International Journal of Pharmaceutics 358 (2008) 91–95

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Novel method for stratum corneum pore size determination using positron annihilation lifetime spectroscopy

Yoshiaki Itoh[a,c,*], Akira Shimazu[b], Yasuyuki Sadzuka[c], Takashi Sonobe[c], Shigeru Itai[c]

[a] Medical Div, Nitto Denko Co., Ltd., Shimohozumi 1-1-2, Ibaraki-shi, Osaka 567–8680, Japan
[b] Core-technology Center, Nitto Denko Co., Ltd., Shimohozumi 1-1-2, Ibaraki-shi, Osaka 567–8680, Japan
[c] School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422–8526, Japan

ARTICLE INFO

Article history:
Received 20 November 2007
Received in revised form 12 February 2008
Accepted 17 February 2008
Available online 10 March 2008

Keywords:
Stratum corneum
Positron annihilation lifetime spectroscopy (PALS)
Ortho-positronium (*o*-Ps)
Positron
^{22}Na

ABSTRACT

Positron annihilation lifetime spectroscopy (PALS) is a powerful tool for the investigation of microstructure. Three main classes of materials, metals, semiconductors and polymers, have been studied by using this technique. But, relatively few investigations have been performed in the biological sciences. PALS provides important information on pore properties and free volume at the molecular level. Our PALS study showed that Yucatan miniature pig stratum corneum separated with heat and trypsin digestion had a longer positron annihilation lifetime than cyclodextrins. This indicates that the stratum corneum has larger pores and/or free volume than cyclodextrins, whose pores have a diameter of 0.5–0.8 nm and a torus height of 0.79 nm. Positron annihilation spectroscopy may be developed as a new technique for the detection of nano-pore properties and free volume in the biological sciences.

© 2008 Published by Elsevier B.V.

1. Introduction

The stratum corneum in the outermost layer of the skin plays an important role in percutaneous absorption and protects deeper tissues from hazardous exposure. The stratum corneum consists of flat keratin-filled corneocytes and a matrix of intercellular lipids. de Jager et al. (2005, 2006a,b) developed a synthetic stratum corneum membrane composed of cholesterol, long-chain free fatty acids and specific ceramides and showed that its permeability closely resembled that of human stratum corneum. The intercellular lipids in the stratum corneum are considered to play an important role in the barrier function of the skin. However, Yu et al. (2003) used fluorescence microscopy to demonstrate the existence of intra-corneocyte diffusion in addition to the lipid multi-lamellar transdermal pathway. Also, enhancers like oleic acid may induce hydrophobic drug localization in the lipid multi-lamellar region and increase the partitioning of hydrophilic drugs into corneocytes. The stratum corneum consists of the intercellular lipid and corneocytes, which form a barrier to permeation. Also, the permeation pathway and the permeability change greatly depending on the physicochemical properties of a specific drug, such as its partition coefficient, molecular size, solubility and so on. To clarify the permeation pathway and to search for the best skin permeation enhancer, we focused our attention on the pores in the stratum corneum. PALS is the perfect technique for measuring the properties of nano-pores in materials. Several techniques can be used to measure pore size, including low-temperature nitrogen absorption and electron microscopy. However, these methods are not sensitive enough to measure pore sizes in the range of ~10 nm, and it is difficult to apply these techniques to materials that are not entirely dry. PALS is highly sensitive in the pore size range 1–10 nm (Thraenert et al., 2006), and with a trace amount of water included in test samples, the technique can be used to measure pore sizes of 0.3–30 nm and also the pore size distribution (Gidley et al., 2006).

The PALS technique uses the positron, the antiparticle of the electron, with the same mass as an electron but with a positive charge. The existence of positrons was postulated in 1928 by Dirac, and positrons were first observed by Anderson in 1932. A positron is emitted in the β^+ decay of radioactive nuclei such as ^{22}Natrium (^{22}Na), ^{65}Zink, ^{68}Gallium and ^{114}Iodid. A radioactive ^{22}Na source with a half-life of 2.6 years is generally used in positron annihilation studies. Measurement of individual positron lifetimes is possible because a 1.27 MeV γ-quantum is emitted from the ^{22}Na source simultaneously with the positron. The positron lifetime was calculated with the time difference between its birth γ-quantum in a β^+-decay and the 0.51 MeV positron annihilation γ-quantum. Positrons, being positively charged, tend to remain distant from atomic nuclei and localize in the pores in materials, so PALS has

* Corresponding author. Tel.: +81 72 621 0283; fax: +81 72 621 0315.
E-mail address: Yoshiaki_itou@gg.nitto.co.jp (Y. Itoh).

0378-5173/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2008.02.016

long been used to study defects in solids. Recently, it has been used to measure the free volume, i.e. the empty space between the molecules, in polymers and biological materials (Kobayashi et al., 1992, 1994; Jean et al., 2006, 2007; Liu et al., 2007). The free volume is related to many physical properties, including the rate of drug diffusion, viscoelasticity, the glass transition temperature, and the dielectric constant. PALS is a powerful tool for measuring the size, number and size distribution of free volume pores in various materials. PALS may therefore provide key information on the properties of the pores and the free volume in the stratum corneum, thus improving our model of drug diffusion at the molecular level.

In this study, we used PALS to measure the pore sizes in Yucatan miniature pig skin, which has a permeability similar to that of human skin.

2. Materials and methods

2.1. Materials

α, β, and γ-Cyclodextrins were obtained from the Wako Chemical Corporation. Yucatan miniature pig skin was obtained from Charles River Laboratories Japan Inc. Sheets of epidermis were removed from pig skin by a 3-min application of hot (60 °C) Dulbecco's PBS(−) solution (Nissui Pharmaceutical Corp.) and were then incubated for 1 min in 0.5% trypsin from porcine pancreas (Sigma Corp., 1000–1500 units/mg) in Dulbecco's PBS(−) solution at room temperature (22 ± 3 °C) to digest the non-cornified cells. After trypsin digestion, the remaining sheet of epidermis was rinsed in water, allowed to air-dry, and was used in this study as stratum corneum.

Polycarbonate (positron annihilation lifetime: 2.103 ± 0.076 ns) was contributed by National Institute of Advanced Industrial Science and Technology (NIST) in Japan.

2.2. PALS experiment

PALS principle is briefly shown in Fig. 1. A positron is emitted in the β^+ decay of ^{22}Na and forms positronium in non-conductive target materials. The lifetime of the positronium is extended in a relatively large pore.

The PALS equipment was purchased from Advanced Measurement Technology, and a sealed positron source (^{22}Na) measuring around 100 coincidence counts per minute was purchased from the Japan Radioisotope Association. A schematic diagram of positron annihilation spectroscopy (PALS) is shown in Fig. 2. The PALS system consists of two photomultipliers (PM) including a scintillator, two signal channel analyzers (SCA), a time-to-amplitude converter (TAC), a multi-channel analyzer (MCA) and a personal computer for data analysis.

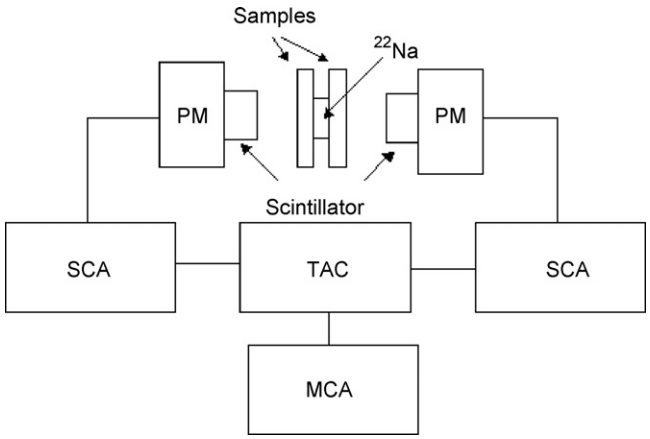

Fig. 2. Schematic diagram of positron annihilation lifetime spectroscopy. Positron lifetime is measured as the time difference between detection of a 1.27 Mev quantum (from β+ decay) and a 0.51 Mev quantum (annihilation process) PM: photo-multiplier, SCA: signal channel analyzer, TAC: time-to-amplitude converter, MCA: multi-channel analyzer.

When a positron is produced by the ^{22}Na source, a 1.27 MeV photon is also produced. When such a photon was detected in the scintillator, it served as a start signal, and the detection of the 0.51 MeV photon produced by the mutual annihilation of a positron and an electron was the stop signal. Signals were enhanced with a photomultiplier, and their energy determined in a signal channel analyzer, then processed in a time-to-amplitude converter and interpreted to yield the positron lifetime by a multiple analyzer.

PALS experiments were carried out at room temperature using 1024 channels with a time resolution of 50 ps. Two samples were platelets measuring 15 mm × 15 mm × 0.5 mm, covered with a thin aluminum foil. The positron source was sandwiched by two samples and each sample was observed for around 1×10^6 coincidence counts, or 10 000 s.

Cyclodextrins and Yucatan miniature pig stratum corneum were covered with thin aluminum foil (10 μm), as the sample thickness must be about 0.5 mm. Two samples were brought into contact with ^{22}Na as in Fig. 2.

3. Results and discussion

PALS has been used to measure the free volume and pore size in polymers and glasses. Polycarbonate was selected as one of the

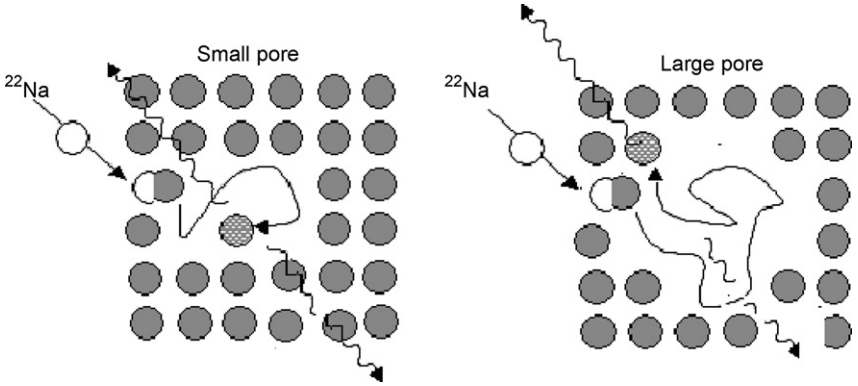

Fig. 1. A sketch of positronium interacting with a pore. A positron is emitted in the β^+ decay of ^{22}Na and forms positronium in the target materials with an electron. The lifetime of the positronium is extended in proportion to the pore size. ●◍◑ show a electron, a electron picked with a positronium and a positronium, respectively.

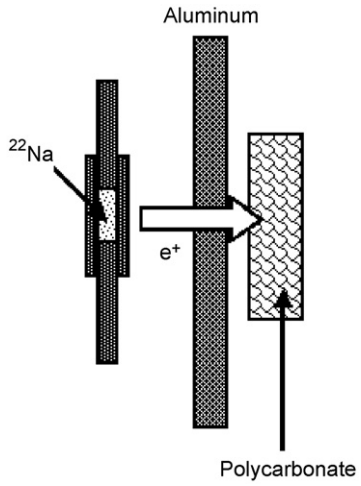

Fig. 3. Schematic diagram of the positron lifetime study involving aluminum and polycarbonate. Polycarbonate has a positron annihilation lifetime of 2.1 ns and was used as a reference material.

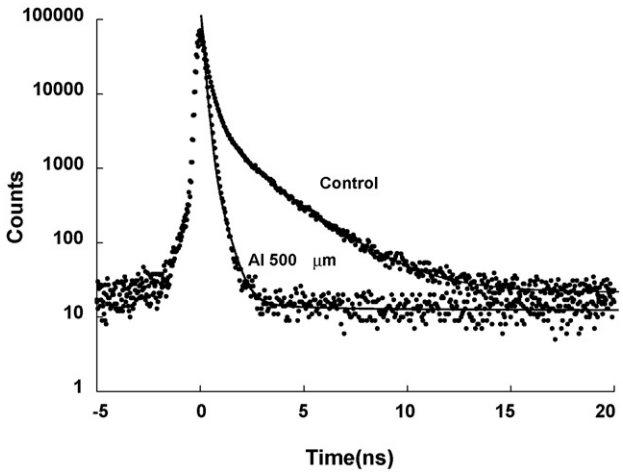

Fig. 4. Positron annihilation lifetime spectra with an aluminum shield. Data indicated by dots, with three fitting curves produced by EXCEL solver. The three spectrum components produced in the polycarbonate (by *p*-Ps, positrons, and *o*-Ps), are indicated by solid lines.

standard materials for PALS measurements, with a positron annihilation lifetime of 2.103 ± 0.076 ns.

Initially we investigated whether aluminum affected our PALS experiments, using a polycarbonate standard as shown in Fig. 3 (one side of Fig. 2) for lapping of samples and fixing the sample thickness. The aluminum was placed between the ^{22}Na source and the polycarbonate standard and we measured the positron annihilation lifetime and intensity. The PALS analysis was performed by non-linear fitting routines after adjusting for source and background with Eq. (1),

$$N(t) = \sum_{i=1}^{n} \frac{I_i}{\tau_i} \exp\left(-\frac{t}{\tau_i}\right) \quad (1)$$

where N is the positron annihilation count in each channel time, T_i is the positron lifetime of i, t is the channel time and I_i is the positron intensity of i. In general, the lifetime spectrum consists of three superimposed exponential decay curves. It is considered that the first lifetime (T_1) is that of para-positronium (*p*-Po), in which the positron and the electron have opposite spins, the second lifetime (T_2) that of positrons prior to annihilation by an electron, and the last lifetime (T_3) is terminated by the pick-off annihilation of ortho-positronium (*o*-Ps), in which they have the parallel spins.

The positron annihilation lifetimes in the polycarbonate standard were 2.2 ns and were unrelated to the thickness of the aluminum (the logarithm of intensities decreased in proportion to the thickness). Fig. 4 shows positron annihilation lifetime spectra with a 500 μm thickness of aluminum and control. The intensity of the *o*-Ps signal was markedly reduced by 500 μm of aluminum. Only a small number of positrons reached the polycarbonate through 0.5 mm of aluminum, confirming that this was strong shielding against positrons. However, we considered that experimental samples demanded the thickness of 0.5 mm and 10 μm aluminum foil had no effect on the PALS measurement, so samples were covered with thin aluminum foil. The pig stratum corneum was adjusted 16 layers and covered with aluminum foil for the PALS measurement.

The positron annihilation spectrum of the Yucatan miniature pig stratum corneum is shown in Fig. 5, showing three exponential components. The third component shows a longer lifetime than when aluminum is the target, which means that *o*-Ps formed in the pig stratum corneum and briefly resided in nano-pores and/or free volume there. The pig stratum corneum showed a positron lifetime of 2.1 ns.

To further clarify the pore size in the pig stratum corneum, we compared its positron lifetime with those of three cyclic saccharides, having rings with six (α-cyclodextrin), seven (β-cyclodextrin), and eight (γ-cyclodextrin) monomer units. Cyclodextrins have nano-scale interior pores and a well characterized structure, and so provided a good standard scale for measuring pore size. We prepared cyclodextrin samples of about 0.5 mm thickness lapped with aluminum foil, as thin aluminum had no effect on positron lifetime.

Table 1 shows the positron annihilation lifetime and positron pore diameter in α, β and γ-cyclodextrins (α, β and γ-CDs). The *o*-Ps lifetime ($T_{o\text{-Ps}}$) in the α, β and γ-CDs were 1.3, 1.6 and 1.9 ns, respectively. The average size of the pores was calculated by the following equation from Tao (1972) and Eldrup et al. (1981) based on the assumption of spherical micro-vacancies and solving for the radius R. $T_{o\text{-Ps}}$ shows the lifetime of *o*-Ps.

$$\tau_{o\text{-Ps}} = 0.5 \times \left[1 - \frac{R}{R+R_0} + \frac{1}{2\pi} \sin\left(2\pi \frac{R}{R+R_0}\right)\right]^{-1} \quad (2)$$

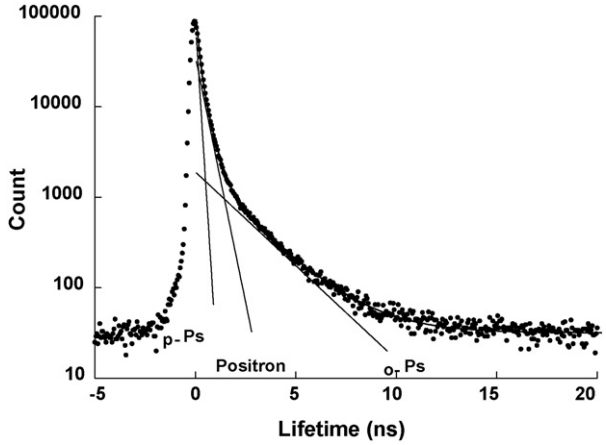

Fig. 5. Positron annihilation lifetime spectrum of pig stratum corneum. Data indicated by dots, with a fitting curve produced by EXCEL solver. The three spectrum components (from *p*-Ps, positrons, and *o*-Ps) are indicated by solid lines.

Table 1
The positron annihilation lifetime, the positron pore diameter and corresponding pore diameter in α, β, and γ-cyclodextrins

	Cyclodextrins		
	α	β	γ
Positron			
Lifetime (ns)	1.3	1.6	1.9
Pore Diameter (nm)	0.43	0.50	0.55
Literature (Parrish, 1987)			
Cavity Diameter (nm)	0.47–0.53	0.60–0.65	0.75–0.83
Cavity Depth (nm)	0.79	0.79	0.79

Experiment was conducted with triplicate.

where the initial factor 0.5 ns is the spin-averaged positronium annihilation lifetime, and the value $R_0 = 0.166$ nm is obtained by the fitting of observed *o*-Ps lifetimes to known mean pore radii in porous materials.

α, β and γ-CDs have cavity diameters of 0.47, 0.60 and 075, respectively, and have identical torus heights of 0.79 nm, respectively. However, the diameters of α, β and γ-CD pores (cavities) determined by positron lifetimes were 0.43, 0.50 and 0.54 nm, respectively. Nakagawa et al. (2000) showed that the topology of the α-CD complex with nitromethane had a diameter of 0.42 nm, which is the almost same as the positron-determined diameter. The cavities in CDs are not cylindrical but toroidal with larger and smaller openings. In general, the cavity diameters were analyzed with the gas absorption method. Both the gas absorption method and the PALS method will show the same diameter for a spherical pore, but the two methods show different diameters for pores with truncated cone shapes. The gas absorption method shows the diameter of a spherical pore volume as being the same as that of a truncated cone, but PALS shows the diameter of the spherical ball that could be placed in the truncated cone. Thus, the gas absorption method reports a larger pore size than PALS. This is one of the unique characteristics of the PALS method, enabling it to determine an actual nano-pore diameter. PALS is a good method to determine the pore sizes in stratum corneum. Table 2 shows the positron lifetimes, positron pore diameter and positron pore volume in pig stratum corneum, polycarbonate, and CDs, where positron pore volume ($V_{o\text{-Ps}}$) was calculated by the following equation.

$$V_{o\text{-Ps}} = \left(\frac{4}{3}\right)\pi R^3 \tag{3}$$

Pig stratum corneum showed a positron lifetime of 2.1 ns, which was longer than that in CDs, α (1.3 ns), β (1.6 ns) and γ (1.9 ns), yielding calculated diameters (by the method of Tao and Eldrup et al.) of 0.43, 0.50 and 0.54 nm. We considered that Yucatan miniature pig stratum layer had at least the pore diameter of 0.59 nm.

A number of mathematical models can predict skin permeability using experimental data sets from various sources. The octanol–water partition coefficient and the molecular weight give the best prediction, and these two factors are considered important descriptors of skin permeability in a quantitative structure permeability relationship. Recently, Lian et al. (2007) showed that the molecular structure descriptors are empirically related to the skin permeability. PALS predicts the actual pore sizes of the stratum corneum, so PALS may clarify the relationship between molecular structure and skin permeability because pore size is directly related to the diffusion of drugs through the stratum corneum.

Cevc (2004) showed that the skin originally had very narrow (approximately 0.4 nm) gaps between cells in the skin barrier and the colloid induced the opening of pores with a diameter of about 30 nm. Aguilella et al. (1994) calculated the radius of negatively charged hydrophilic transdermal pores to be approximately 20 nm. Yoshida and Roberts (1993) reported a pore radius of 0.2 nm and Ruddy and Hadzija (1992) estimated a radius of 3.6 nm. The reported pore radius always depends on a model's assumptions, and may deviate from the actual value. No direct method predicts the real pore radius for the stratum corneum. But PALS provides the real pore radius in stratum corneum and also provides important information on the pore properties and free volume in the stratum corneum that will determine drug diffusion at the molecular level.

We showed that Yucatan miniature pig stratum corneum had a real pore size of 0.59 nm and had no other pores with sizes in the range observed by PALS spectra. We cannot at this point determine whether the pores are in the corneocytes and/or the matrix of intercellular lipids, or whether an enhancer might change the pore size. However, PALS is sensitive to measure pore sizes on the order of ~10 nm, unlike nitrogen absorption and electron microscopy. PALS may be a useful new tool in the biological sciences for investigating the properties of molecular pores and free volume. We have no information on the function of the pores measured by the positrons in our study. However, Kobayashi et al. (1992, 1994) showed the relationship between the diffusion coefficient and positron pore size in polymers. PALS is the only tool that can measure the real pore sizes and free volume of the stratum corneum, therefore PALS may uniquely provide useful information that may predict the skin permeability of drugs and help us to optimize transdermal delivery.

Table 2
Positron annihilation lifetime, positron pore diameter and volume in pig stratum corneum, polycarbonate and cyclodextrins

Sample	Lifetime (ns)	Positron pore diameter (nm)	Positron pore volume (nm^3)
Polycarbonate	2.2	0.60	0.110
Pig skin	2.1	0.59	0.098
α-Cyclodextrin	1.3	0.43	0.041
β-Cyclodextrin	1.6	0.50	0.064
γ-Cyclodextrin	1.9	0.54	0.084

Experiment was conducted with triplicate.

References

Aguilella, M.J., Kontturi, K., Murtomaki, L., Ramirez, P., 1994. Estimation of the pore size and charge density in human cadaver skin. J. Control. Release 32, 249–257.

Cevc, G., 2004. Lipid vesicle and other colloids as drug carriers on the skin. Adv. Drug Deliv. Rev. 56, 675–711.

de Jager, M., Gooris, G.S., Ponec, M., Bouwstra, J.A., 2005. Lipid mixtures prepared with well-defined synthetic ceramides closely mimic the unique stratum corneum lipid phase behavior. J. Lipid. Res. 46, 2649–2656.

de Jager, M., Groenink, W., Bielsa i Guivernau, R., Andersson, E., Angelova, N., Ponec, M., Bouwstra, J., 2006a. A novel in vitro percutaneous penetration model: evaluation of barrier properties with *p*-aminobenzoic acid and two of its derivatives. Pharm. Res. 23, 951–960.

de Jager, M., Groenink, W., van der Spek, J., Janmaat, C., Gooris, G., Ponec, M., Bouwstra, J., 2006b. Preparation and characterization of a stratum corneum substitute for in vitro percutaneous penetration studies. Biochim. Biophys. Acta 1758, 636–644.

Eldrup, M., Lightbody, D., Sherwood, J.N., 1981. The temperature dependence of positron lifetimes in solid pivalic acid. Chem. Phys. 63, 51–58.

Gidley, D.W., Peng, H.G., Vallery, R.S., 2006. Positron annihilation as a method to characterize porous materials. Annu. Rev. Mater. Res. 36, 49–79.

Jean, Y.C., Li, Y., Lin, G., Chen, H., Zhang, J., Gadzia, J.E., 2006. Applications of slow positrons to cancer research: search for selectivity of positron annihilation to skin cancer. Appl. Surf. Sci. 252, 3166–3171.

Jean, Y.C., Chen, H., Lin, G., Gadzia, E., 2007. Life science research using positron annihilation spectroscopy: UV-irradiated mouse skin. Radiat. Phys. Chem. 76, 70–75.

Kobayashi, Y., Hirata, K., Hattori, S., Sasuga, T., 1992. Correlation between the orthopositronium pick-off annihilation lifetime and the free volume in molecular liquid and polymers. Jpn. Bull. Chem. Soc. Jpn. 65, 160.

Kobayashi, Y., Hirata, K., Hattori, S., Sasuga, T., 1994. Evaluation of polymer free volume by positron annihilation and gas diffusivity measurements. Polymer 35, 925.

Lian, G., Chen, L., Han, L., 2007. An evaluation of mathematical models for predicting skin permeability. J. Pharm. Sci. 97, 584–598.

Liu, G., Chen, H., Chakka, L., Gadzia, J.E., Jean, Y.C., 2007. Applications of positron annihilation to dermatology and skin cancer. Physica. Status. Solidi. 4, 3912–3915.
Nakagawa, T., Immel, S., Lichtenthaler, F.W., Linder, H.J., 2000. Topography of the 1:1 α-cyclodextrin-nitromethane inclusion complex. Carbohydr. Res. 324, 141–146.
Parrish, M.A., 1987. Cyclodextrins, a review. Specialty Chem. 7, 366–380.
Ruddy, S.B., Hadzija, B.W., 1992. Iontophoretic permeability polyethylene glycols through hairless rat skin: application hydrodynamic theory for hindered transport thought liquid filled pores. Drug Des. Discov. 8, 207–224.
Tao, S.J., 1972. Positronium annihilation in molecular substances. J. Chem. Phys. 56, 5499–5510.
Thraenert, S., Hassan, E.M., Krause-Rehberg, 2006. Ortho-positronium lifetime measurement—positron source activity and statistics. Nucl. Method Phys. Res. B248, 336–339.
Yoshida, N.H., Roberts, M.S., 1993. Solute molecular size and transdermal iontophoresis across excited human skin. J. Control. Release 25, 177–195.
Yu, B., Kim, K.H., So, P.T., Blankschtein, D., Langer, R., 2003. Visualization of oleic acid-induced transdermal diffusion pathways using two-photon fluorescence microscopy. J. Invest. Dermatol. 120, 448–455.