Investigation of *in vitro* and *in vivo* skin permeability using laser scanning microscopy

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Abstract

Understanding the skin permeability, especially, live human skin permeability is important for several reasons. Firstly, transdermal (through skin) drug delivery is proclaimed as a key delivery mechanism of the future. It offers a targeted drug delivery to the ailment (e.g. muscular pain), with the potential for lower dosages and increased patient compliance. Secondly, skin is the largest organ of an organism serving to protect it from environmental assault. Therefore, thorough understanding of the skin permeability to molecules, macromolecules, and nanoparticles will serve to optimise targeted drug delivery strategies, and minimise potentially hazardous environmental impact.

In this thesis, these important tasks were addressed by applying emerging optical imaging techniques, e.g. Fluorescence Confocal Scanning Microscopy (FCSM) and Fluorescence Multiphoton Microscopy (FMM), to study of the skin permeability, its kinetics and penetration pathways, with a particular focus on *in vivo* imaging. These microscopes are especially suitable for imaging of a thick and heterogeneous tissue such as skin. They provide real-time imaging of distribution of endogenous (intrinsic to tissue) and exogenous (externally introduced) fluorophores, as well as luminescent nanoparticles within superficial skin layers *in vivo* and *in vitro* on the subcellular scale. Thus, both three-dimensional skin architecture and exogenous fluorophores/nanoparticles can be simultaneously visualised. Of critical importance is to answer a question, whether molecules and nanoparticles under study are absorbed sub-dermally, passing *stratum corneum*, the topmost layer of the skin?

As a result of multidisciplinary collaborative team investigations, particularly useful nanoparticle transdermal models have been identified and employed: surface-modified quantum dots (CdSe/ZnS) and metal oxide wide bandgap nanostructures, such as zinc oxide (ZnO-nano) and titanium dioxide (TiO₂-nano) nanoparticles. It appeared that ZnO-nano exhibited remarkable in skin in FMM imaging context. It is explained by its enhanced optical non-linear and serendipitous spectral properties.

Due to the widespread use of ZnO-nano in skin care products, this work may find useful applications *in vivo* non-invasive imaging of nanoparticle transdermal penetration. The overall outcome from the optical and electron microscopy imaging studies was that, in human *in vivo*, nanoparticles stayed in *stratum corneum* and accumulated into skin folds and/or hair follicle

roots of human skin, with a remarkable exception of skin treated with chemical enhancers. The current body of evidence, including contributions of this study, suggests that the form of sunscreen-based nanoparticles studied here is unlikely to result in safety concerns.

In order to demonstrate an approach for investigation of the permeation pathways and kinetics of low-molecular weight drugs and toxins, permeation of organic dye Rhodamine B (Rh:B) via intercellular (through lipid) and intracellular pathways (through corneocytes) was investigated, using FMM and its derivative, fluorescence recovery after photo-bleaching (FRAP) techniques. As a result of this study, the diffusion coefficient of this exogenous fluorophore has been determined, which turned out to be in a good agreement with the tabulated value.

Declaration

The work has not been submitted for a higher degree to any other university or institution.

Signature _____

The skin used in experiments was applied from liposuction plastic surgery by Princess Alexander hospital. Instruments used in this project were supplied by Centre for Microscopy and Microanalysis in University of Queensland (Transmission Electronic Microscope and Scanning Electronic Microscope, for nanoparticle distribution and size determination study); Australian Institute for Bioengineering and Nanotechnology in University of Queensland (Zeiss 510 FCSM and FMM); Therapeutic Research Unit, The School of Medicine, The University of Queensland, Princess Alexandra Hospital (DermaInspect *in vivo* FMM); School of Physics, The University of Queensland (In-house built FMM, Olympus BX50); Centre for Advanced Light Microscopy (Zeiss 510 FCSM). The running costs for this project were covered from the funds of my principal supervisor, Associate Prof. Andrei Zvyagin, and associate supervisor, Prof. Michael Roberts. The University of Queensland provided the major portion of the infrastructure support to this project. For the last year of my PhD research project, Macquarie University provided me postgraduate (MQRES) scholarship.

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Glossary

Medical and Nanomaterial Terminology

Stratum corneum (SC)	Outermost layer of the epidermis (the outermost layer of the skin)
Stratum lucidum	A thin, clear layer of dead skin cells in the epidermis such as that on
	the palms of the hands and the soles of the feet
Stratum granulosum	The highest layer in the epidermis where living cells are found
Stratum spinosum	A multi-layered arrangement of cuboidal cells that sits beneath the
	stratum granulosum
Stratum basale	The layer of keratinocytes that lies at the base of the epidermis
	immediately above the dermis
Mitosis	The process by which a eukaryotic cell separates the chromosomes
	in its cell nucleus, into two identical sets in two daughter nuclei
Strata	The plural of stratum (the geological formation)
Cuboidal cells	Epithelial cells having a cube-like shape
Desmosome	Also known as macula adherens or macula adherents, is a cell
	structure specialized for cell-to-cell adhesion
Melanocytes	Cells located in the bottom layer (the stratum basale) of the skin's
	epidermis and in the middle layer of the eye
Basement membrane	A structure that supports overlying epithelial or endothelial cells
Sweat glands	The skin contains two different groups of sweat glands: apocrine
	sweat glands and merocrine sweat glands. Both gland types contain
	myoepithelial cells, and specialized epithelial cells located between
	the gland cells and the underlying basal lamina
Apocrine glands	Apocrine sweat glands develop during the early- to mid-puberty ages
ripotrino grando	within the age range of 13 to 15 days, and release more than normal
	amounts of sweat for approximately a month, regulating and
	releasing normal amounts of sweat after a certain period of time.
	They are located wherever there is body hair
C 1 1 1	
Sebaceous glands	Glands found in the skin of mammals
Sebum	Sebaceous glands secrete an oily substance called sebum that is
	made of fat (lipids) and the debris of dead fat-producing cells
Lymph nodes	An organ consisting of many types of cells, and is a part of the
	lymphatic system

Trans-appendageal	Through hair follicles or sweat ducts of skin
Extracellular matrices	The extracellular part of animal tissue that usually provides
	structural support to the cells in addition to performing various
	other important functions
Cervical mucus	A viscous, slimy mixture of mucins, water, electrolytes, epithelial
	cells, and leukocytes that is secreted by glands lining the nasal,
	esophageal, and other body cavities and serves primarily to protect
	and lubricate surfaces, located in neck
Ceramide	A family of lipid molecules, found in high concentrations within
	the cell membrane of cells and composed of sphingosine and a fatty
	acid
Desquamans	The shedding of the outer layers of the skin
Cholesterol	A lipid found in the cell membranes of all animal tissues, and it is
	transported in the blood plasma of all animals
Pilosebaceous	The structure consisting of hair, hair follicle, arrector pili muscle,
	and sebaceous gland
Interstitial	Situated between the cells of a structure or part
Epidermis	Outermost layer of the skin
Dermis	A layer of skin beneath the epidermis that consists of connective
	tissue, and cushions the body from stress and strain
Reticular dermis	Dermis which has the form of the net
Lipid bilayers	A membrane composed of lipid molecules (usually phospholipids)
Corneocyte	Dead cells in stratum corneum, a protein complex that is made of
	tiny threads of keratin in an organized matrix
Proinflammatory cytoki	nes Signalling proteins and glycoproteins which are capable of
	promoting inflammation
Porcine	Pigs, also called hogs or swine
Irritation potentia	The possibility of becoming irritated
Cytotoxicity	The quality of being toxic to cells
Percutaneous	Any medical procedure where access to inner organs or other tissue
:	is done via needle-puncture of the skin, rather than by using an
	"open" approach where inner organs or tissue are exposed (typically
	with the use of a scalpel)

Transdermal	Applied to the skin, usually as part of an adhesive patch, for absorption
	into the bloodstream
In vivo	Refers to experimentation within, or using an entire organism
In vitro	Refers to experimentation involving tissues removed from an organism
Exogenous	Refers to an action or object coming from outside a system
Endogenous	"Arising from within", the opposite of exogenous.
Flavoproteins	Proteins that contain a nucleic acid derivative of riboflavin: the flavin
	adenine dinucleotide (FAD) or flavin mononucleotide (FMN)

Reduced pyridine nucleotides (NAD[P]H) Zinc Oxide nanoparticle (ZnO-nano) Titanium dioxide nanoparticle (TiO₂-nano) Quantum dot/Quantum dots (Qdot/Qdots)

Optical Physics terminology

Nonlinear optical microscopy (NLOM) Fluorescence multiphoton microscopy (FMM) Fluorescence confocal scanning microscopy (FCSM) Fluorescence recovery after photo-bleaching (FRAP) Fluorescence correlation spectroscopy (FCS) Fluorescence lifetime imaging (FLIM) Scanning electron microscope (SEM) Energy Dispersive Spectroscopy (EDS) Transmission electron microscope (TEM) Numerical Aperture (NA) Two dimensions (2D) Three dimensions (3D) Ultra violet bandA (UVA) Ultra violet bandB (UVB) Photoluminescence (PL) Second harmonic signal (SHG) Photomultiplier (PMT) Time-correlation single-photon counting module (TCSPC) Signal-to-noise ratio (SNG)

Femtosecond (fs) Picoseconds (ps) Nanoseconds (ns) Nanometre (nm) Micrometre (µm)

GM	Unit of absolute value of two-photon action cross-section
	1 GM $= 10^{-50}$ cm ⁴ s/photon
Photobleaching	Photochemical destruction of a fluorophore
Excitation	Excited state of an atom
Emission	The process by which the energy of a photon is released by another
	entity
Dichroic	A dichroic material is either one which causes visible light to be
	split up into distinct beams of different wavelengths (colours) (not
	to be confused with dispersion), or one in which light rays having
	different polarizations are absorbed by different amounts
Epi-fluorescence	Fluorescence detected by eye piece
Bohr radius	In the simplest atom, hydrogen, a single electron orbits, and the
	smallest possible orbit for the electron, which with the lowest
	energy, the distance from the nucleus called the Bohr radius
Maximum permissible ex	bosure The highest power or energy density (in W/cm^2 or J/cm^2)
	of a light source that is considered safe, i.e. that has a
	negligible probability for creating a damage
Therapeutic window	An index for estimation of drug dosage which can treat
	disease effectively while staying within the safety range
Basophilic granules	Microscopic appearance of a small particle or grain

Chemistry terminology RhodamineB (Rh:B) Caprylic capric triglycerides (CCT) Polyethylene glycol (PEG) Phosphate buffered saline (PBS) Hydrochloric acid (HCl) Weight by weight (w/w) Micromolar (µM) Gram (g) Dalton (Da) Grams per moles

pН	The measure of the acidity or alkalinity of a solution
Hydrodynamic diame	ters The size of molecules of liquids in motion
Lipophilic	Refers to the ability of a chemical compound to dissolve in fats, oils,
	lipids, and non-polar solvents such as hexane or toluene
Hydrophilic	A physical property of a molecule that can transiently bond with water
	(H ₂ O) through hydrogen bonding
Formaldehyde	A colourless, toxic, potentially carcinogenic, water-soluble gas, $\mathrm{CH}_2\mathrm{O}$,
	having a suffocating odour, usually derived from methyl alcohol by
	oxidation: used chiefly in aqueous solution, as a disinfectant and
	preservative, and in the manufacture of various resins and plastics
Octinoxate	Octyl methoxycinnamate (INCI), or octinoxate (USAN), is an organic
	compound that is an ingredient in some sunscreens and lip balms. It is
	an ester formed from methoxycinnamic acid and 2-ethylhexanol. It is a
	clear liquid that is insoluble in water
Terpenes	A large and varied class of hydrocarbons, produced primarily by a wide
-	variety of plants, particularly conifers, though also by some insects
	such as swallowtail butterflies, which emit terpenes from their
	osmeterium
Laurocapram	The penetration enhancer in cosmetic preparations and
	personal-care products
Oleic Acid	A monounsaturated omega-9 fatty acid found in various animal and
	vegetable sources
OCT	For embedding tissues to be cryosectioned
Amine	Organic compounds and functional groups that contain a basic
	nitrogen atom with a lone pair, usually written -NH ₂
Carboxylic	Organic acids characterized by the presence of a carboxyl group,
	which has the formula $-C(=O)OH$, usually written $-COOH$ or $-CO_2H$

Passivation shell Formation of a hard non-reactive surface film that inhibits further corrosion