bio-protocol

Published: Apr 05, 2022

Ex-vivo Skin Permeability Tests of Nanoparticles for Microscopy Imaging

Nethanel Friedman[1], Sharon Merims[2], Jhonatan Elia[3] and Ofra Benny[1, *]

[1]The Institute for Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University, Jerusalem, Israel
[2]Sharet institute of oncology, Hadassah Ein-Karem hospital, Jerusalem, Israel
[3]Department of plastic and aesthetic surgery, Hadassah Ein-Karem hospital, Jerusalem, Israel
*For correspondence: Ofra.benny@mail.huji.ac.il

Abstract

Delivery of drugs through the skin is a major challenge in the field of drug delivery systems. Quantification of materials, and specifically nanoparticles, within the skin layers is essential for the development of advanced topical and transdermal delivery systems. We have developed a technique for *ex-vivo* segmentation and evaluation of human skin samples treated with fluorescent nanoparticles. The method is based on horizontal cryosections of skin samples, followed by confocal microscopy and image analysis. This protocol is relatively simple to perform with basic histological tools, thus it can serve for various dermatology assays.

Keywords: Horizontal cryosectioning, Fluorescent nanoparticles, Skin permeability, Confocal microscopy, Nanoparticles penetration depth

This protocol was validated in: Nanomedicine (2021), DOI: 10.1016/j.nano.2021.102414

Background

Penetration of nanoparticles through the skin, and specifically via hair follicles, have emerged as a potential delivery route for drugs. Nanoparticles can serve as carriers for drugs for topical treatments and systemic delivery, directed to the blood vessels at the hair bulb. Various types of nanoparticles were studied for follicular drug delivery, differing from each other by composing materials, size, and shapes (Costa *et al*., 2021; Patzelt and Lademann, 2013). While our method was developed with gold nanoparticles, this can be relevant for particles made of different materials, such as polystyrene, lipids, *etc.,* and varied fluorescent labeling.

One of the most significant characterizations for follicular nanocarriers is the particles' penetration depth. Determination of penetration depth can be performed using several methods, for instance histology followed by confocal microscopy and tape striping (Escobar-Chávez *et al*., 2008; Lohan *et al*., 2017). In our manuscript (Friedman *et al*., 2021), we have studied the follicular penetration of fluorescently labeled gold nanoparticles, and the impact of particle shape on penetration depth. To assess the depth of penetration into human skin, we have developed a method for the incubation of skin samples with nanoparticles, and the sectioning of samples horizontally and vertically for further confocal microscopy analysis.

In the method detailed here, we are using human skin samples and polymeric nanoparticles with fluorescent labeling. Serial horizontal sectioning of the samples is performed for the evaluation and quantification of the particles in each section using confocal microscopy imaging.

Materials and Reagents

1. 24-well cell-culture treated plate (ThermoFisher, catalog number: 142475)
2. Ethanol 70%
3. 5 × 5 sterile gauze pads
4. 8 × 8 mm Peel away embedding mold (Electron Microscopy Sciences, catalog number: 70180)
5. Superfrost Plus microscope slides (75 × 25 mm, Fisher Scientific, catalog number: 12-550-15)
6. Microscope cover glass (24 × 60 mm, Marienfeld, catalog number: 0101242)
7. Dulbecco′s Modified Eagle′s Medium, high glucose (DMEM) (Sigma-Aldrich, catalog number: D5796)
8. Dulbecco's-Phosphate-buffered saline (DPBS) (pH 6.7–7) (ThermoFisher, catalog number: 14200075)
9. Incubator 37°C, CO_2 5% (ThermoFisher, Heracell 150i, catalog number: 50116048)
10. OCT compound (Scigen, catalog number: 4586)
11. Formaldehyde 35% (Bio-Lab, catalog number: 67505)
12. ImmEdge Hydrophobic Barrier PAP Pen (Vector Laboratories, catalog number: H-4000)
13. DAPI (Sigma-Aldrich, catalog number: D9542)
14. Fluoromount mounting medium (Sigma-Aldrich, catalog number: F4680)

Equipment

1. Dissecting scissors, 9.5 cm, straight (WPI, catalog number: 503244)
2. Dressing forceps, 12.5 cm, serrated (WPI, catalog number: 501217)
3. Tweezers
4. Small beaker
5. Cryostat (Leica CM1950)
6. Glass staining jar (Normax, supplied by Alexred, catalog number: NR 4431000)

Software

1. ImageJ freeware (http://rsbweb.nih.gov/ij/) – image analysis

Procedure

A. Skin sample preparation

1. Harvest skin and maintain it in DMEM with 1% Penicillin/Streptomycin at 4°C until use.
2. For storage, use a plate with a cover in a suitable size for your skin sample. Adjust the medium volume to moisturize the dermis. Do not cover the external skin layers (epidermis) with the medium (see the example in **Figure 1a**).
3. Use the full-thickness human skin within 24 h of harvesting.
4. Remove fats with dissecting scissors and forceps gently. Do not leave any fat remains, and do not harm the dermis.
5. Remove hair's edges with scissors.
6. Cut ~2.5 cm^2 square skin samples.
7. Sterilize the skin samples in a small beaker with 70% ethanol solution for 1 min.
8. After sterilization, place the samples in PBS 1× for 10 s, to neutralize the ethanol.

B. Treatment with nanoparticles

1. Take a 24-well plate and fill a well with DMEM, up to the well walls.
2. Select a skin sample with dense hair follicles and place it on the well edge, with the dermis facing the medium. The skin sample should surround the well edges without falling into it (**Figure 1**).
3. Apply the desired fluorescent nanoparticles in the required form (solution, ointment, gel, *etc.*) on the skin samples.
4. Cover the plate and incubate the tissue in an incubator at 37°C for 24 h.
5. Take the skin samples from the plate and remove excess particles ointment/solution with a sterile gauze pad soaked with PBS.

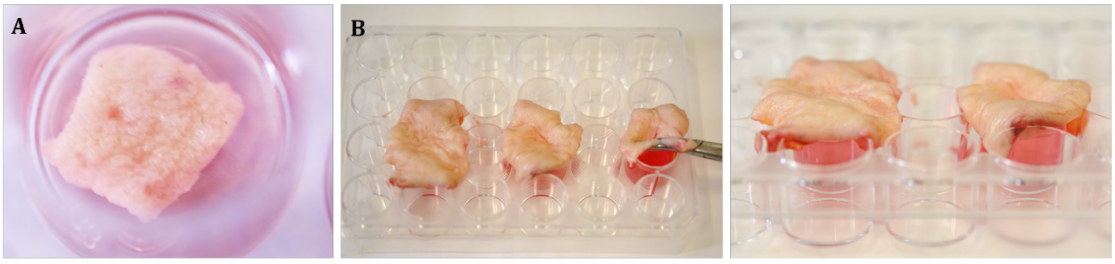

Figure 1. Skin storage and experimental setup.
A. Skin samples storage before use within 24 h from harvesting. Skin sample should be placed in a vessel at a suitable size with DMEM in such volume that it does not cover the epidermis. **B.** Skin samples, cleaned from fats, are placed on 24 well plate edges for treatment. The well is filled with DMEM, to nourish the sample during incubation.
Taken from previous work with the permission of the publisher (*Nanomedicine: Nanotechnology, Biology and Medicine*. License number 5195761380089).

C. Cryo-sectioning—sample preparation and sectioning

1. Freeze the skin samples in OCT, using dry ice in an embedding mold. We recommend using one mold per sample.
2. For horizontal sectioning, place the sample with the epidermis towards the bottom, and cover it with OCT.
3. Set the chamber temperature to -21°C, and the specimen head temperature to -29°C.
4. Fix the sample on the disc.
5. Prepare a set of positive charged slides, and number each slide. Each slide will contain 2–3 sections. The numbers should be consecutive and represent the depth of the skin sample.
6. Cut the first 100 µm from the skin, to achieve a flat section that can be applied on microscope slides.
7. Cut sections at the thickness of 14 µm, according to the slides prepared in step C5. Repeat this step to the desired depth.

D. Sample staining for confocal microscopy

1. Fix slides in a glass staining jar with 4% formaldehyde for 20 min.
2. Wash three times with PBS 1× for 5 min, by dipping in a glass staining jar.
3. Use a PAP pen to mark a square around the sample.
4. Stain samples with 100 µL of DAPI (0.1 µg/mL) for 10 min.
5. Mount slides with one drop of fluoromount media per sample and cover with a cover glass.
6. Image samples with confocal microscopy (**Figure 2**).
7. Analyze images using ImageJ software.
8. Split image channels for each skin layer (Image>Color>Split Channels).
9. To measure fluorescence intensity and reduce background for further analysis (**Figure 2c, 2d**), choose the required area with the oval selector and use the measurement tool (Analyze>Measure).
10. Repeat step D9 with the oval selector on empty area, for background intensity measurement. In your data analysis, reduce the background intensity from your measurements.

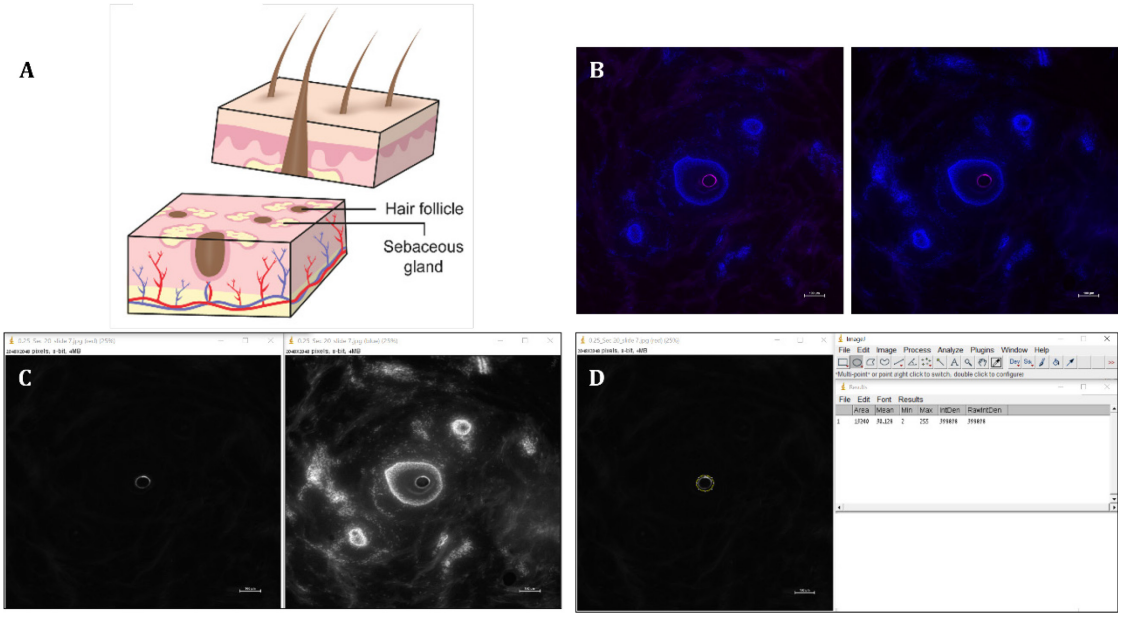

Figure 2. Skin samples horizontal microscopy.
A. Illustration of horizontal skin segmenting **B.** Two consecutive skin samples in the horizontal orientation under confocal fluorescent imaging. Blue DAPI staining reveals hair follicles as circles in the center. Fluorescent

nanoparticles accumulate around the hair (purple). **C.** Image analysis by ImageJ is used to split channels and calculate fluorescent intensity to quantify particle accumulation per section. (**D**). Taken from previous work with the permission of the publisher (*Nanomedicine: Nanotechnology, Biology and Medicine*. License number 5195761380089).

Acknowledgments

This project was funded by a Kamin fund of the Israel Innovation Authority, and by an Israel Science Foundation (ISF) grant. This protocol served as in our previous work "Physical properties of gold nanoparticles affect skin penetration via hair follicles" (Friedman *et al*., 2021).

Competing interests

The authors declare no competing interests.

Ethics

Human full-thickness skin samples were received from patients undergoing routine plastic surgery procedures in the plastic surgery unit at "Hadassah Medical Center" after receiving written consent, with approval by the Hadassah Research Ethics Committee, in adherence to Helsinki guidelines (0348-17-HMO).

References

Costa, C., Cavaco-Paulo, A. and Matama, T. (2021). Mapping hair follicle-targeted delivery by particle systems: What has science accomplished so far? *Int J Pharm* 610: 121273.

Escobar-Chávez, J. J., Merino-Sanjuan, V., Lopez-Cervantes, M., Urban-Morlan, Z., Pinon-Segundo, E., Quintanar-Guerrero, D. and Ganem-Quintanar, A. (2008). The tape-stripping technique as a method for drug quantification in skin. *J Pharm Pharm Sci* 11(1): 104-130.

Friedman, N., Dagan, A., Elia, J., Merims, S. and Benny, O. (2021). Physical properties of gold nanoparticles affect skin penetration via hair follicles. *Nanomedicine* 36: 102414.

Lohan, S. B., Saeidpour, S., Solik, A., Schanzer, S., Richter, H., Dong, P., Darvin, M. E., Bodmeier, R., Patzelt, A., Zoubari, G., *et al*. (2017). Investigation of the cutaneous penetration behavior of dexamethasone loaded to nano-sized lipid particles by EPR spectroscopy, and confocal Raman and laser scanning microscopy. *Eur J Pharm Biopharm* 116: 102-110.

Patzelt, A. and Lademann, J. (2013). Drug delivery to hair follicles. *Expert Opin Drug Deliv* 10(6): 787-797.