissue‐tolerable plasma.* Experimental dermatology, 2011. **20**(6): p. 488-490.
14. Lademann, O., et al., *Stimulation of the penetration of particles into the skin by plasma tissue interaction.* Laser Physics Letters, 2011. **8**(10): p. 758-764.

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„Ich, Miriam Ulmer, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: The Penetration, Efficacy and Potential of Antiseptic Nanoparticle-Emulsions selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Anteilserklärung an den erfolgten Publikationen

Miriam Ulmer hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: M. Ulmer, A. Patzelt, T. Vergou, J. Lademann, H. Richter, A. Kramer, G. Müller, W. Sterry, B. Lange-Asschenfeldt, *In vitro investigation of the follicular penetration of porcine ear skin using a nanoparticle-emulsion containing the antiseptic polihexanide*, Laser Physics Letters, 2012.

Beitrag im Einzelnen (bitte kurz ausführen):

a) Vorbereitungen von Gewebsproben
b) Durchführung der Versuche (Biopsien, Laser Scan Microscopy)
c) Erhebung und Auswertung der statistischen Daten
   Verfassen des Publikationsentwurfes, Abstimmung mit dem korrespondierenen Autor und allen Koautoren

Publikation 2: M. Ulmer, A. Patzelt, T. Vergou, H. Richter, G. Müller, A. Kramer, W. Sterry, V. Czaika, J. Lademann, *In vivo investigation of the efficiency of a nanoparticle-emulsion containing polihexanide on the human skin*, European Journal of Pharmaceutics and Biopharmaceutics, 2012.

Beitrag im Einzelnen (bitte kurz ausführen):

a) Administrative Vorgänge (Ethikkommission, Probandengewinnung, Screening und Aufklärung
b) Herstellen der endgültigen Versuchslösungen
c) Durchführung und Auswertung der Versuche
d) Statistische Auswertung in Zusammenarbeit mit dem statistischen Institut der Charité
e) Verfassen des Publikationsentwurfes, Abstimmung mit dem korrespondierenden Autor und allen Koautoren

Publikation 3: M. Ulmer, J. Lademann, A. Patzelt, F. Knorr, A. Kramer, T. Koburger, O. Assadian, G. Daeschlein, B. Lange-Asschenfeldt, *Review: New strategies for preoperative skin antisepsis*, Skin Pharmacology and Physiology, 2014.

Beitrag im Einzelnen (bitte kurz ausführen):

a) Literaturrecherchen, fachliche Korrespondenz mit den relevanten Instituten
b) Verfassen des Publikationsentwurfes zum Thema partikulärer Desinfektionsmittel

c) Abstimmung mit den Autoren bezüglich der therapeutischen Anwendung von hautverträglichem Plasma (TTP)
d) Verfassen der Endfassung des Manuskripts

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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Liste der Ausgewählten Publikationen / List of chosen publications

Authors: M. Ulmer, A. Patzelt, T. Vergou, J. Lademann, H. Richter, A. Kramer, G. Müller, W. Sterry, B. Lange-Asschenfeldt

Title: *In vitro investigation of the follicular penetration of porcine ear skin using a nanoparticle-emulsion containing the antiseptic polihexanide.*

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Title: *In vivo investigation of the efficiency of a nanoparticle-emulsion containing polihexanide on the human skin.*

Journal: European Journal of Pharmaceutics and Biopharmaceutics

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Authors: M. Ulmer, J. Lademann, A. Patzelt, F. Knorr, A. Kramer, T. Koburger, O. Assadian, G. Daeschlein, B. Lange-Asschenfeldt

Title: *Review: New strategies for preoperative skin antisepsis.*

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Publikationsliste / List of publications and impact factors

Authors: M. Ulmer, A. Patzelt, T. Vergou, J. Lademann, H. Richter, A. Kramer, G. Müller, W. Sterry, B. Lange-Asschenfeldt
Title: *In vitro investigation of the follicular penetration of porcine ear skin using a nanoparticle-emulsion containing the antiseptic polihexanide.*
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Authors: M. Ulmer, A. Patzelt, T. Vergou, H. Richter, G. Müller, A. Kramer, W. Sterry, V. Czaika, J. Lademann
Title: *In vivo investigation of the efficiency of a nanoparticle-emulsion containing polihexanide on the human skin.*
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Authors: M. Ulmer, J. Lademann, A. Patzelt, F. Knorr, A. Kramer, T. Koburger, O. Assadian, G. Daeschlein, B. Lange-Asschenfeldt
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