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Dermatokinetics of Nanoparticles (25 nm)

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Abstract

Conflicting reports exist on the dermal penetration of nano-sized particles from topical formulations and cosmetic products. This study evaluated the penetration capacity of nanoparticles in the skin membrane and the disposition of nanoparticles from the dermis. Quantum dots with no surface charge and of hydrodynamic diameter ~25 nm were selected as model nanoparticles for the study. *In vitro* permeation studies were carried out using excised porcine skin in a Franz diffusion cell for 24 h and the amount of quantum dots permeated into and through the skin was measured. The intensity of quantum dots in the skin layers was determined by fluorescence microscopy. *In vivo* studies were carried out by injecting quantum dots of different concentrations (0.024 or 0.048 or 0.08 nmol) into the dermis regions and the amount of quantum dots cleared from the skin tissues was determined. Preliminary *in vitro* permeation studies indicated quantum dots of ~25 nm size can permeate the skin with greater amount of quantum dots retained in the skin (~73% of the total amount delivered). Fluorescence microscopy studies signified greater retention of quantum dots in the stratum corneum layer and just underneath. Dermatokinetics studies revealed that the disposition of the nanoparticles from the dermis followed first order exponential kinetics (K=0.038/h).

Key words: Quantum dots, permeation, fluorescence microscopy, kinetics, disposition

Introduction

Nanotechnology-based therapeutic products are gaining significant attention in recent times. The efficiency of many therapeutic products has been demonstrated as a result of nano-sizing (Lamprecht *et al*., 2005; Csaba *et al*., 2006; Lee *et al*., 2006; Sinha *et al*., 2006; Bechet *et al*., 2008). There are several nano-based targeted drug delivery systems developed to localize the drug in affected tissues (Wang *et al*., 2008). Similarly, in the dermatological area, nano technology based topical formulations are being developed for more efficient delivery of drugs across the skin (Kim *et al*., 2008; Sheihet et al., 2008; Tahara *et al*., 2008). In cosmetics, pure sun blockers such as titanium dioxide, zinc oxide and talc have been reduced to the nano size level for better protective effects (Nohynek *et al*., 2008). However, conflicting reports exist on the permeation of these nano fined particles across the skin barrier. Several studies have shown that the nano systems placed on the skin can penetrate across the skin layers (Ryman-Rasmussen *et al*., 2006; Chu *et al*., 2007) while numerous reports have shown localization of the nano systems at the stratum corneum (SC) layer and follicular regions (Lademann *et al*., 1997; Schulz *et al*., 2002; Lademann *et al*., 2007; Zvyagin *et al*., 2008). Quantum dots of different sizes have been used to study the penetrability of nanoparticles across the layers of skin. Vogt et al have showed that particles of 40 nm size have penetrated through the depth of 225 µm or in the region of epidermis or papillary dermis (Vogt *et al*., 2006). Ryman-Rasmussen et al demonstrated that nanoparticles permeated across the skin into the receiver compartment in the *in vitro* Franz diffusion cells. Kohli and Alpar have shown that the penetrability of nanoparticles across the skin depends not only on the size but also on the charge present on the surface of the particles (Kohli *et al*., 2004). In general, most of the studies have demonstrated the penetrability of nanoparticles in the size range of <40 nm regardless of the surface charge across the SC via follicular as well as intercellular pathways. It is likely that the

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particles in this size range would be able to penetrate better if the intactness of the upper most layer, SC is compromised. These observations are exciting as well as concerning in different perspectives. For instance, when one would intend to deliver drugs into the deeper layers of skin using a nanoparticulate based drug delivery formulation, deeper penetration of the nanosystem would be desirable. Provided the skeleton of nanoparticulate system is biodegradable, there is less concern about accumulation of nanoparticles in the skin layers, as they degrade eventually. On the other hand, when the sunscreen agents and other aesthetic products are designed in the nanosized systems, they are intended to retain on the skin to provide relatively better protection and appearance of the skin as compared to their conventional counter products. But when the nano ingredients in these formulations pave their path through the skin and into the systemic circulation, there would be a cause for concern. For example, titanium dioxide which is a sunscreen agent has also been shown to be genotoxic to cells exposed to ultraviolet-A radiation (Dodd and Jha, 2009). Similarly, systemic absorption of benzophenone-3, which is a common ingredient in the cosmetic agents, would cause severe untoward effects in children and adults (Jiang, et al., 1999). On the other hand, nanoparticles uptake by cells and improved transfection rates was also reported (Peng *et al*., 2009). Therefore, in the present study we reassessed penetrability of nanoparticles through the skin. Quantum dots with no surface charge of hydrodynamic diameter ~25 nm were selected as model nanoparticles and their penetrability through the porcine skin and dermatokinetics was studied in rat model.

Materials and Methods

Materials

Poly ethylene glycol coated non functional surfaced quantum dots (12 nmol/mL; Evi Tags™) was procured from Evident Technologies, NewYork, USA. Material system is CdSe/ZnS, hydrodynamic diameter ~25 nm and the full width at half maximum (FWHM) diameter is <35 nm. The quantum dots were stored in dark at 4^0C and the dilutions were carried out in deionized water.

Analytical method

The amount of quantum dots was quantified by measuring the fluorescence emission intensity at 555 nm with the excitation at 420 nm using a Perkin Elmer spectrofluorimeter (LS 55). Quantum dots in the skin were estimated after extracting it from the skin using a standardized procedure. Skin samples dissolved in sodium hydroxide or 0.9% sodium chloride solution was used as blank. The sensitivity of the fluorimetric method was 0.0001 nmol/mL and the linearity was between 0.0001-0.01 nmol/mL (R^2 = 0.99). The method was validated by determination of linearity, precision, and accuracy.

Porcine Skin

Porcine belly skin was obtained from local abattoir of freshly slaughtered pigs, and the fat adhering to the dermis side was removed using a scalpel. The skin was washed with water and stored at -20^0C and was used within a week. The AC electrical resistance of the full thickness skin was measured by placing a load resistor R_L (100 kΩ) in series with the skin. The voltage drop across the whole circuit (V_0) and across the skin (V_S) was measured using an electrical set up consisting of a waveform generator and a digital multimeter (Agilent Technologies, Santa Clara, CA). For measuring resistance, voltage of 100 mV was applied at 10 Hz and the skin resistance in kΩ was approximated from the formula

$$R_S = \frac{V_S R_L}{V_0 - V_S}$$

where R_S is the skin resistance and R_L is the load resistor in kΩ. Skin with resistance >30kΩ.cm^2 was used in the *in vitro* permeation studies.

Recovery of quantum dots from skin

Quantum dots (0.008-0.036 nmol; 20 µl) were injected into porcine or rat skin sections (~0.7 sq. cm) and kept aside for 24 h. The skin was cut into fine pieces (~1 mm), soaked in vials containing 1 M sodium hydroxide (3 mL) and kept in shaker water bath at 37 ± 0.5^0C for 24 h to dissolve the tissue. The vials were then vortexed for 3 min, centrifuged (5,000 rpm) and the supernatant was analyzed by spectroflourimetry. The recovery was found to be 83.19 ± 5% and 78.62 ± 6% in porcine and rat skin, respectively. In another set of experiments, skin sections (porcine and rat) were extracted similarly without injecting quantum dots to assess the skin fluorescence under similar conditions.

In vitro permeability studies

The permeation studies were carried out for a period of 24 h using a Franz diffusion cell (Logan Instruments Ltd., Somerset, NJ) using excised porcine skin (~2 mm). The skin was mounted between the half-cells with the dermis in contact with the receptor fluid (0.9% sodium chloride solution) and was equilibrated for 1 h. The receiver compartment had a volume of 5 mL and the area available for diffusion was 0.64 cm^2. The top of the donor compartment was covered with parafilm to prevent the evaporation of the vehicle. Quantum dots (500 µL; 0.12 nmol/mL) were placed in the donor compartment. The receiver compartment was stirred at 600 rpm with a 3-mm magnetic stir bar at room temperature. Similarly, control experiments were run in parallel by placing water in the donor to determine the amount of skin fluorescence compounds leached into the receiver. The receiver was analyzed using spectroflourimetry.

The amount of quantum dots retained in the skin was determined after the *in vitro* diffusion studies. Briefly, the skin was washed with water (1 mL) five times and the

active diffusion area was excised, cut into fine pieces, soaked in vial containing 1 M sodium hydroxide (3 mL) and kept in shaker water bath at 37 ± 0.5^0C for 24 h. Then the vials were vortex for 3 min, centrifuged (5,000 rpm) and the supernatant was analyzed by spectroflourimetry.

Fluorescent microscopy studies

Microscopy studies were carried out following the permeation studies. Immediately after the permeation experiments the active diffusion area of the skin was separated and frozen. Sections of 30 µm thicknesses were cut perpendicular to the skin surface using a Leica 1800 cryostat (International Medical equipment, San Marcos, CA). Quantum dots immobilized in the skin layers were illuminated using 25 – 50 µW of the 457 nm output from a Coherent 200 Ar ion laser and a Nikon TE2000 research grade inverted microscope. Emission images were recorded using a PI/Acton Photonmax EMCCD camera. Emission from specific locations within the skin was also simultaneously dispersed using a PI/Acton grating spectrometer and the resulting spectra recorded with a Princeton Instruments IRY-512 photodiode array to confirm the presence of quantum dots as opposed to skin autofluorescence. In addition, only the interior region (~5 µm from either transverse surface) of the skin was chosen for study to avoid the possibility of any surface QD contamination from the sectioning knife.

Dermatokinetics of quantum dots in rats

In vivo dermatokinetics of quantum dots were carried out in sprague-dawley rats (200–250 g) under ketamine (80 mg/kg) and xylazine (10 mg/kg) anesthesia administered intraperitoneally (Institutional Animal Care and Use Committee (IACUC), University of Mississippi, Protocol # 09-012). Depending on the duration of the study, rats were divided into two groups. The first group (n=14) was used for the first three time points (0, 4 and 8 h) while the second group (n=9) was used for 16 and 24 h studies. The injection region was shaved using trimmer and the area (~1 cm^2) was demarcated with permanent marker. Quantum dots (20 µL) of different concentrations (0.024 or 0.048 or 0.08 nmol) were injected into the demarcated regions of the dermis. The amount of quantum dots in the skin was determined at different time points (0, 4, 8, 16 and 24 h) after separating the skin tissue corresponding to the injection site (~1 cm^2) using 8 mm biopsy punch. Control experiments were carried out by injecting the same concentration of the quantum dots in excised rat skin *in vitro*. The amount of quantum dots cleared from the skin tissues was determined after extracting the quantum dots by method described above.

Statistical analysis

Statistical analysis was carried out by T-test (Graphpad prism 5, graphpad software, Inc., CA, USA). *P* value < 0.05 was considered statistically significant. The data points provided in the graphs are an average of three trials. The error bars represent the standard deviation (s.d.).

Results and discussion

Numerous attempts have been made to assess the penetration of nanosized particles into the skin by applying topical formulations. Results in the literature indicate that the particles <40 nm in size are capable of permeating into the dermis region through the follicular route (Shim *et al*., 2004). Potential capability for nanoparticles (10 nm) to traverse the human full thickness skin barrier and reaching the viable epidermis was demonstrated by Baroli *et al*., 2007. Gamer *et al*., 2006 reported that the microfine zinc oxide (~80 nm) and titanium dioxide (~160 nm) could not diffuse through the porcine SC layer. On the other hand, there are some reports which indicate that the elastic particles with higher particle size (100-150 nm) are capable to permeate the SC rapidly and reach the viable epidermis, although the rigid particle with similar size were unable to permeate (van den Bergh *et al*., 1999; Honeywell-Nguyen *et al*., 2004). In addition, larger particles of FITC-conjugated dextran bead (0.5 -1 µm) were reported to penetrate into the epidermis and occasionally to the dermis when the skin was flexed for 30 min (Tinkle *et al*., 2003). We believe that the inconsistency in results could be attributed to the type of skin, experimental condition, nanoparticles size and shape, presence of other components in donor etc. However, invariably the reports in the literature suggests that the particles <40 nm size are capable of diffusing through the skin layer; hence we selected particles of hydrodynamic diameter 25 nm size to investigate the penetration and clearance of nanoparticles from the skin. Quantum dots are used as model nanoparticulate system. They are semiconductor nanocrystals with intense and photostable fluorescence, which make them easy to detect with high accuracy and reliability, and possess good biocompatibility. Structurally they have a metalloid core surrounded by a shell and possess higher stability during *in vivo* studies. The coating of the metalloid with biocompatible materials or functional groups makes it bioactive and reduces the toxicity. However, separation of the coating layer from the core may generally leads to toxicity. The rapid advances in nanotechnology have made the possibility to prepare various types of intact particles with diverse properties and are less toxic. Thus the applications of these particles are extended to various fields including cell targeting, drug delivery, tissue engineering etc. Application of these particles is also extended in diagnosis and therapy of skin disorders. The current study assessed the penetrability of the nanoparticles (~25 nm) across the full thickness porcine skin (~2 mm) in 24 h. Further, the amount of particles retained in the skin during the process was also assessed. Quantification of quantum dots in the skin as well as in the receptor was carried out by fluorescence spectroscopy. While developing the estimation of quantum dots by fluorescence spectroscopy, we observed a higher amount of fluorescence in skin when the excitation was below 350 nm.

However, above this value the intensity of skin fluorescence was reduced and the excitation at 420 nm the fluorescence was almost negligible. Further this method was validated for linearity (0.0001- 0.01 nmol/mL), precision, and accuracy (relative mean error-1.68- 7.45%).

Skin from the same region and same animal was used for the permeation studies to minimize variations. Figure.1 represents the percentage of quantum dots permeated into and through the full thickness skin in 24 h. It is evident from the figure that relatively a greater amount of quantum dot was recovered from the skin ($P<0.005$) than that delivered into the receiver. Nevertheless, these results obtained from the current study supports the earlier report suggesting that the nanoparticles with a size ~ 25 nm could permeate through the skin.

In the next step, after the permeation study the skin was sectioned and viewed using fluorescence microscopy employing the 457 nm line of an Ar ion laser to assess the intensity of the quantum dots in different layers of the skin. Emission images were recorded using a highly sensitive EMCCD camera capable of single molecule and single quantum dot detection and imaging (Hassey *et al*., 2006; Odoi *et al*., 2006; Hammer *et al*., 2006). Emission from specific locations within the skin was also simultaneously dispersed using a grating spectrometer and the resulting spectra recorded with a photodiode array to confirm the presence of quantum dots as opposed to skin autofluorescence. This imaging setup was chosen as an alternative to confocal microscopy due to its sensitivity and very low detection limits (down to single quantum dot). The untreated skin was used as control.

The total fluorescence images along with the dispersed emission spectra of the quantum dot treated and untreated skin are depicted in Figure.2. It is evident from the figure that the greater amount of quantum dots was retained in the SC layer and just underneath. The intensity was greatly reduced in the dermis region (~200-1000 µm) and not apparent in the lower layers (~1400- 2000 µm). In lower skin regions, the skin autofluorescence becomes the dominant emitter and is the only source of emission. The microscopic studies additionally support that the nanoparticles used in this study could effectively permeate the skin. But these studies could not clearly reveal the actual pathway of permeation of nanoparticles. The earlier reports demonstrated different pathways in each case. Fluorescence and laser microscopy studies carried out by Vogt and co-workers, 2007, revealed that the particles of 40 nm penetrated predominantly via the follicular routes into the dermis. Further there are other reports which suggest that the possible routes for the permeation of microspheres into the skin are pilosebaceous pores or sweat gland pores (Rolland *et al*., 1993; Mordon *et al*., 2003). Moreover, in porcine skin the vertical and lateral gaps between corneocytes are reported to be ~ 19 nm (van der Merwe *et al*., 2006), suggesting that particles <19 nm core length or width could permeate through the corneal lateral intercellular lipidic matrix. However, it is well known that the intercellular spaces in the SC are occupied by lipids. So far, no work clearly demonstrated how the nanoparticles permeate across these lipidic pathways.

Transcutaneous drug diffusion generally involves the hydrophobic pathways through the least resistant intercellular lipidic matrix (Cevc and Vierl, 2007). Considering the density of distribution of nanoparticles in the layers of epidermis, it appears that the pathways of absorption of nanoparticles of size 25 nm is not very different from the pathways of permeation of molecular permeant. Therefore, it can be speculated that the potential pathways for absorption of nanoparticles could be a combination of intercellular spaces, follicular and pilosebaceous as well.

Anatomically, below the epidermis is the papillary dermis layer which possesses large number of micro circulatory capillary blood vessels and lymphatic vessels. It comprises two horizontal networks, an upper plexus/ network contains terminal arteriole from which the capillary loop arise, while the lower is formed by the perforating vessels from the underlying layers. The density of the capillary loops in this region is considerably higher than the lower layers with an estimated internal diameter >3 µm. However, the lower reticular layer is devoid of cellular or vascular tissues and consists of dense collagenous and elastic connective tissue. Below the dermis is the subcutaneous tissue, which is composed of connective tissue and fatty tissue, followed by muscle tissues.

It is well known that molecules that diffused through the epidermis are cleared from the dermis region of the skin. This is either due to dermal microcirculation or drug metabolism in the skin or migration into deeper tissues such as subcutis and muscle. Further, it is described that the clearance rate of molecules from skin depends on the blood perfusion rate in the tissue, diffusivity of the molecules, size of the molecules and the concentration of the molecules at that region. A review of the literature suggests that intradermally injected large molecules such as proteins and large sugars, are cleared exclusively by lymph vessels as well (Reed *et al*., 1993; Renkin *et al*., 1994; Ikomi *et al*., 1996). However, the clearance of nanoparticles from the skin has not been investigated so far. It is thus important to investigate the dermatokinetics of nanoparticles determine the fate of nanoparticles post permeation onto the skin. Therefore, the kinetics of escape of nanoparticles from the site of administration/penetration into skin was investigated in this study. Three different concentrations (0.024 or 0.048 or 0.08 nmol) of nanoparticles were injected into the dermis region and the amount recovered from the skin at different time intervals were measured. Control experiments were carried out in parallel by injecting the same concentration of the quantum dots in the freshly excised skin *in vitro* to assess the loss of fluorescence with time. Figure.3

represents the amount of quantum dots recovered at different time intervals in the skin. The control experiments showed that the nanoparticles did not undergo significant loss of fluorescence in the skin (data not shown). The *in vivo* disposition curves could be fit to a monoexponential equation indicating that the dermal disposition of nanoparticles followed first order kinetics (K = 0.038/h). This is similar to the disposition of several drug molecules when administered via intracutaneous administration (Connor *et al*., 1985; Yoshida *et al*., 2002). The kinetics observed in the current study suggests that the nanoparticles in the size range of ~25 nm are picked up by the dermal circulation and is typically based on the concentration of the molecules in that region, which is of greater importance in drug delivery and assessing the potential for toxicity and interactions with biological systems.

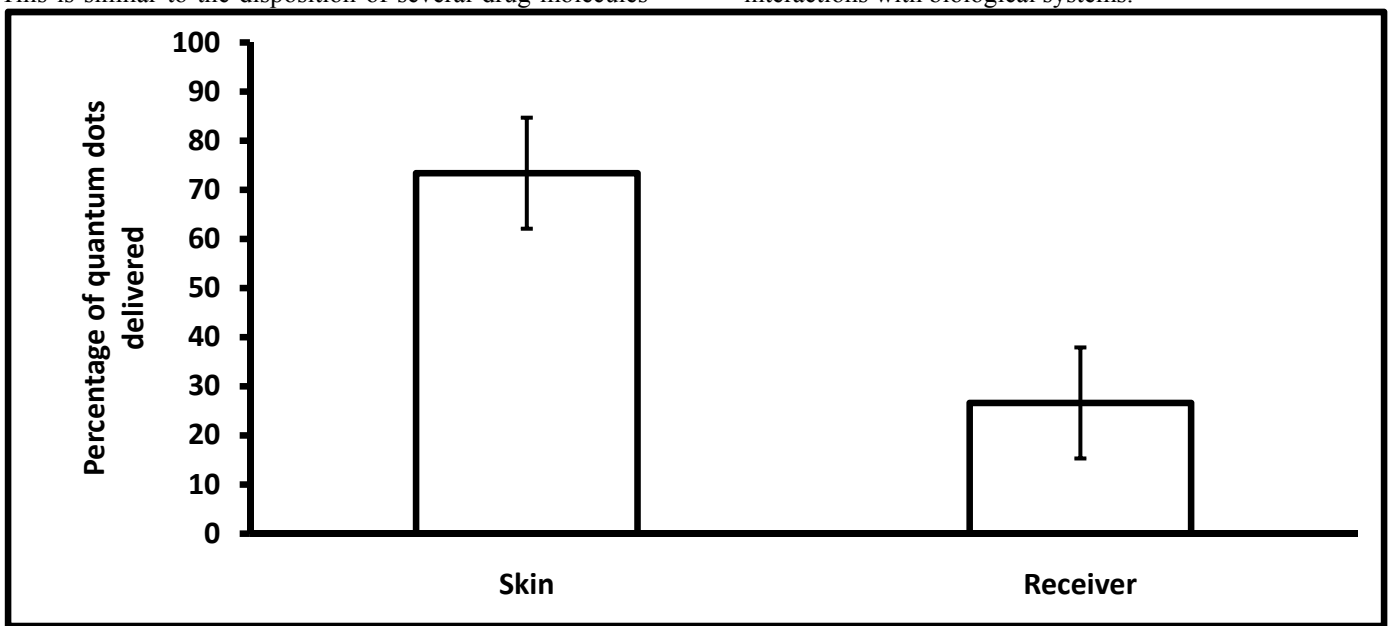

Figure.1 Fraction of quantum dots recovered from the skin and permeated across the excised fullthickness porcine skin after 24 h. Permeation study was carried out by placing quantum dots (500 µL; 0.12 nmol/mL) in the donor chamber and the area available for diffusion was 0.64 cm^2. Each data point is mean of three trials ± SD.

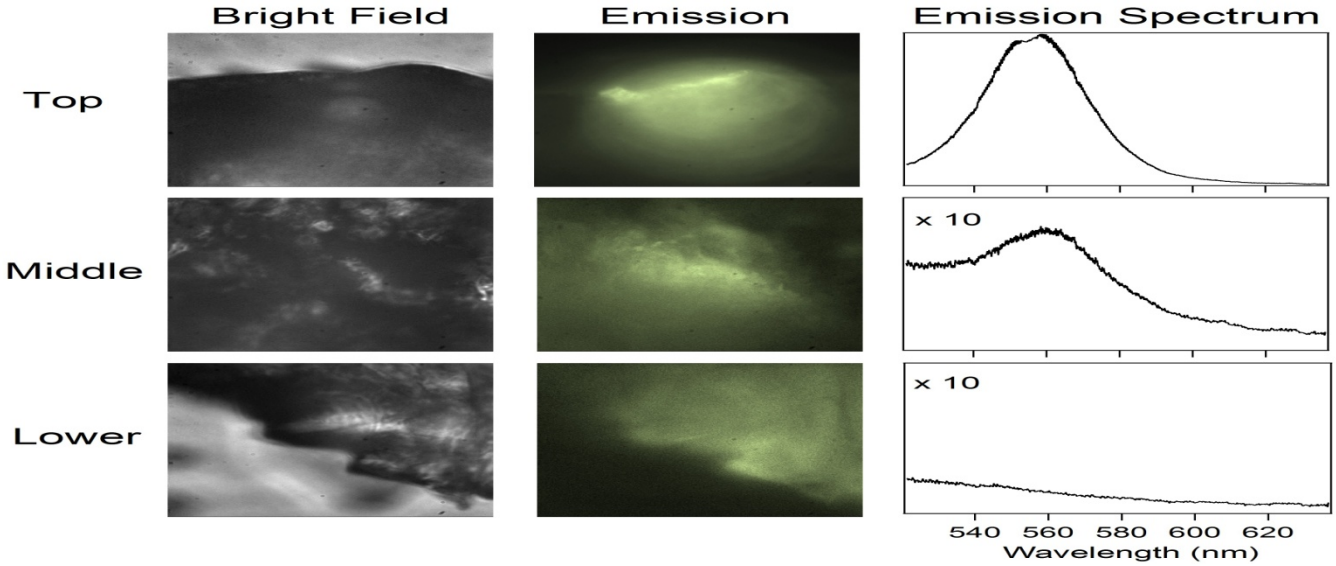

Figure.2 Bright field, fluorescence emission and emission spectra from skin segments (at different depth from the skin surface) treated with quantum dots. The top layer represents the stratum corneum (0 – 65 µm), middle layer is the dermis region (~200-1000 µm) and the lower subcutaneous layer (~1400- 2000 µm). The higher intensity of fluorescence in the top layer could be attributed to the relatively higher amount of deposition of the quantum dots in the stratum corneum, while the fluorescence intensity reduced in the underneath layers.

Conclusion

In the current study, the penetration of the nanosized particles (~25 nm) into and across intact skin was further established. The fluorescence microscopic studies indicate higher intensity of fluorescence in the top skin layer and just underneath due to the relatively higher amount of deposition of the quantum dots. Further, the disposition of the nanoparticles from the dermis followed a first order exponential decay. This observation has its own significance from the drug delivery and toxicological perspectives.

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