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Research paper

Stability of polylactic acid particles and release of fluorochromes upon topical application on human skin explants

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ABSTRACT

Particle-based drug delivery systems allow the controlled and targeted release of incorporated active compounds to the skin and are promising tools to improve the efficacy of topical therapies. In this study we investigated the stability and release properties of biodegradable polylactic acid (PLA) particles upon topical application on human skin explants. PLA particles loaded with the hydrophilic fluorochrome 4-Di-2-Asp (DiAsp-PLA) were compared to PLA particles loaded with the lipophilic fluorochrome Bodipy 630/650 (BP-PLA). Changes of the particle morphology after their incubation on skin surface were investigated by means of electron microscopy while fluorescence microscopy and flow cytometry were used to evaluate particle penetration in hair follicles and fluorochrome release. We found that BP-PLA particles released rapidly the loaded fluorochrome and lost the particulate morphology within a few hours after application on skin surface. On the contrary, DiAsp-PLA particles maintained the particulate morphology, accumulated in hair follicles, and allowed a constant release of the incorporated fluorochrome for up to 16 h. These results show that, once applied to skin surface, PLA particles release the incorporated fluorochromes in a time-dependent manner and suggest the perspective to modulate particle stability and release properties by incorporating excipients with different degree of lipophilicity.

1. Introduction

During the last years, carrier systems have proved to be a matter of intense medical and pharmaceutical research due to the need for innovative, non-invasive, low-risk, as well as selective and more effective diagnostic tools and therapeutic approaches. Providing enhanced bioavailability and limited side effects of incorporated substances through novel administration methods and penetration pathways [1], nanoparticles are going to find broad application in both biotechnology and medicine fields [2–7]. Microspheres and nanoparticles have already been investigated for use in diagnostic and treatment of cancer, in cardiovascular medicine, pneumology, neurology, ophthalmology, immunology, and dermatology [8–15]. Based on their preferential penetration and accumulation in hair follicles (HFs) [16,17] along with the perspective to release the incorporated substances selectively to epidermis, dermis, or sebaceous glands [2,6,15], particles represent promising carrier systems for local drug delivery in dermatology. A variety of particle types and preparation methods have been introduced so far [18–28] in order to influence particle biodistribution. Multiple studies suggest that selective skin penetration and specific targeting effects can be achieved by modifying particle size, lipophilicity, surface charge, and stability [18–28].

Biodegradable particles represent a highly interesting option in the development of carrier systems for skin targeting strategies. In fact, reports on the possible translocation of nanoparticles across the skin barrier have fostered safety concerns for the use of micro- and nanoparticles in dermatological applications [29]. Since polylactic acid (PLA) is a biodegradable polymer with low toxicity, having already found medical use in the reconstructive medicine

Abbreviations: PLA, polylactic acid; DiAsp, 4-(4-diethylaminostyryl)-N-methylpyridinium iodide; BP, bodipy 630/650 methyl bromide; EM, electron microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; HFs, hair follicles; MFI, mean fluorescence intensity.

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(e.g., stents, joint replacement), PLA particles seem to be a promising carrier system for topical as well as transdermal therapy strategies.

In a model of barrier-disrupted skin, we have found that polystyrene (PS) particles (Fluospheres, Molecular Probes, 40 nm) applied on skin surface accumulated in HFs could translocate to the viable epidermis and, as a consequence, were internalized by Langerhans cells [30]. On the contrary, in a successive study we found that PLA particles were not associated with Langerhans cells after topical application on barrier-disrupted skin. Particle accumulation in HFs was followed by particle aggregation and destabilization along with the diffusion of the incorporated fluorochrome to the skin [15]. Interestingly, such destabilization only occurred after PLA particle contact with the skin surface or a lipophilic solvent but not when particles were dispersed in aqueous solvents. We therefore hypothesized that the physicochemical nature of the constituting polymer influences particle stability in the different environments. Nevertheless, those incorporated substances (e.g., fluorochromes or drugs) intercalated between the polymer strands, having affinity for hydrophobic environments and being free to diffuse out of the particles, might also influence PLA particle stability on skin surface. In our previous work, we used PLA particles loaded with high lipophilic dyes (nile red and coumarin-6) having high affinity to cell membranes and lipophilic skin areas. In this study, we investigated whether the incorporation of a moderately lipophilic fluorochrome (Bodipy 630/650, BP, $\log P = -1.109$[2]) or a hydrophilic one (4-Di-2-Asp, DiAsp, $\log P = 0.755$[2]) can improve the stability of PLA particles on skin surface. The two PLA particle formulations, DiAsp-PLA and BP-PLA, were therefore compared with regard to skin penetration and fluorochromes release kinetics. Furthermore, electron microscopy was used to detect particle distribution and morphology after topical application on human skin explants.

2. Material and methods

2.1. Preparation of PLA particles

Two PLA particle formulations were investigated. BP-PLA particles were loaded with 0.2% (w/w) 8-bromomethyl-4,4-difluoro-3,5-bis-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene (Bodipy 630/650 methyl bromide, Invitrogen, France), and DiAsp-PLA particles were loaded with 0.2% (w/w) (4-(4-diethylaminostyryl)-N-methylpyridinium iodide (4-Di-2-Asp, Invitrogen, France). Poly (D,L-lactic acid) 50,000 g/mol was purchased from Phusis (Grenoble, France). Particles were synthesized as described in Lamalle-Bernard et al. [31]. This method allows the preparation of anionic PLA nanoparticles without addition of any surfactant. Briefly, the polymer was dissolved in acetone at a concentration of 2% w/w together with the fluorescent dyes. This solution was added to an aqueous solution under moderate stirring and the mass transfer of acetone into the continuous aqueous phase resulted in the formation of PLA nanoparticles by precipitation. The solvents were then evaporated under reduced pressure at room temperature. No residual solvent was detected by proton NMR spectra from dissolved particle samples. Nanoparticle size distribution was determined by quasi-elastic light scattering at 25 °C, at a scattering angle of 90°, using a Zeta sizer 3000HS (Malvern instruments, UK). Particle average size was 207 nm (polydispersity index 0.067) for Di-Asp-PLA particles and 164 nm (polydispersity index 0.045) for BP-PLA particles. Zeta potential of -50 ± 3 mV and -55 ± 5 mV was measured for Di-Asp-PLA and BP-PLA particles, respectively.

[2] Calculated with Molinspiration Property Calculator: http://www.molinspiration.com/cgi-bin/properties.

The final PLA concentration was between 60 and 70 mg/ml depending on the batch and was precisely measured by weighing the wet and dried materials. Solutions were protected from light throughout the experimental procedure. Particles were stored after synthesis at 4 °C.

2.2. Scanning electron microscopy (SEM)

PLA and PS (200 nm carboxylate-modified microspheres, Fluospheres®, Invitrogen, USA) particle dispersions in phosphate buffer were dried on gold electron microscopy (EM) grids coated by a ~300 nm polyvinyl formal film (Formvar, Polysciences Inc., MW 24,000–40,000 g/mol). SEM images were recorded immediately after drying and after 72 h with a Hitachi S-4000 SEM using an acceleration voltage of 20 kV. The images were analyzed with the Digital Image Processing System from Point Electronic GmbH.

2.3. Tissue samples and topical particle application

Human skin (retroauricular region, breast, and abdomen) was obtained within 24 h after surgical excision from healthy volunteers undergoing plastic surgery. Volunteers had signed an informed consent approved by the Institutional Ethics Committee of the Medical Faculty of the Charité-Universitätsmedizin Berlin and in accordance with the ethical rules stated in the Declaration of Helsinki Principles. Particle application on skin explants was performed according to established procedures [15]. Prior to the application of particles, cyanoacrylate skin surface stripping (CSSS) was performed once as described previously [17] using superglue (UHU GmbH, Buehl/Baden, Germany). Experiments were done on retroauricular skin samples with size of 2×2 cm = 4 cm^2 for the evaluation of skin penetration and on breast and abdomen skin with size of 4×4 cm = 16 cm^2 for the flow cytometry analyses of isolated epidermal cells. Shortly before the transcutaneous application of particles, dispersions in PBS (pH = 7.4) were prepared by dilution of the stock solutions and vigorous vortexing. The particle dispersions were applied on the surface of each skin sample (20 µl/cm^2) at a concentration of 0.1% w/v, which corresponded to 3.47 E+11 particles per ml in case of BP-PLA particles and 1.72 E+11 particles per ml in case of DiAsp-PLA. Particle suspensions were applied centrally on each skin sample, leaving safety margins of 0.5 cm to the border of the tissue in order to avoid sideways non-specific penetration of tested particles into the tissue. Samples were then placed in a humidified chamber and incubated at 37 °C, 5% CO_2, 100% humidity over 2, 4, 8, 12, and 16 h. In follicular penetration experiments, skin samples from three different donors were used for each incubation time. For epidermal cell isolation, abdominal or breast skin samples from five different donors were used.

2.4. Transmission electron microscopy (TEM)

After PLA particle application on human skin explants and incubation (2, 4, 8, and 16 h) in humidified chambers (37 °C, 5% CO_2, 100% humidity), samples of particles were re-collected from skin by placing 400 nm copper TEM grids coated by a ~15 nm carbon film (Quantifoil) on the skin and pressing them slightly against the skin using a cover slip. Transmission electron microscopy (TEM) images were recorded with a Zeiss EM 10 CR. with an acceleration voltage of 80 kV. The images were analyzed with the software Simple PCI from C-Images.

2.5. Adhesive tape stripping

After particles incubation (2, 4, 8, and 16 h) on excised human skin, adhesive tape stripping was performed five times on the

tissue samples using transparent tape (Tesafilm®, Hamburg, Germany). The tapes were affixed on a glass slide and subsequently observed with a fluorescence microscope. 4-Di-2-Asp was detected using a BX60F3 Olympus microscope (Hamburg, Germany) with 470–490 nm band pass and 550 nm long pass filters, while Bodipy 630/650 was detected using an AxioPlan microscope (Zeiss, Germany) using Texas Red filters. Pictures of at least ten different areas per sample were taken with a CCD camera (Olympus, Germany).

2.6. Cryosections and fluorescence microscopy

The penetration profile of the two PLA particle formulations in HFs and the time-dependent diffusion of fluorochromes into skin after particle topical application were investigated on skin cryosections by fluorescence microscopy. After skin incubation, adhesive tape stripping was performed five times to remove the particles that had not penetrated into HFs and to reduce the amount of free particles on skin surface. A skin area of 1 cm^2 was excised and split into four blocks which were frozen in liquid nitrogen. Cryosections of 5 μm thickness were prepared from each block using a microtome (2800 Frigocut-N, Reichert-Jung, Heidelberg, Germany). To avoid counting the same hair follicle twice or more, once obtained a central section of a hair follicle, we excluded the next 2–5 sections. Horizontal sections of hair follicles were excluded from the analysis. As in previous studies of our group [17], cutting was performed with fresh blades from the dermis side toward the epidermis in order to avoid dislocation of particles from the skin surface on the section. Cryosections from three different donors were screened by fluorescence microscopy. 4-Di-2-Asp was detected using a BX60F3 Olympus microscope (Hamburg, Germany) with 470–490 nm band pass and 550 nm long pass filters, while Bodipy 630/650 was detected using an AxioPlan microscope (Zeiss, Germany) using Texas Red filters.

2.7. Evaluation of particles and fluorochrome penetration depth

A semi-quantitative analysis of skin cryosections was performed in order to evaluate the penetration depth of DiAsp-PLA particles in HFs. For each incubation time, a number of 148–356 cryosections and at least 150 HFs from three different donors were analyzed. Terminal hairs were excluded from the study and only sections with vellus and intermediate hair follicles were evaluated. HFs were classified, according to the maximal penetration depth reached by the particles: *Superficial Accumulation, Middle Epidermis, Complete Epidermis*, and *Deep Penetration*. Similarly, the depth of BP penetration in skin after topical application of BP-PLA particles was evaluated performing a semi-quantitative analysis of skin cryosections. For each incubation time, a number of 300–500 cryosections from three different donors were evaluated. Cryosections were classified according to the maximal dye penetration depth into: *Superficial Accumulation, Middle Epidermis, Complete Epidermis*, and *Deep Penetration*.

2.8. Isolation of epidermal cells and flow cytometry

After 16 h of particle incubation on skin surface, adhesive tape stripping was performed five times to remove superficial particles. Skin samples from at least four different donors were chopped into 3 mm^2 slices and digested with dispase (2.4 U/ml Dispase I, Roche, Germany) over 2.5 h at 37 °C in order to detach the epidermis layer. Trypsin digestion (0.025% trypsin, 1.5 mM $CaCl_2$ in PBS) was then performed over 15 min in order to separate epidermis cells. Cells were collected after filtration and centrifugation at 216 g for 10 min. Fluorescence microscopy pictures of at least 15 cells per sample were taken by means of an Axioplan microscope (Zeiss, Germany). Flow cytometry analysis was carried out using FACS Calibur and CellQuests software (Becton Dickinson, Heidelberg, Germany). A total of 20,000 events per sample were collected. For data analysis, FCS-Express 3.1 software (De Novo Software, USA) was used.

3. Results

3.1. PLA particles stability upon deposition on Formvar films

In order to test the stability of the different PLA formulation on a lipophilic surface, particles were incubated on electron microcopy grids covered with Formvar films and their integrity was investigated by means of scanning electron microscopy. Shortly after deposition on Formvar-coated EM grids, unlabeled PLA particles as well as fluorochrome-loaded PLA particles appeared as homogeneously dispersed single or agglomerated particles (Fig. 1A). However, when particle dispersions were incubated for several hours on the polymer film surfaces, the different particle formulations exhibited different degree of stability. Especially, we noticed an irreversible destabilization of BP-PLA particles. Unlabeled PLA and DiAsp-PLA particle were agglomerated but kept the particulate morphology, whereas most of BP-PLA particles had lost their particulate form and only few single intact particles were imaged (Fig. 1B). FITC-labeled PS particles (FITC-PS), which in our previous studies [15,30] were found to accumulate in HFs without release of the loaded fluorochrome, were used as control particles. As expected, they kept the particulate state upon incubation on Formvar films and formed ordered single layers on the Formvar polymer surface (Fig. 1A and B). The fluorescence images of the same particles shortly after deposition on Formvar films (Fig. 1C) and 72 h after incubation on the lipophilic polymer (Fig. 1D) allowed assessing that the different particle formulations had undergone different degree of aggregation, forming structures similar to those already observed in our previous study [15]. Only electron microscopy allowed us to compare particle morphology and assess the destabilization and loss of the particulate state for BP-PLA samples after incubation on the polymer surface.

Following these findings, we hypothesized that upon incubation on skin surface, PLA particles loaded with lipophilic fluorochromes may undergo similar destabilization as observed after their deposition on Formvar films. To analyze changes in PLA particles' morphology after their incubation on skin surface, particles were collected after incubation on skin surface by placing EM grids on the skin samples and pressing them slightly using a cover slip. The recovered material was analyzed by SEM shortly after its collection. Particles were identified in all EM grids. However, it was not possible to obtain sharp high magnification images of the recovered PLA and PS particles. The particles were probably embedded in lipids or other biological material which had been collected from skin as well. The biological material entrapped part of the scattered electrons hindering the collection of distinct high magnification images (data not shown). This effect did not allow us to perform an efficient SEM analysis of PLA particles' stability after their incubation on skin surface. This was achieved by means of TEM as reported in the next chapter. Nevertheless, we can conclude that the SEM analysis of particles incubated on Formvar film surface was useful to assess aggregation and changes in the particulate state of different PLA particle formulations upon incubation on a hydrophobic surface similar to skin.

3.2. PLA particles distribution and stability upon incubation on skin surface

Aggregation and changes in the morphology of PLA particles after different incubation times on human skin explants were

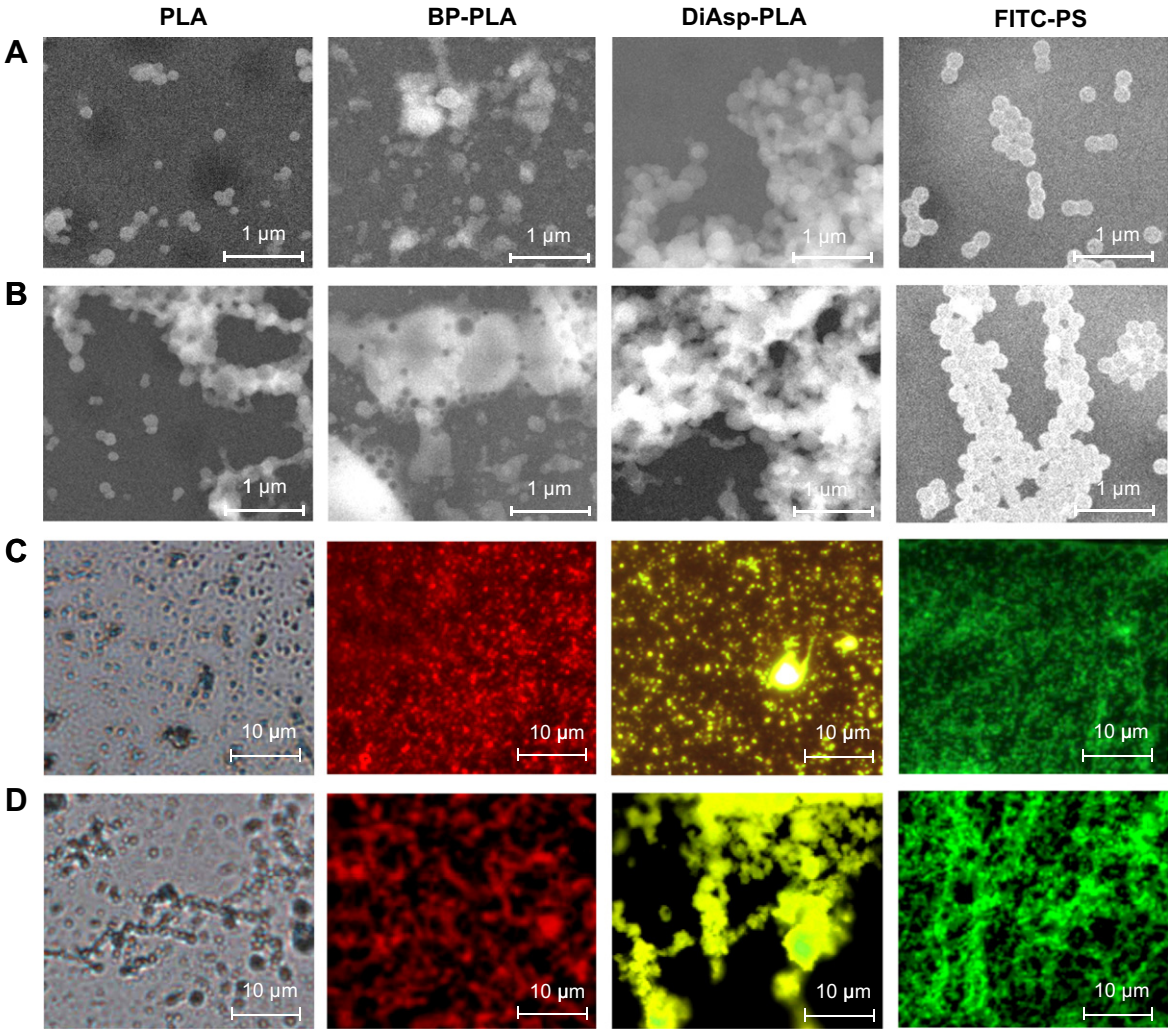

Fig. 1. PLA particles loaded with different fluorochromes displayed different stability upon deposition on the surface of hydrophobic polymer films. Representative scanning electron microscopy (SEM) (A and B) and fluorescence microscopy (C and D) images of particles incubated on Formvar films show that BP-PLA samples lost the particulate morphology upon incubation on the polymer surface forming non-particulate structures, whereas unlabeled PLA, DiAsp-PLA, and FITC-PS particles kept their particulate state. Particle dispersions in PBS were applied on EM grids coated by Formvar polymer films and images of the particles were taken shortly after their deposition on the film surface (A and C) or after 72 h (B and D). FITC-labeled polystyrene particles (FITC-PS), which were shown to be stable on skin surface [15,30], served as control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

assessed by means of transmission electron microscopy (TEM). Whereas the SEM analysis of PLA particles collected from skin was not successful, TEM images were sufficiently focused allowing an adequate analysis of sample particulate morphology. In addition, the distribution of PLA particles on the surface of the same skin samples was investigated by collecting and analyzing the outermost skin layers using fivefold adhesive tape stripping and fluorescence microscopy.

DiAsp-PLA particles were homogeneously distributed on the outermost layers of the stratum corneum (Fig. 2A, tapes 1 and 2) and deeper penetration within the stratum corneum was observed only in correspondence to skin furrows and HFs (Fig 2A, tapes 4 and 5). Small aggregates of DiAsp-PLA particles were observed homogeneously distributed on skin surface predominately after 2 and 4 h of incubation, whereas bigger particle agglomerates were observed after longer incubation times (8 and 16 h). The TEM analysis confirmed the presence of particle aggregates at all incubation times (Fig. 2A, TEM). Only few non-particulate structures were detected among aggregated particles, whereas most of the DiAsp-PLA particles kept the particulate state even after 16 h of incubation on skin surface. Different distribution patterns on skin surface and destabilization were observed for the BP-PLA particle formulations. Dispersed BP-PLA particles were hardly detectable by means of conventional fluorescence microscopy because of their small size (164 nm). However, a granular pattern of fluorescence could be detected in the first layers of the stratum corneum after 2 and 4 h of incubation (Fig. 2B, tapes 1 and 2). No particle aggregates similar to those of DiAsp-PLA particles were observed. On the contrary, corneocytes and especially the intercellular spaces of the stratum corneum showed an intense fluorescence, which decreased after longer incubation times and was barely detectable after 16 h (Fig 2B). The TEM analysis of BP-PLA samples showed aggregated particles after 2 and 4 h, whereas a mixture of particles and non-particulate structures were detected after 8 h. Finally, after 16 h of incubation, almost only non-particulate structures were observed besides few aggregated particles. The structures formed by destabilized BP-PLA particles on the skin surface were similar to those observed by means of SEM after particle incubation on Formvar films (Fig. 1B).

We can therefore summarize that BP-PLA particles destabilize within a few hours after application on the skin surface. Concomitant to the particle destabilization release of the loaded fluorochrome occurred. On the contrary, DiAsp-PLA particles formed aggregates on skin surface but were longer stable than BP-PLA

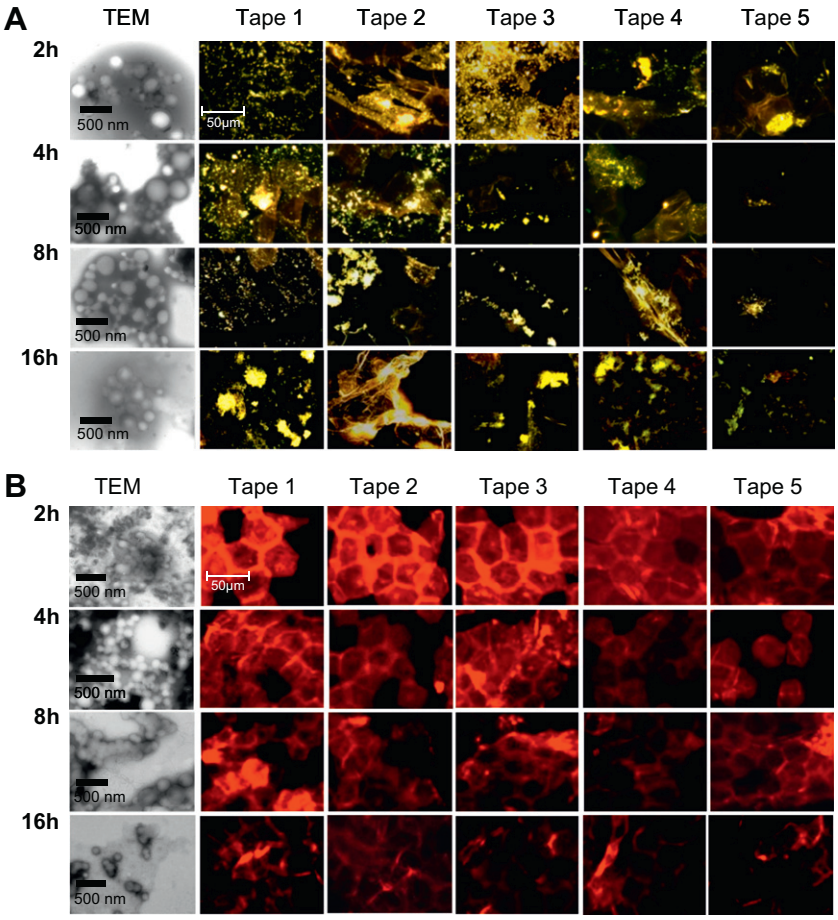

Fig. 2. Transmission electron microscopy (TEM) and fluorescence microscopy (tapes 1–5) images of DiAsp-PLA (A) and BP-PLA (B) particles collected from the surface of human skin explants after different incubation times (2, 4, 8, and 16 h). This selection of fluorescence microscopy images shows the different distribution patterns for the two PLA particle formulations on the skin surface. TEM images show that DiAsp-PLA particles were longer stable than BP-PLA particles, which after 16 h of incubation on skin explants had completely lost their particulate morphology. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

particles. These results confirmed our assumption that the stability of PLA particles upon contact with skin is influenced by the type of incorporated substance.

3.3. DiAsp-PLA particles penetration in hair follicles and fluorochrome release upon topical application on human skin explants

The penetration of DiAsp-PLA particles in HFs and the diffusion of released fluorochrome were evaluated in cryosections of human skin samples as described in materials and methods and in Fig. 3A. Dispersed single particles as well as particle aggregates were observed in HF openings and canals. Two hours after incubation, DiAsp-PLA particles had penetrated in 61 ± 3% of all observed HFs, whereas in the remaining observed HFs particles were accumulated only in the HFs openings (*Superficial Accumulation*, Fig. 3A and B). Approximately in 24 ± 8% of all observed HFs DiAsp-PLA particles had penetrated deep into the HFs infundibulum (Fig. 3B). After 4 h of incubation, the total number of HFs with penetrated particles had only slightly increased but the percentage of HFs with deep penetrated particles had increased (*Deeper Penetration*, 36 ± 5%). Interestingly enough, after 8 h of incubation the percentage of HFs with penetrated particles decreased (41 ± 5%). Especially, the number of HFs with deep penetrated particles (*Deep Penetration)* decreased strongly (18 ± 7%). After 12 h, we observed a second increase of HFs with penetrated particles (65 ± 6%) with percentages of deep penetrated particles similar to those after 4 h of incubation. Finally, after 16 h the percentages of HFs with particles penetrated to the end of the epidermis and deeper decreased again (33 ± 1% and 18 ± 2%, respectively). Especially, DiAsp-PLA particles which were penetrated in HFs canals provided for a constant dye release to the surrounding epithelium during the whole observation time (16 h). In most of the HFs sections with penetrated particles, the released fluorescent dye reached an average penetration depth corresponding to the end of the HFs infundibulum (225 ± 34 µm according to Vogt et al., [32]). Diffuse yellow fluorescence due to released DiAsp was observed also in the interfollicular SC and epidermis and was detectable in all samples with maximal fluorescence intensity after 4 h of incubation (Fig. 3C).

We conclude that during the observation time, DiAsp-PLA particles accumulated in HFs openings and canals providing for a constant release of fluorochromes to the skin. During the first 2 h, most of DiAsp-PLA particles accumulated in the HFs openings whereas at 4 h, particles penetrated in the HFs canals while releasing the fluorescent dye (Fig 2c, 4 h). After 8 h, the deeply penetrated particles had released most of the loaded fluorochrome and were no longer microscopically detectable. This resulted in a decrease of particle-positive HFs. However, during a longer incubation time, additional DiAsp-PLA particles from the superficial infundibulum (HFs openings) penetrated along the HFs canal resulting in a second increase of particle-positive HFs (Fig. 3B, 12 h) and in a further release of the fluorochrome.

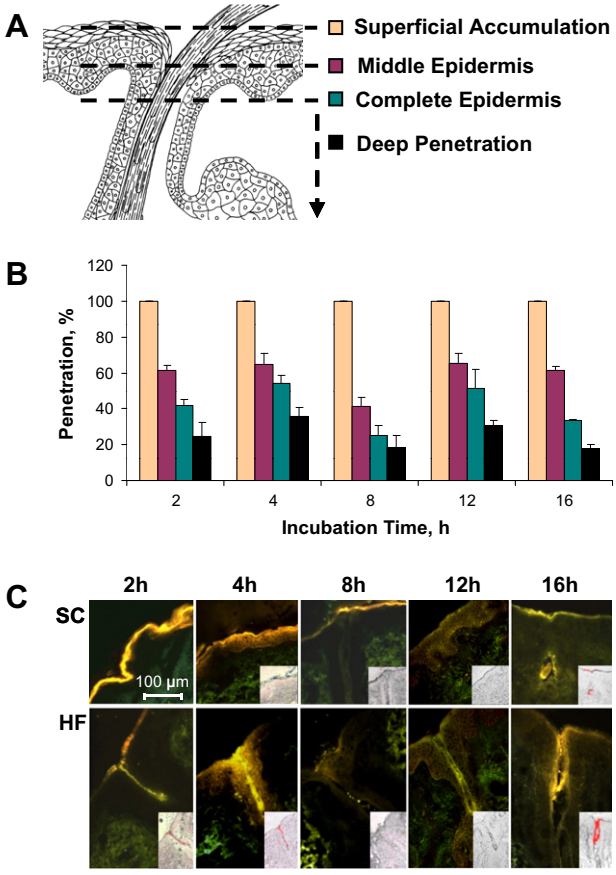

Fig. 3. Semi-quantitative analysis of DiAsp-PLA particle accumulation in HFs. For each incubation time, a number of 148–356 cryosections and at least 150 HFs, from three different donors, were examined. (A) HFs were classified according to the maximal penetration depth reached by the applied particles. (B) The penetration of DiAsp-PLA particles into HFs was investigated at different time-points after topical application of the particles on human skin explants. (C) Representative fluorescence microscopy images of skin cryosections after topical treatment with DiAsp-PLA particles. The images show the time-dependent diffusion of DiAsp into stratum corneum (SC) and the accumulation of particles in hair follicles (HF). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

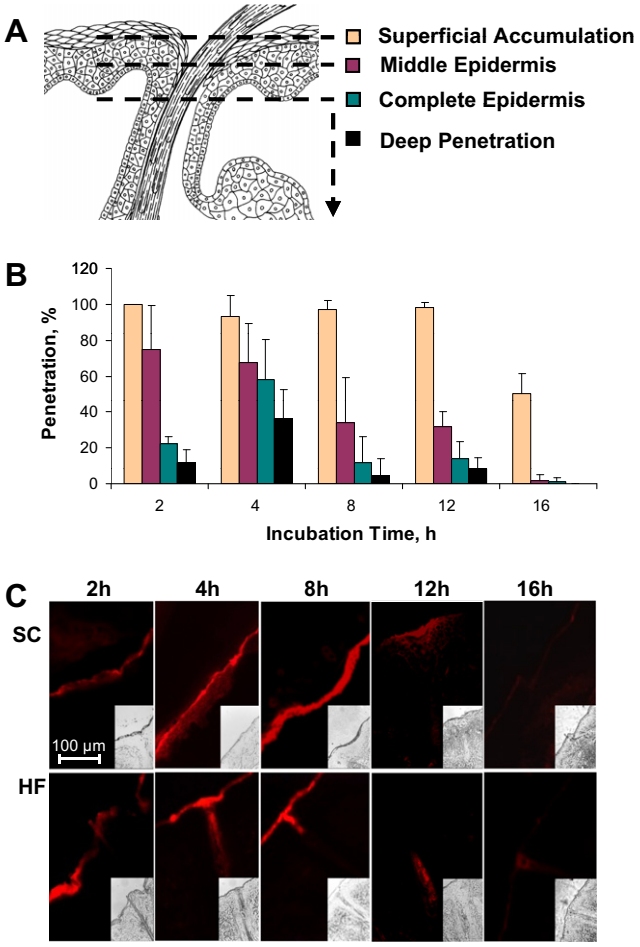

Fig. 4. Semi-quantitative analysis of BP penetration in skin after topical application of BP-PLA particles on human skin explants. For each incubation time, a number of 300–500 cryosections and at least 150 HFs, from three different donors, were examined. (A) Each skin cryosection was classified according to the depth reached by the released dye. (B) The penetration of BP into skin was investigated at different time-points after BP-PLA particle topical application. (C) Representative cryosections of skin after topical treatment with BP-PLA particles. The highest fluorescence intensity was observed within the stratum corneum (SC) and in hair follicle (HF) canals. BP diffused to SC and epidermis within the first 2 h, persisted up to 8 h, and was almost completely cleared after 16 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. BP skin penetration upon topical application of BP-PLA particles on human skin explants

The penetration depth of BP-PLA particles and released BP after particle topical application was evaluated in skin cryosections as described in Materials and Methods and in Fig. 4A. The identification of BP-PLA particles in the HFs canal was technically difficult since neither single particles nor particle aggregates were clearly distinguishable from the sebum which had been stained by the diffused BP. BP fluorescence intensity was detected in the stratum corneum in 92–100% of all analyzed cryosections after 2, 4, and 8 h, whereas after 16 h, only 54 ± 11% of cryosections showed fluorescence at this level (Fig. 4B). The highest number of skin sections with BP penetration to the m*iddle epidermis* was observed after 2 h (75 ± 25%), whereas the highest percentage of cryosections with dye penetrated to the entire epidermis and deeper were detected after 4 h of incubation (58 ± 22% and 36 ± 16%, respectively). Already after 8 h the fluorescence intensity of BP decreased, especially in the dermis and epidermis. Finally, after 16 h, no BP fluorescence intensity was detected in HFs canals and epidermis, with exception for marginal fluorescence intensity in the SC (Fig. 4B and C). At this time point, the dye had diffused to the dermis where homogeneous, low intensity BP fluorescence was detected. BP fluorescence persisted longer especially in skin areas with HFs where particles had accumulated. In correspondence to HFs, BP had diffused to the perifollicular epithelia along all the infundibulum (Fig. 4C, HF). BP fluorescence was also observed in association with sebaceous glands especially in intermediate HFs after 2, 4, and 8 h (data not shown). We conclude that BP-PLA particle formulation rapidly released the loaded fluorochrome upon application on skin surface and that the dye diffused to the epidermis and then the dermis within a few hours after release. In addition, these data confirm that different dye release kinetics are associated with different PLA particle formulations.

3.5. Analysis of epidermis cells isolated after topical application of particle on human skin explants

Epidermal cells isolated from skin explants previously incubated with PLA particles were analyzed by means of flow cytometry and fluorescence microscopy to verify the penetration of released fluorochromes in skin and to investigate the possible translocation of PLA particles to the epidermis layers. The flow

cytometry analysis of epidermis cells from skin treated with BP-PLA and DiAsp-PLA particles was compared with that of cells from untreated control skin. Flow cytometry histograms and mean fluorescence intensity (MFI) values of both DiAsp-PLA and BP-PLA samples were shifted with respect to control samples (Fig. 5A and B). The average MFI of three independent experiments (three different donors) was 15.2 ± 6.2 for BP-PLA particle and 12.6 ± 7.1 for DiAsp-PLA particle, which were significantly higher than that of the skin controls (MFI = 5.3 ± 0.6 and 6.6 ± 0.8, respectively). The histograms of cells isolated from untreated skin and then incubated with the same particles formulations were also shifted with respect to the control samples (Fig. 5C and d). However, these cells observed by means of fluorescence microscopy showed a granular pattern of fluorescence intensity, which is typical of internalized particles (Fig. 5C and D). On the contrary, the fluorescence microscopy analysis of cells isolated from skin after topical application of PLA particles (Fig. 5A and B) revealed a diffuse fluorescence intensity with patterns clearly distinguishable from those observed in isolated cells after incubation with particles (Fig. 5C and D).

We can conclude that topical applied PLA particles did not translocate to the epidermis, but released the loaded fluorochromes which diffused into skin through the epidermal cell layers.

4. Discussion

During the last years, various approaches to optimize particle bioavailability, stability, and drug release features have been introduced. One of the techniques to enhance the stability of hydrophobic particles is coating with hydrophilic polymers, which allows for longer availability in serum or tissues. Chan et al. [22] designed triple-layer doxorubicin-loaded particles (PLGA–lecithin–PEG), showing that a variation of lipid/polymer mass ratio and PLGA-polymer length could have an impact on drug release and that PEG coating contributes to prolonged stability of the particles. Similar results have been reported by Jain et al. [13] in a study with pegylated biodegradable particles for mucosal delivery of hepatitis B vaccine. It was postulated that PEG stabilizes polymeric particles, allowing for a prolonged release of the vaccine.

The incorporation of substances with stabilizing ability might be another option to control the stability and drug release properties of particulate carriers. The advantage would be the possibility to use biocompatible excipients, avoiding cross-linking, or the use of non-biodegradable polymers for the coating of particles. We previously reported that PLA particles loaded with highly hydrophobic dyes (nile red and coumarin-6) were stable in aqueous dispersions but aggregated and destabilized with rapid release of the incorporated fluorochrome upon contact with lipophilic areas of the skin [15]. We hypothesized that the instability of PLA particles on skin surface might be influenced by the physicochemical properties of the incorporated dye. The purpose of this study was therefore to investigate PLA particles loaded with moderately hydrophilic or hydrophobic fluorochromes, Bodipy 630/650 (BP), and 4-Di-2-Asp (DiAsp), respectively, in order to determine if the type of encapsulated substance might influence the stability of PLA particles on skin surface. Particle morphology, distribution on skin surface, and release of fluorochromes from the two PLA particle formulations were evaluated. Special emphasis was given to particle accumulation in HFs and diffusion of the released fluorochrome to the different epidermal layers. Thereby, we aimed to improve particle penetration in HFs and the kinetics of dye release with the goal to use PLA particles as drug delivery systems for dermatological applications.

The results of this study confirmed that the stability of PLA particles on skin surface can be modulated by the type of incorporated substance. Markedly different stability and release properties were observed for the two PLA particle formulations upon contact with skin. DiAsp-PLA particles were found to form aggregates which were homogeneously distributed on skin surface or accumulated in HFs canals maintaining the particulate morphology for up to 16 h. On the contrary, BP-PLA particles had the tendency to destabilize with loss of the particulate state and release of the loaded dye (Figs. 1 and 2).

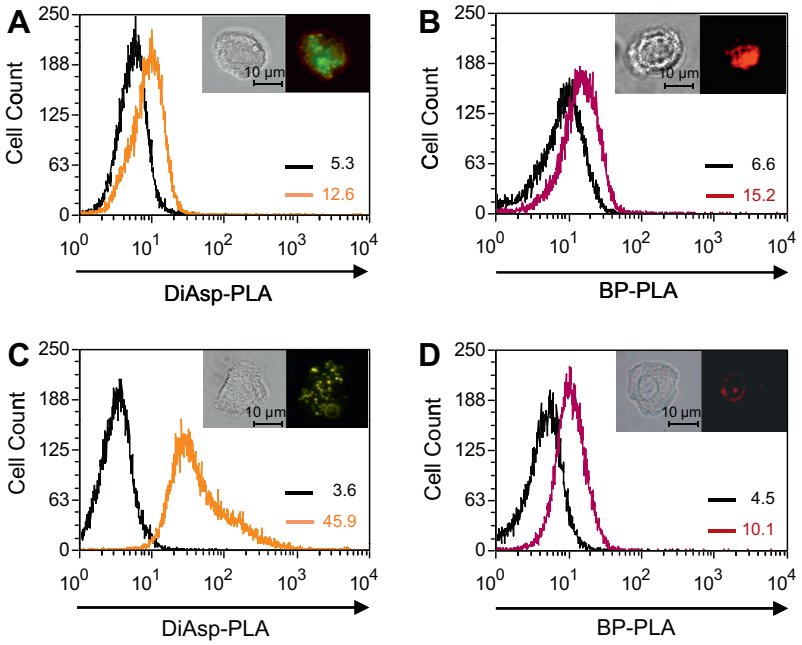

Fig. 5. PLA particles did not penetrate to the epidermis cell layers. Flow cytometry and fluorescence microscopy revealed that cells isolated from skin treated for 16 h with Di-Asp-PLA (A) and BP-PLA (B) particle formulations were homogeneously labeled by the fluorochromes released from the topically applied particles. On the contrary, PLA particles were stable in aqueous media and epidermis cells incubated *in vitro* with Di-Asp-PLA (C) and BP-PLA (D) particle displayed a granular fluorescence pattern typical for internalized particles. Mean fluorescence intensity of controls (black line) and samples (gray lines) of at least 4 different donors is shown in each panel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In order to investigate the behavior of particles upon contact with skin, we used electron microscopy to visualize particles re-collected after different incubation times on skin surface. The structures formed by destabilized BP-PLA particles on the polymer film surface (Fig. 1) were comparable to those formed by the same particles on skin surface and observed by means of fluorescence microscopy and TEM (Fig. 2). Formvar films are made of poly (vinyl formal), which is a hydrophobic polymer [33] and partially resembles skin lipophilic areas (i.e., ceramides, sebum). Formvar films reproduce the hydrophobic environment of skin while representing a simplified system with respect to skin. They are therefore a suitable model to study the behavior of particles upon interaction with a hydrophobic surface similar to skin. The TEM analyses of particles collected from skin allowed us to detect changes in the particulate state of BP-PLA particles upon topical application. The recording of TEM images was complicated by the presence of biological material collected from skin as well. Moreover, images had to be collected rapidly to avoid the collapse of PLA particles after a few minutes of exposure to the high-energy electron beam (80 kV). On the contrary, the SEM analysis of particles deposited on polymer films allowed us to study the morphological changes of samples free of possible interfering materials and using an electron beam at lower energy (20 kV). These results proved that the structures observed by means of TEM in samples collected from skin were due to destabilized PLA particles and not to other organic material collected from skin as well. The fact that PLA particle stability upon incubation on Formvar films was comparable to that of particles on skin surface shows that PLA particles destabilization is mainly due to contact with hydrophobic regions. The affinity of the fluorochromes to the hydrophobic regions of skin might play an important role. Bodipy is a lipophilic molecule. It showed high affinity to the hydrophobic regions of the stratum corneum (Fig. 2B, 2 h tapes 1–3) and upon BP-PLA particles deposition on skin surface it diffused rapidly to the skin (Fig. 4). The rapid dye leakage might have contributed to particles destabilization and loss of the particulate state. On the contrary, the water-soluble DiAsp, having less affinity for skin hydrophobic structures, diffused slowly out of the particles and might have had a stabilizing effect.

As a consequence of their higher stability, DiAsp-PLA particles were found to have a better HFs penetration profile than previously investigated PLA particles: DiAsp-PLA penetrated in 65 ± 5% whereas coumarin-6 and nile red-loaded PLA particles were detected in 48 ± 14% and 49 ± 8% of all observed HFs, respectively [15]. We observed deeply penetrated particles in a significant number of HFs at all incubation times, as well as moderate but constant fluorescent dye release during the same period. Interestingly enough, we found a biphasic penetration of DiAsp-PLA particles, with maximal penetration of particles after 4 h and 12 h and a temporary decrease of HFs with penetrated particles (particle-positive HFs) 8 h after particle topical application. We hypothesize that after 8 h of incubation those particles which had penetrated deep into the HFs had released most of the fluorescent dye, and consequently, were no longer detectable by means of fluorescence microscopy. The further penetration of particles from the HFs openings, taking place between 8 and 12 h after topical application of particle, is thought to be responsible for the second increase of particle-positive HFs. We therefore assume that part of the DiAsp-PLA particles remain stable in the HFs openings and can still penetrate within the HF canals 8–16 h after application. This hypothesis is supported by the adhesive tape stripping and TEM results, where significant amounts of DiAsp-PLA particles were detected on skin surface even after 8 and 16 h of incubation. These observations underline the role of skin furrows and HFs as reservoir, allowing for a prolonged particle retention in the skin and thus for a prolonged drug release.

BP-PLA particles demonstrated a rather different dye-release profile than DiAsp-PLA particles. BP fluorescence intensity on skin surface (Fig. 2, tapes 1–5), and in skin cryosections (Fig. 4) was detected mostly 2 and 4 h after particle topical application, indicating that the release of fluorochrome from BP-PLA particles starts upon particle contact with skin. The highest fraction of skin sections with BP diffused into the epidermis was found 4 h after particle application (Fig. 4B). At this time point, the samples had still the particulate shape (Fig. 2, TEM). However, after 8 h, destabilized particles were detected together with a decrease of BP fluorescence intensity in the skin. An almost complete clearance of the dye from the epidermis and lost of particle morphology were observed after 16 h of incubation (Fig. 4). The fact that the first destabilized particles were visible by means of TEM in concomitance to dye clearance from the epidermis would indicate that after a first release of dye from intact particles, particle destabilization occurred causing a rapid release of the remaining fluorochrome and its diffusion to skin within a few hours.

The PLA particle formulation investigated in our previous study was loaded with nile red (NR-PLA) and coumarin-6 (Coum-6-PLA) [15] which are highly hydrophobic fluorochromes with a high affinity to lipids. When comparing the stability and dye release features of the four different PLA particle formulations, DiAsp-PLA, BP-PLA, and the previously studied NR-PLA and Coum-6-PLA, it is evident that PLA particles loaded with the hydrophilic fluorochrome 2-Di-4-Asp were the most stable ones, maintaining the particulate morphology over 16 h. This resulted in enhanced HFs penetration and prolonged fluorochrome release kinetics. It is important to note that the different size of the investigated particles did not influence particle stability upon contact with skin. In fact, the most stable DiAsp-PLA particles had an average size of 207 nm and the less stable particle formulations, BP-PLA, NR-PLA, and Coum-6-PLA, had sizes above and below 200 nm (164 nm, 228 nm, and 365, respectively).

The flow cytometry analysis of isolated epidermis cells, showing shifted values of MFI in epidermal cells after incubation with PLA particles, confirmed our previous results on dye penetration into keratinocytes 16 h after topical application of PLA particles [15]. Furthermore, we showed that the shift in fluorescence intensity was exclusively due to the released fluorochromes and not to particles translocated to the deeper epidermal layers. In fact, the fluorescence analysis of cells isolated from skin incubated with particles never showed cells associated with particles (Fig. 5). The aggregation of PLA particles upon deposition on skin surface and accumulation in HFs probably hindered the translocation of particles to the viable epidermis. The fact that PLA particles did not translocate through CSSS damaged skin barrier is an important matter in relation to the safety of PLA particles.

5. Conclusions

The results of this study confirm our previous findings that PLA particles preferentially accumulate in HFs after topical application, form aggregates, and release the incorporated substances. PLA particle stability upon topical application, fluorochrome diffusion kinetics, and clearance depended on the lipophilicity of the incorporated fluorochromes. These results show that PLA particle formulations, despite being stable aqueous dispersion, lose their particulate morphology upon contact with skin. Particle stability could be improved by modifying the chemical structure and lipophilicity of the loaded fluorochromes. This suggests the possibility to load PLA particles with drugs having the adequate physicochemical properties or to use excipients in order to control the stability of particles upon topical application and modulate the release of loaded drugs. DiAsp-PLA particles were stable after topical application

and exhibited a constant and prolonged release of the loaded fluorochrome. Providing a constant drug release allows for an improved control of drug dosage and thus improved compliance of the patient. On the other hand, particles with release properties like those of BP-PLA particles, which exhibited a rapid release and diffusion of loaded fluorochrome to the epidermis and then dermis, might be beneficial in topical treatments where high local therapeutic drug concentration and rapid drug clearance are required. The biodegradability of PLA particles and the absence of particle translocation into epidermis cells minimize potential hazardous effects. Characteristics such as feasible large-scale preparation, biodegradability, and the possibility to manufacture particles formulation with different release abilities make of PLA particles the optimal drug delivery systems for local dermatotherapy.

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