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**PASSIVE AND ACTIVE STRATEGIES FOR
TRANSDERMAL DRUG DELIVERY WITH
NANOTECHNOLOGY AND TEMPORAL PRESSURE**

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PASSIVE AND ACTIVE STRATEGIES FOR TRANSDERMAL DRUG DELIVERY

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**Interdisciplinary Graduate School
NTU-Northwestern Institute of Nanomedicine**

A thesis submitted to the Nanyang Technological University in
partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Lio Chin Shiu, Daniel

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

30th December 2019

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Date

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Assistant Professor Xu Chenjie

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal, and 2 papers under review where I was the first and/or corresponding author.

Chapter 3 is published as Daniel Chin Shiuan Lio, Chenghao Liu, Christian Wiraja, Beiying Qiu, Chee Wai Fhu, Xiaomeng Wang, and Chenjie Xu., 2018. Molecular Beacon Gold Nanosensors for Leucine-Rich Alpha-2-Glycoprotein-1 Detection in Pathological Angiogenesis. ACS Sensors, 3(9), pp.1647-1655

The contributions of the co-authors are as follows:

- A/Prof Chenjie Xu provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by A/Prof Chenjie Xu and Assoc Prof Xiaomeng Wang.
- I co-designed the study with A/Prof Chenjie Xu and performed all the laboratory work at the School of Chemical and Biomedical Engineering (with Dr. Christian Wiraja) and the Lee Kong Chian School of Medicine (with Chenghao Liu, Beiying Qiu). I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me in School of Chemical and Biomedical Engineering.

Chapter 4 is currently under preparation.

The contributions of the co-authors are as follows:

- A/Prof Chenjie Xu, and I conceived the project and wrote the manuscript.
- Myo Myo Swe Oo was a FYP who was trained by me in synthesis of nanoparticles, cell culture, and fluorescence imaging.
- Mengjia Zheng and Chew Wan Ting Sharon assisted in SEM.
- I, Chenghao Liu, Myo Myo Swe Oo, Christian Wiraja, Teo Hui Yen, Melissa performed cell culture, in vivo experiments, RT-PCR, Western Blotting.
- Assoc Prof Xiaomeng Wang provided suggestions for in *vivo* experiments.

Chapter 5 is currently under review by *Nature Communications*.

The contributions of the co-authors are as follows:

- A/Prof Chenjie Xu and Prof David Becker provided the inspiration to the project.
- A/Prof Chenjie Xu, Prof David Becker, Assoc Prof Xiaomeng and I conceived and designed the experiment.
- I conducted the experiments and optimized the procedures.
- Kwek Sheng Yi, Milton assisted in the high-power imaging of mouse skin and staining of Connexin 43 and Occludin.

- Rui Ning Chia assisted in preparing the STZ-induced diabetic mice.
- Mohideen was a FYP who assisted in the cryosectioning, imaging, and analysis of the dextran molecules.
- Hao Chang assisted in preparing the summary diagram.

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List of Publications

First/ co-first author publications

- 1) **Daniel Chin Shiuan Lio**, Chenghao Liu, Christian Wiraja, Beiying Qiu, Chee Wai Fhu, Xiaomeng Wang*, Chenjie Xu*. Molecular Beacon Gold Nanosensors for Leucine-Rich Alpha-2-Glycoprotein-1 Detection in Pathological Angiogenesis, ACS Sensors, 2018, 3(9), 1647-1655, DOI: 10.1021/acssensors.8b00321
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- 4) **Daniel Chin Shiuan Lio[#]**, Cheng Hao Liu[#], Myo Myo Swe Oo, Christian Wiraja, Melissa Hui Yen Teo, Mengjia Zheng, Sharon Wan Ting Chew, Xiaomeng Wang, Chenjie Xu*. Transdermal delivery of small interfering RNAs with topically-applied mesoporous silica nanoparticles for facile skin cancer treatment. (*manuscript under review by Nanoscale*)

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- 8) Razina Z. Seenii, **Daniel Lio**, Christian Wiraja, Yuchun Liu, Bee Tin Goh, Chenjie Xu*. Decreasing Skin Electric Resistance using PEDOT: PSS-Containing Conductive Microneedles in Transdermal Drug Delivery (*manuscript in preparation*)
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- 2) **D. C. S. Lio**, R. N. Chia, M. S. Y. Kwek, C. Wiraja, C. Hao, S. M. A. Khadir, X. Wang*, D. L. Becker*, C. Xu*, “Transdermal Delivery of Nano- and Macro molecules via Temporal Pressure with Insulin as Proof of Concept”; Poster, 12th Annual Symposium on Nanotechnology, 2019, February, Singapore (Top 3 Poster Award)
- 3) **D. C. S. Lio**, R. N. Chia, M. S. Y. Kwek, C. Wiraja, C. Hao, S. M. A. Khadir, X. Wang*, D. L. Becker*, C. Xu*, “Transdermal Delivery of Nano- and Macro molecules via Temporal Pressure with Insulin as Proof of Concept”; Poster and Oral, NTU SCBE PhD Symposium 2019, 2019, March, Singapore (Bronze Prize)
- 4) **D. C. S. Lio**, R. N. Chia, M. S. Y. Kwek, C. Wiraja, C. Hao, S. M. A. Khadir, X. Wang*, D. L. Becker*, C. Xu*, “Temporal pressure enhanced transdermal delivery through micropore formation”; Oral,

Japan-Singapore International Skin Conference, 2019, April,
Singapore

- 5) **D. C. S. Lio**, R. N. Chia, M. S. Y. Kwek, C. Wiraja, C. Hao, S. M. A. Khadir, X. Wang*, D. L. Becker*, C. Xu*, "A New Methodology for Transdermal Drug Delivery Based on Temporal Pressure"; Oral, 10th International Conference on Materials for Advanced Technologies, 2019, June, Singapore
- 6) **D. C. S. Lio**, R. N. Chia, M. S. Y. Kwek, C. Wiraja, C. Hao, S. M. A. Khadir, X. Wang*, D. L. Becker*, C. Xu*, A Novel Transdermal Delivery Method For Nano- And Macro Molecules Via Temporal Pressure Technology Inspired By Traditional Chinese Medicine's Tui Na, Biomimetics in Bioengineering Conference, 2019, August, Brisbane, Australia

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Summary

The field of transdermal drug delivery (TDD) has made an important contribution to the medical practice because of its avoidance of first-pass effect of the liver and to achieve a more localized drug delivery. However, TDD has yet to achieve its potential as an alternative to oral delivery and hypodermic injections due to the strong skin barrier to substances over 500Da in size.

Over the years, many studies have been strategically performed to enhance TDD efficacy while preserving the skin barrier integrity. These strategies can be categorized into passive and active methods. Passive strategies for TDD taps on the hydrophobic properties and surface charge of the skin to modify the drug formulations to achieve modification of the stratum corneum for enhance penetration. However, these strategies face potential skin irritation. Active strategies on the other hand utilize external forces to achieve a stronger disruption of the stratum corneum for improved penetration of drugs of size bigger than 500 Da. However, these technologies are usually bulky, and administrations are mostly performed by healthcare professionals.

In the first half of this thesis, nanotechnology is proposed as a compatible and excellent carrier to achieve passive delivery of genetic

materials for non-invasive detection and regulation of skin biomarkers. Specifically, the nanoparticles were fabricated by incorporating molecular beacons (MB), oligonucleotide-based hairpin probes with specific target gene recognition ability, onto metal nanoparticles (gold nanorods, AuNR). Tagged on the surface of the AuNR through gold-thiol bonds, these structures can be effectively internalized by human keratinocyte cells. Subsequently, the fluorescence signal of the MB was compared in different *in vitro* conditions against RT-PCR and Western-Blot to confirm its specificity. Last but not least, these nanosensors were topically applied on the skin for effective penetration into the epidermis tissue to achieve non-invasive detection of skin biomarker.

In addition, mesoporous silica nanoparticles (MSNPs) is also proposed in this thesis as a versatile and highly compatible polymeric nanocarrier for MB delivery across the skin. Specifically, MBs were incorporated into the pores of MSNPs, followed by a coating of positively charged poly-L-lysine to improve the cellular uptake (MSNPs-MB-PLL). The uptake efficiency of MSNPs-MB-PLL, specificity and functionality of released MB were tested in human skin squamous carcinoma cells (SCC) with promising results. Subsequently, we topically delivered MSNPs-PLL with Cy5 into SCC xenograft model and observed good penetration of NPs through the trans-follicular route into the SCC tumor. Finally, topically applied

MSNPs-siRNA-PLL demonstrated 2-fold suppression of TGF β R-1 gene in SCC.

Studies have shown that the size of NPs is the limiting factor to the extent of TDD. Therefore, active strategy will be explored in the second half of the thesis. For the first time, temporal pressure is proposed as a promising alternative to hypodermic injection. The temporal pressure was achieved with the use of magnets and tested *in vivo*. Optimization was performed with different pressure forces to achieve effective TDD while protecting the skin integrity (0.28MPa for 1- or 5-mins application). Subsequently, temporal pressure demonstrated delivery of molecules up to 200 kDa and nanoparticles up to 500 nm. To prove the translatability, temporal pressure was applied to deliver insulin as a proof of concept in normal and diabetic mice. Effective delivery of insulin was achieved with 80% reduction in blood glucose in just 30 mins, for a period of 6 hrs.

In conclusion, nanotechnology and temporal pressure-based strategies are introduced in this thesis for the field of transdermal drug delivery. Especially for delivery of genetic therapy, nanotechnology can facilitate the transdermal delivery of various therapeutic approaches. On the other hand, temporal pressure serves as an attractive alternative to

hypodermic injection, achieving effective transdermal delivery similar to microneedles.

Chapter 1. Introduction

1.1. Background and Scope

Transdermal Drug Delivery (TDD) surfaces as an attractive alternative to oral delivery of drugs and potentially can be a replacement for hypodermic injection. The global market for TDD reached \$7 billion in 2015, with an increase of \$238 million just within a year¹. Compared to traditional oral delivery, TDD is advantageous for first-pass effect of the liver which prematurely metabolizes drugs². Clinically, hypodermic injection is conventionally the quickest drug administration to the systemic circulation, however the drug effectiveness is often short lived, and the process is invasive and painful which generate dangerous biohazard and environmental waste and pose risk of disease transmission if needles were to be reused. TDD received vast attention due to the ability to administer drug through a controlled release of active agent to the systemic circulation.

TDD is complicated because the skin serves as a natural barrier. A study dated back in 1924 by Rein found that stratum corneum and epidermis are the major contributors to resistance in transdermal delivery³. As such, TDD has evolved through three generations over the years (Table 1.1). The first generation consisted mainly of today's patches with little or no enhancement, suitable for low molecular weight drugs at low doses². Second generation saw the identification of skin penetration enhancers. However, it

was a struggle to achieve balance between improved deliveries while preventing damage to deeper skin tissues. In the third generation, advanced technologies enabled TDD of a variety of drugs including macromolecules (DNA, proteins) and virus-based vaccines through targeted permeabilization of skin's stratum corneum. These complex technologies (microneedles, electroporation, cavitation ultrasound) require the supervision of medically trained professionals⁴. The three generations of technologies can also be further classified into two categories (Table 1.1), passive (patches, gels, nanoparticles) and active (microneedles, electroporation, iontophoresis, ultrasound) strategies⁵.

Generations >>>	1 st Topical app	2 nd Chemical enhancer	2 nd Nanoparticles	2 nd Ionto- phoresis	3 rd Electro- poration	3 rd Cavitation Ultrasound	3 rd Thermal ablation	3 rd Micro- needles
Skin permeability	✗	✓	✓	✓	✓	✓	✓	✓
Prevent Skin Irritation	✓	✗	✗	✗	✗	✗	✗	✗
Delivery of large, hydrophilic molecules	✗	✗	✓	✗	✓	✓	✓	✓
Cost	✓	✓	✓	✗	✗	✗	✗	✓
Convenience	✓	✓	✓	✗	✗	✗	✗	✓
Self-admin	✓	✓	✓	✓	✗	✗	✗	✓
Type	Passive Strategy			Active Strategy				

Table 1. 1. Summary Table of current transdermal drug delivery technologies

While many TDD technologies have surfaced to achieve painless and convenient drug delivery, most technologies are hindered by the use of bulky equipment, inability to penetrate the skin barrier, poor loading efficiency, and require the presence of healthcare professional to administer the treatment⁶. Therefore, there is a need for TDD technologies which can improve loading efficiencies, effectively penetrate skin barrier, and provide user-friendly experience for the users.

To address this need, passive and active strategies for transdermal drug delivery are designed and evaluated in this thesis. Briefly, the passive strategies utilizes two nanomaterials (1) gold nanorods (AuNR) which received many interests over its inertness⁷, biocompatibility⁸, and ability to penetrate the skin without any enhancers⁹⁻¹⁰. (2) FDA-approved material silica, specifically mesoporous silica nanoparticles (MSNPs)¹¹ which received many interest over its tuneable pore size, good loading efficiency, and excellent compatibility with skin cells. However, the penetration of these nanoparticles is still limited by their size¹² when delivered via a passive method. Therefore, the third technology which will be discussed in this thesis is based on active strategies, termed “temporal pressure” which was not reported elsewhere. Briefly, the enhanced TDD was achieved through application of pressure on the skin for a short period of time, which caused molecular changes in the epidermal cells to allow penetration of drugs across

the skin. Taking into consideration that temporal pressure was not reported elsewhere, this thesis will cover the discovery, as well as the optimization process to achieve an efficient yet safe procedure.

Comprehensively, the scope of this work includes the design and synthesis of AuNR, MSNPs, and as a proof of concept, molecular beacons (MB) an oligonucleotide-based sensor will be used as the model nucleic acids to be delivered *in vitro* and *in vivo*. To prove the functionality of MB post-delivery, validation of these MB signal generation against gold-standard method were performed.

On the other hand, the efficacy of temporal pressure was proven by delivering Dalton and nanometres range molecules, and to prove its translatability, insulin was used as the model drug to be delivered transdermally. To evaluate the safety of this technology, the influence of temporal pressure on the skin will be discussed in this thesis.

1.2. Overarching Thesis Goal to develop novel transdermal drug delivery methods

Designing Passive and Active Strategies for Enhanced Transdermal Drug Delivery

1.2.1. Passive Strategy

1.2.1.1. Application of molecular beacon bounded gold nanosensors to monitor Lrg1 expression in pathological angiogenesis

Angiogenesis, a process of new blood vessel formation, contributes to the development and progression of several diseases. Leucine-rich alpha-2-glycoprotein-1 (Lrg1) is an emerging biomarker for angiogenesis. Its expression is up-regulated in human skin of pathological angiogenesis. However, there is no existing sensor that allows visualization and monitoring of Lrg1 expression noninvasively and in real time. Methods to evaluate such successful gene expression are typically invasive methods including PCR, western blotting, or immunostaining assays. Herein, we report a nucleic acid-gold nanorod-based nanosensor for noninvasive monitoring of cellular Lrg1 expression in angiogenesis. After the designing and fabricating MB nanosensor targeted towards Lrg1 marker, the MBs are covalently

conjugated onto gold nanorods, which prequench the fluorophores on the MBs. Upon intracellular entry and endosomal escape, the complexes interact with cellular Lrg1 mRNA through hybridization of the loop area of the molecular beacons. This complexation distances the fluorophores from nanorod and restores the prequenched fluorescence. The reliability of this platform is confirmed by examining the increased Lrg1 expression in migrating human skin keratinocytes and the Lrg1 gene expression in the mouse skin epidermis.

1.2.1.2. Synthesis and transdermal delivery of small interfering RNAs with topically-applied mesoporous silica nanoparticles (MSNPs) for facile skin cancer treatment

RNAi therapies utilize RNA such as small interfering RNAs (siRNAs) to interfere with the expression of specific genes related with the disorders. Encouraging results were reported for several gene related skin diseases. However, these studies also revealed the technical hurdles to overcome the skin's barrier in a patient-friendly and clinically-relevant manner. These siRNA are negatively charged and big in size, hindering the penetration into the skin. Thus, we tap on MSNPs as the second passive strategy delivery tool for transdermal delivery. We studied the encapsulation within MSNPs

with MB as the model oligonucleotide. To improve these MSNPs-MB penetrations in vitro and in vivo, we coated a positive layer of poly-L-lysine (PLL) to produce MSNPs-MB-PLL. To evaluate the efficacy of these nanoparticles (NPs), we studied the cellular uptake efficiency, functionality and specificity of MBs post-delivery in skin squamous cell carcinoma. Last but not least, we demonstrated the efficacy of MSNPs-PLL by delivering TGF β R-1 siRNA (MSNPs-siRNA-PLL) to a squamous cell carcinoma xenograft model in vivo via a topical cream and compared it against MSNPs-scramble siRNA-PLL.

1.2.2. Active Strategy

1.2.2.1. Transdermal Delivery of Nano and Macro molecules via Temporal Pressure with Insulin as Proof of Concept

Despite the development of passive strategies in the previous 2 sections to improve transdermal delivery of drugs, the penetration of drugs is still limited by the size of it. As such, the transdermal delivery field saw the emergence of active strategies which involve the use of external energy to act as a driving force for drug penetration into the skin. These active strategy methods include electroporation, laser ablation, iontophoresis, etc.

Nonetheless, these physical means are accomplished through utilization of additional, specialized equipment alongside trained users to modulate skin permeability, thus presenting challenges in terms of patient convenience and home-use. Another technology worth mentioning is the microneedle, which has received vast attention recently. However, due to their small sizes, the amount of drugs could be delivered is usually within microgram range.

Herein, we report a new methodology that utilizes the temporal pressure to enhance the penetration of all kinds of drugs ranging from small molecules to protein and nanoparticles. This portion of the thesis will demonstrate the discovery of temporal, optimization of application procedure, and the molecular changes in skin barrier which led to the improved delivery. A range of fluorescent molecules (3kDa – 20kDa) and fluorescent nanoparticles (20nm – 500nm) were used to study the extent of improvement in transdermal delivery in C57/Bl6J mice. In Vivo Imaging System (IVIS) was used to image the amount of penetrated fluorescent particles, followed by histology to study the penetration depth and haematoxylin and eosin staining to study the damage done to the skin.

Following the successful demonstration of temporal in improving transdermal drug delivery, the last part of the thesis project aimed to demonstrate the clinical relevance and efficacy in delivering drugs across the

skin. As a proof of concept, insulin was chosen as the model drug to be delivered transdermally. The initial study of insulin was performed in normal C57/Bl6J mice to study the penetration efficiency into the systemic circulation. Thereafter, a 5 days delivery experiment was performed in diabetic mice model to demonstrate the efficacy of temporal pressure assisted insulin delivery in comparison to subcutaneous injection, solid microneedles, and dissolving microneedles.

Chapter 2. Literature Review

2.1. Transdermal Drug Delivery

2.1.1. Significance of Transdermal Drug Delivery (TDD)

Invented in 1853 by Charles Gabriel Pravaz and Alexander Wood, the needle and syringe technology is one of the breakthrough inventions to eliminate many technical difficulties for drug delivery¹³. According to World Health Organization, about 16 billion injections worldwide are administered every year. While injections remain till date as the most efficient method and gold standard of drug administration, it is often cited in literature as the most painful part of minor procedures¹⁴⁻¹⁵. Furthermore, studies show that a remarkable 20% of the population has a fear for injections and 10% of them suffer from Trypanophobia, an excessive or irrational fear of injections¹⁶⁻¹⁷. This unnecessary fear affects medication compliance and treatment outcome. For example, 50.8% of Type II Diabetes patients are unwilling to initiate insulin therapy as pain was associated with it¹⁸.

In the healthcare environment, needle and stick injuries post as major hazards that can expose healthcare workers to infections e.g. Hepatitis B and C and HIV. An astonishing figure of 600,000 to one million needle stick injuries occur annually in America alone, of which approximately 16,000 of these needles are infected with HIV¹⁹. After the use of needles for injection,

the management of these needle waste post a burden for many healthcare institutions, and when misuse of not disposed in the correct manner, is a very dangerous tool for transmissions of diseases²⁰.

Oral delivery of drugs remains another commonly used route for medication. However, the oral drug generally becomes active when it passes from the gastrointestinal tract and the liver into the blood, termed first-pass effects²¹. This causes majority of the drug to be retained or digested by enzymes, leading to undesirable drug interactions at major organ sites and inefficient usage of drugs²².

To address these concerns, Transdermal Drug Delivery (TDD) became a popular research entity as it represents an attractive alternative to oral delivery and conventional hypodermic injection. In particular, TDD avoids first-pass effect of metabolism associated with oral route^{2, 23}, resulting in improved bioavailability and prolonged release of certain medications. Furthermore, TDD also has various advantages over hypodermic injections²⁴⁻²⁵, which are painless administration, eliminate the need for disposal of sharp waste, and minimize the risk of disease transmission. In 1979, the first TDD technology was Scopolamine, a patch designed to deliver alkaloid.

The global transdermal drug delivery system market is expected to reach USD 81.4 billion by 2024²⁶, with great anticipation to incorporate TDD in the future treatments (Figure 2.1). The key players in the TDD field include Noven Pharmaceuticals, Inc who received FDA approval in 2014 for its estradiol transdermal system, and Boehringer Ingelheim GmbH in 2015 for their clinical trials in bioequivalence of transdermal clonidine administration²⁶.

Transdermal Drug Delivery System Market, by Technology, 2013 - 2024 (USD Billion)

Figure 2.1. Transdermal Drug Delivery System Market, by Technology, 2013 – 2024 (USD Billion), Figure Courtesy of Global Market Research Insights Report.

2.1.2. Current passive and active strategies for effective Transdermal Drug Delivery

Advances in transdermal drug delivery (TDD) can be categorized under three generations². Briefly, the first generation of systems include drug loaded patches for simple diffusion across the skin, second generation yielded additional advances for small molecules by increasing skin permeability, and third generation enable TDD through permeabilization of skin's stratum corneum. These three generations of TDD systems can be further classified in two main types of systems, namely Passive and Active strategies for enhanced TDD⁵.

Passive approaches in enhancing TDD include optimization of formulation and stratum corneum modification, and influence of drug and vehicle interactions⁵. Firstly, stratum corneum modification includes one of the most used passive approaches in the TDD field, chemical penetration enhancers. These enhancers are molecules that causes reversible changes of the skin's barrier properties without permanent damage to the cells²⁷. Secondly, correct interactions of drugs and vehicles with the skin can promote effective penetration of drugs across the skin barrier²⁸. This include the use of prodrugs²⁹, ion pairing³⁰, eutectic³¹ or supersaturated systems³². In recent years, nanotechnology has emerged as a promising tool for TDD⁸.

¹⁰. The ability to control the size, surface properties, loading efficiency, release profile, and stimuli responsive release are crucial factors for the nanoparticle's transdermal penetration and drug efficacy³³⁻³⁴ .

On the other hand, active approaches to enhancing TDD targets its effects to the stratum corneum by disrupting them. This enables more effective transdermal drug delivery while still protecting the deeper tissues². These active approach technologies can be categorized into Electrical-assisted, Mechanical-assisted, and others. Electrical-assisted TDD facilitates the transport of molecules across the skin using an electromotive force and high voltage gradients across the skin to momentarily decrease the skin's electrical resistance. Technologies include iontophoresis, electroporation, radiofrequency, and magnetophoresis.

Mechanical-assisted TDD involves the use of mechanical forces to induce disruption of skin barrier. TDD of different drugs of differing lipophilicity and molecular weight, e.g. proteins, peptides and oligonucleotides, has been shown to be improved via active approach. This includes abrasion, skin stretching, skin puncture, perforation, and the recent popular microneedles. While these technologies proved to be effective, prime issues to be addressed include safety, ease of handling, and cost-effectiveness.

2.2. Passive Strategy

2.2.1. Chemical-based systems

Till date, more than 360 chemical penetration enhancers (CPE) have been identified to modify the stratum corneum barrier to facilitate molecular delivery³⁵. An ideal CPE should be 1) non-toxic and non-allergenic, 2) work rapidly, 3) work unidirectionally, and 4) when removed, the skin's barrier properties should return back to normal quickly. Examples of these CPE include terpenes, sulphoxides, laurocapram, pyrrolidones, fatty acid and fatty alcohol, alcohol and glycol, surfactants, urea, etc³⁶.

The use of CPE has proven, in several cases, to improve passive diffusion of small therapeutics. Chemical such as Dimethyl Sulfoxide (DMSO) utilizes its amphiphilic nature to interact with the cell membranes³⁷, enhancing TDD of both hydrophobic and hydrophilic molecules. Interestingly, DMSO was found to penetrate the skin at a rate of 176g/m³, compared to 14.8g/m³ for water³⁸. In addition, different concentrations of DMSO have different impact on the cell membrane. At low concentrations (2.5-7.5 mol %), it induces expansion and thinning of the lipid membrane, increasing the fluidity of the hydrophobic region of the membrane. On the other hand, high concentrations of DMSO (10-25 mol %) induces formation of transient "water pores", a result of the movement of water molecules into the membrane³⁷.

These penetration enhancement characteristics of DMSO allow it to be an attractive penetration enhancer for drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs)³⁹.

Another commonly used CPE is the alcohol, e.g. ethanol, 1-octanol, 1-hexanol, 1-decanol, and lauryl alcohol. Several studies demonstrated the mechanism of alcohol enhanced TDD through lipid extraction that occurs due to lipid solubilization, therefore increasing the porosity of the stratum corneum, leading to improved penetration of hydrophilic drugs⁴⁰⁻⁴¹.

While CPEs have proved to be effective, a common drawback of them is that their efficiency is often accompanied by skin irritation due to their mechanism of action, disrupting the order of stratum corneum lipid bilayers or corneocytes structure organization^{35, 42}. Studies have shown that the mechanism of CPEs are one of the etiological factors to dryness and barrier disruption in skin conditions. These allow the entrance of allergens and toxins that can caused inflammation⁴³. Common skin conditions associated with this infection include atopic dermatitis, psoriasis, eczema, acne vulgaris, etc.

2.2.2. *Drug and vehicle interactions*

Given the properties of stratum corneum being lipophilic, negatively-charged, etc, drugs can be modified to enhance the transdermal drug delivery. These vehicle enhancements are chosen based on the pharmacological or physiochemical properties, e.g. prodrugs and ion pairing.

Prodrug design is a method used to increase the solubility or permeability of a drug compound, resulting in permeability enhancement²⁹. This can be achieved through 1) increase lipophilicity through alkyl-chain lengthening, and 2) introducing PEGylation to the compound, such as esters, carbonates, carbamates and ether, which can readily cross the skin ⁴⁴. The mechanism behind this strategy is to allow the prodrug to facilitate partitioning of the molecule into the stratum corneum. Once in the skin, the prodrug may be cleaved in the skin to release the active compound. A study was performed to enhance transdermal delivery of Cytarabine, an anticancer drug. It was found that by modifying the drug with N-Hexyl amide 10, the penetration was improved to a molecular flux, J_{ss} of 89.0 nmol/cm²/h, as compared to 3.7 nmol/cm²/h of Cytarabine⁴⁴.

Ion pairing is formed by a pair of oppositely charged ions held together by Coulomb attraction. This strategy has emerged as an effective approach for enhancing solubility of ionic molecules in non-polar solvents and

addressing the poor bioavailability of hydrophilic molecules. The mechanism behind the ion pair strategy involves forming an ion-pair such that the neutralized charges for the complex are achieved to partition into and permeate through the stratum corneum. The ion-pair then dissociates in the aqueous microenvironment of the epidermis, releasing the active drug compound to diffuse within the epidermal and der

mal tissues⁴⁵. Ivaturi and Kim demonstrated that methotrexate: L-arginine ion paired system has a partition coefficient of 24 times greater than that of methotrexate alone. The permeation of methotrexate: L-arginine across the rabbit nasal mucosa was approximately three times greater as compared to control.

While these drug and vehicle interaction strategies are promising, tedious research studies are required to ensure that the key physiochemical parameters of the compound, e.g. molecular weight and partition coefficient are of recommended range. The molecular weight is still limited to ~600 Dalton size molecules. Furthermore, the chemicals used in enhancing the penetration may cause irritation to the skin⁴⁴.

2.2.3. Nanotechnology and Drug Interactions

In recent years, nanotechnology has been prevalent in the field of transdermal drug delivery, finding inherent popularity in the field of cosmeceutical skin care on the basis on their compatibility and practical properties⁴⁶. These nanoparticles (NPs) approximately the size of 100nm or below, can be distinguished, based on the synthesis on these nanomaterials used in dermatology and cosmetology. These include nanospheres, nanocapsules, nanoemulsions, and nanocarriers (niosomes) (Figure 2.2). In addition, inorganic and metal nanoparticles e.g. silver and gold nanoparticles are used as a carrier for drugs for skin diseases⁴⁷. In this thesis, metal and polymeric nanoparticles will be discussed.

Metal nanoparticles are tiny materials made of pure metals⁴⁸ e.g. gold, platinum, silver, etc. At nano size, these materials have unique physical and chemical properties due to their high surface area and nanoscale size. For example, silver nanoparticles (AgNP) are well known for their antimicrobial property which is useful in controlling colonization and proliferation of pathogens including multidrug-resistant organisms⁴⁹. Ahmadi et al. demonstrated that AgNP, together with tetracycline achieved faster wound closure (100% in 12 days) as compared to AgNP or tetracycline alone. Furthermore, decreased surface bacterial (*Pseudomonas aeruginosa*)

concentration, and significant decrease in deep skin bacteria counts were achieved with AgNP and tetracycline as compared to control⁵⁰. Interestingly, many studies have proven that AgNP are good penetrants of skin^{12, 49}. Tak et al. demonstrated the penetration of AgNPs in blood capillaries in hairless mice after five days of treatment. Whereas a 30 hrs study performed in vitro demonstrated different penetration profile of rod-shaped, spherical, and triangular AgNPs until the dermal, epidermal, and stratum corneum layer respectively. Thus, penetration of AgNPs to desired skin site can be controlled by different shapes of AgNPs to achieve targeted delivery⁴⁹.

Another nanomaterial which is extensively researched in recent years is the gold nanoparticles (AuNP). Different shape and sizes of AuNP have related surface plasmon resonance which enables quenching of fluorescence. In addition, their excellent biocompatibility and easy modification on the surface enables a wide range of applications such as diagnosis, targeted delivery, and therapy. Nanoflares is a technology based on gold nanoparticles consisting of a dense shell of duplex DNA, one called the recognition strand complementary to mRNA target of interest, and the other called the flare strand, consists of a fluorophore⁵¹. In the absence of complimentary mRNA, the fluorophore remains quench by the AuNP due to close proximity. When mRNA is present, it binds to the recognition strand, displacing the flare strand, resulting in fluorescence turn-on⁵². This

technology was demonstrated by Yeo et al. for abnormal scar detection. In particular, CTGF-targeting Nanoflares were found to penetrate past the epidermis, into and distribute in the dermis, detecting hypertrophic scar fibroblasts in an ex vivo model of human skin⁵³. Furthermore, these AuNP can be modified with small interfering RNA duplexes to achieve RNAi-based therapy for personalized, topically delivered gene therapy of cutaneous tumors, skin inflammation, and genetic skin disorders⁹.

Figure 2.2. Common types of nanostructures. (Reprinted from APL Bioengineering, Vol 3, Chiara Martinelli et al., Nanostructured carriers as innovative tools for cancer diagnosis and therapy, Copyright (2019), with permission from APL Bioengineering).

2.3. Active Strategy

2.3.1. Electrical assisted systems

Mentioned in previous section, active strategy enables stronger disruption of stratum corneum, therefore allowing more effective transdermal drug delivery. In this section, electrical systems will be discussed, which include the commonly explored iontophoresis and electrophoresis (Figure 2.3). The enhanced TDD via iontophoresis is attributed to a combination of topical application of charged drug and application of low-level electric current ($0.5\text{mA}/\text{cm}^2$). The mechanism behind this technology is based on electromigration; when the current is applied, the charged drug migrates to the electrode of the opposite polarity, at a short distance away on the skin surface^{6, 54}. In addition, neutral molecules can penetrate the skin through convective flow as a result of electro-osmotic forces when the electric current is applied⁵⁵.

Iontophoresis is FDA approved to deliver lidocaine for local dermal analgesia and fentanyl HCL for acute postoperative pain. These two products are marketed by Endo Pharmaceuticals and Alza respectively². Other iontophoresis driven drugs include pilocarpine to induce sweat for the diagnostic workup for cystic fibrosis⁵⁶, and tap water to treat palmoplantar hyperhidrosis via accumulation of H^+ ions within the sweat duct⁵⁷. The

limitation of iontophoresis however, is the occurrence of tingling, itching, and skin irritation (erythema and odema) experienced during current application⁵⁸.

Electrophoresis on the other hand, uses pulses of high voltage and short duration to create transient aqueous pores in the lipid bilayer of cell membrane, facilitating uptake of various drug. In addition, the electric pulses rapidly decrease the resistance of stratum corneum to allow the penetration of drugs past the superficial skin barrier. The extent of drug delivery can be controlled by the amplitude, length and number of electric pulses, and the common ones include exponentially decaying pulses or square-wave pulses. Zhao et al. used electrophoresis (15 pulses at 1ms long and 1sec interval and 100V per skin passage) to demonstrate the delivery of a model peptide vaccine into the skin of mice to generate a strong cytotoxic T-lymphocyte response⁵⁹. In another study, Vanbever et al. demonstrated increased transdermal delivery of metoprolol through hairless rat skin via electrophoresis with an exponentially decaying pulse as compared to passive diffusion. In addition, they showed that pulse voltage and pulse time were contributory factors to enhance cumulative transport⁶⁰.

While electrophoresis has proved effective over past decades, there are still many concerns to be address. These include the safety of

transdermal electrophoresis e.g. long term alterations of skin structure and electrical properties following use of high voltage pulses⁶¹. Others include pain, increase in skin hydration, disorganization of SC layer, transient increase in the blood flow, and skin irritation⁶².

Figure 2. 3. Technologies which utilize active methods to achieve enhance skin penetration. (Reprinted from frontiers in Bioengineering and Biotechnology, Vol 6, Article 15, Sabine Szunerits et al., Heat: A Highly Efficient Skin Enhancer for Transdermal Drug Delivery, Copyright (2018), with permission from Frontiers).

2.3.2. Mechanical Systems

Mechanical systems tap on the use of physical means to breach or bypass the SC barrier. Famous examples of such method include microneedles (MN), needleless injection and microdermabrasion (Figure 2.3).

MN-based delivery involves an array of needles with ~400 needles of ~150 to 1000µm in length and ~50 to 80µm in diameter, used to pierce the upper skin layers non-invasively to create a physical pathway for molecules. There are three main categories of MN which include solid, hollow⁵, and water-soluble microneedles². Solid MN can be applied to create pores on the skin before a drug formulation is applied onto the region⁶³. In addition, these solid MN can be coated with a drug before delivering them into the skin⁶⁴. Hollow MN, on the other hand, deliver the drugs through the needle interiors via convective flow or pressure driven flow for a rapid outcome. It has been used to effectively deliver insulin to diabetic rats, achieving a 53% drop in glucose level and 0.43 ng/ml of insulin in the systemic circulation⁶⁵.

In recent years, many different polymers have been used to fabricate MN, including poly(vinyl alcohol) (PVA), poly(vinylpyrrolidone) (PVP), dextran, carboxymethyl cellulose (CMC), chondroitin sulfate and sugars. The use of water-soluble materials to fabricate water soluble MN enables low cost manufacturing and eliminates the potential risk of biohazardous sharp waste

to the user⁶⁶. Yu et al. developed a glucose-responsive insulin MN integrated with hypoxia-sensitive hyaluronic acid (HS-HA) vesicles containing insulin and GOx for stimuli triggered control release of insulin into the skin⁶⁷. While MN is the current state of the art technology, it still has some limitations which include insufficient needle penetration, breaking of delicate needles, and the limited amount of drugs loaded in the small MN patch^{5, 68}.

Another method to remove the stratum corneum is microdermabrasion which can be achieved through the use of sandpaper. Commonly used for cosmetic purpose, the abrasive mechanism has shown to improve skin permeability to drugs e.g. lidocaine and 5-fluorouracil². Furthermore, microdermabrasion also enable the delivery of vaccine where where study showed strong immune responses to several vaccines delivered topically in combination with enterotoxin of *Escherichia coli*⁶⁹. However, studies have reported the formation of micro-blisters, and topical creams or ointments should be used with caution because of the thinning of stratum corneum⁷⁰.

Needle-free injection, also commonly known as jet injectors have been used since 1990 to deliver medications⁷¹ such as local anesthesia, intralesional corticosteroids⁷², Intralesional bleomycin⁷³, and aminolevulinic acid⁷⁴. Despite its accomplishment, it is not widely accepted in routine

practices due to risk of infection, pain, injury of operator's finger, etc. Furthermore, technical difficulties such as clogging of the injector, splash and splatter are among other factors that discouraged healthcare professionals from using them.

2.3.3. Other physical systems

In addition to the mentioned technologies, a number of other physical approaches in TDD include ultrasound and laser radiation. Ultrasound is an oscillating pressure wave at a frequency above upper limit of human hearing capacity. It was first discovered to enhance skin permeation when ultrasound was used as a massaging tool to deliver anti-inflammatory drugs into the skin⁷⁵. The range of frequencies studied for TDD is in the range of 20kHz to 16MHz to deliver insulin, mannitol, glucose, morphine, lidocaine, etc⁷⁶. The enhancement is achieved through a combination of acoustic cavitation and thermal effects⁷⁷. Acoustic cavitation refers to the formation of small gaseous bubbles in the coupling medium which, upon their collapse by the acoustic pressure, frequency and bubble size, emits shock waves, thus disrupting the stratum corneum. This mechanism causes an increase in the pore size, leading to enhanced TDD⁷⁸. In addition, the absorption of ultrasound energy and scattering effects in the skin transform into thermal energy, which recent studies have shown to be a significant mechanism for increased flux of hydrophilic permeants⁷⁹. While ultrasound mediated TDD has proven to be effective, it has been limited by associated skin tissue heating which can damage the deeper tissue.

Lasers have been widely used for medical diagnosis and therapeutic purposes. The erbium:yttrium–aluminum–garnet (Er:YAG) laser (wavelength=2940 nm) has been studied to cause ablation of the stratum corneum with minimal damage to the skin⁸⁰. In addition, researches have shown that laser mediated TDD can enhance and control TDD, including nalbuphine, indomethacin, 5-fluor-ouracil, vitamin C, lidocaine⁸¹⁻⁸⁴. The enhancement is achieved by the skin barrier disruption, which extent can be controlled by parameters such as wavelength, pulse length, pulse energy, pulse number and pulse repetition frequency⁵. However, concerns over the use of lasers for TDD include risk of mutagenic effects, skin lesion (ulcers), and deep penetration of radiation may affect the collagen fibers⁸⁵.

In summary, both passive and active strategies play important roles in TDD. Nanotechnology has emerged as a promising passive platform for TDD due to its biocompatibility and different unique properties. Encouraged by works of Chad Mirkin et al, this thesis aims to further explore the use of AuNP as a transdermal carrier for early diagnosis of pathological angiogenesis. In addition, few studies have been performed to explore the role of mesoporous silica nanoparticles (MSNP) in gene delivery for TDD. Therefore, this thesis will study MSNP as a polymeric nanocarrier for potential gene delivery in skin disorders. Last but not least, amidst the many active strategies mentioned, almost all the technologies are accomplished

through the utilization of additional, specialized equipment alongside trained users to modulate skin permeability, thus presenting challenges in terms of patient convenience and home-use. Therefore, the final two chapters of this thesis will focus on the discovery of a technology termed Temporal Pressure. In addition to overcoming such drawbacks of bulky equipment and complexness of equipment usage, temporal pressure also promotes the feasibility of a one-step strategy for enhancing TDD.

Chapter 3. Molecular Beacon Gold

Nanosensors for Leucine-Rich Alpha-2-Glycoprotein-1 Detection in

Pathological Angiogenesis

Daniel Chin Shiuan Lio, Chenghao Liu, Christian Wiraja, Beiying Qiu, Chee Wai Fhu, Xiaomeng Wang, and Chenjie Xu., 2018. Molecular Beacon Gold Nanosensors for Leucine-Rich Alpha-2-Glycoprotein-1 Detection in Pathological Angiogenesis. ACS Sensors, 3(9), pp.1647-1655

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3.1. Introduction

Angiogenesis, a process of new blood vessel formation, contributes to the development and progression of several diseases including skin cancers, proliferative diabetic retinopathy, and chronic wound healing⁸⁶. This complex process is regulated through a balance of proangiogenic and antiangiogenic molecules, which is often derailed in diseases⁸⁷. Repetitive monitoring of angiogenic gene expression patterns in vivo would allow the dynamic tracking of biological changes during disease progression or upon treatment⁸⁸.

Although vascular endothelial growth factors (VEGFs) and their receptors play a key role in angiogenesis⁸⁹⁻⁹¹, other factors also contribute to angiogenesis through extensive crosstalk with each other⁹²⁻⁹⁴. For example, TGF- β 1 signalling has been reported to be involved in multiple steps during angiogenesis⁹⁵, and can promote or inhibit new blood vessel formation, which is highly dependent on factors, such as ligand concentration, its bioavailability, presence of other regulatory factors⁹⁶. Our team et al. have discovered Leucine-rich alpha-2- glycoprotein 1 (Lrg1), as a promising therapeutic target in the diagnosis and treatment of angiogenesis-related diseases⁹⁷⁻⁹⁸. It was found to mediate vessel growth through regulating the endothelial TGF- β 1 signalling. Recently, Sng et al. found that down-

regulation of Lrg1 plays a role in skin fibrosis⁹⁹. Therefore, monitoring the Lrg1 expression can be a promising platform for early detection of diseases.

The expression of Lrg1 in cells and animal models is currently examined through classic techniques such as reverse transcription polymerase chain reaction (RT-PCR), western blotting, and immunostaining^{97, 100-101}. While they provide extremely sensitive and definitive validation, it comes with a major drawback in the need to sacrifice multiple animals to obtain statistically significant observation, as in the case for any end-point analysis method. Therefore, it is always desirable to have a noninvasive and real-time imaging method for in vivo monitoring of Lrg1 expression, which could potentially be related to the activation of angiogenic switch, leading to pathological angiogenesis.

In recent years, gold nanoparticles received numerous attentions for its photodynamic properties and ease of surface modification¹⁰²⁻¹⁰³. These include photodynamic therapy¹⁰⁴, non-invasive radiofrequency ablation for cancer¹⁰⁵⁻¹⁰⁶, modulation of angiogenesis¹⁰⁷, ease of conjugating drugs¹⁰⁸ and nucleic acids¹⁰⁹ through gold-thiol bonds for delivery. In the field of non-invasive biomarker detection, oligonucleotides-functionalized gold nanomaterials such as Spherical Nucleic Acids (SNAs) have surfaced as a reliable technique for the detection of the abnormal expression of disease

biomarkers^{9, 53, 110-114}. These biocompatible complexes can easily enter living cells through scavenger receptor-mediated endocytosis despite its negatively charged surface¹¹⁵, which cannot be achieved by the pure oligonucleotide-based probes (e.g., molecular beacon (MB)). The oligonucleotides on the particles are resistant to nuclease degradation due to steric effect from their tight packing¹¹⁶ and more importantly, they can detect and quantify cellular gene in nanomolar sensitivity¹¹⁷⁻¹¹⁸. SNAs have been used to sort aggressive melanoma cells within heterogeneous cell lines via the detection of Nodal (a biomarker for tumor progression and localized in vascular networks)¹¹⁹. Such sorting can be achieved through the hybridization of designed oligonucleotide and complementary Nodal mRNA sequence, releasing fluorescence signals within the cell which are detectable through fluorescence imaging. To allow more precise detection of biomarkers, these nanoparticles could be multiplexed with target gene and control sequence to improve cell sorting and quantification^{51, 120-121}. In the field of transdermal drug delivery (TDD), SNAs demonstrated great cellular penetration of >100% in skin keratinocytes within hours, accompanying effective suppression of epidermal growth factor receptor (EGFR) with EGFR siRNA-conjugated SNAs. This led to a reduction of epidermal thickness by almost 40% in mice without detectable toxicity¹²². The use of gold nanoparticles to overcome skin barrier for drug delivery was also supported

by Francesca et al, where gold nanoparticles of size ~13nm were found to penetrate and present in all three layers (epidermis, dermis and subcutaneous layers) of the skin via TEM imaging¹²³. A computational modelling simulation revealed that the kinetics and thermodynamics of gold nanoparticles enabled the permeation of gold nanoparticles through the skin barrier¹²⁴.

Based on these pioneering works, we hypothesize that oligonucleotide-gold complexes would also allow the noninvasive and real-time monitoring of cellular Lrg1 expression for the diagnosis of pathological angiogenesis. As a proof-of concept, we built one model system by complexing MB with gold nanorod (Figure 3.1). MBs, single stranded hairpin shaped oligonucleotides, with only one fluorophore are specifically designed to recognize the exon sequences of Lrg1 mRNA. They are conjugated onto gold nanorods through thiol-gold bond^{8, 114}. The complexation brings the dyes in close proximity to the gold nanorods, resulting in their quenching. Gold nanorods exhibit intense and narrow optical absorption peaks in the far-red and near-infrared (NIR) wavelength regions, owing to the excitation of longitudinal plasmons. This permits the use of NIR dyes for deep tissue imaging. In addition, gold nanorods are recently discovered to be a good contrast agent for photoacoustic imaging¹²⁵. This provides the potential utilization of multimodality imaging in the future for more comprehensive

analysis. The synthesized complexes are approximately 68 nm in diameter with quenching efficiency greater than 90%. In this thesis, the specificity toward the Lrg1 mRNA and ability to penetrate the skin are examined with in vitro human skin keratinocytes.

Figure 3.1. Schematic diagram of MB-gold nanorod nanosensors for the noninvasive detection of the angiogenesis biomarker, Lrg1 in pathological angiogenesis. (Top left, clockwise): the nanosensors are designed as a potential diagnostic tool for pathological angiogenesis. In particular, abnormal blood vessel growth results from the high Lrg1 expression in the endothelium cells lining the blood vessels. Upon the cellular uptake of nanosensors, Lrg1 mRNA will bind to the complementary MB strands. The binding will distance the fluorophore from the gold nanorod, leading to the

restoration of fluorescence. Therefore, the cellular Lrg1 mRNA expression is revealed and quantified.

3.2. Materials and Methods

All experiments were done with nuclease-free water. Chemicals and solvents were purchased from Sigma-Aldrich unless specifically mentioned. The hydrodynamic diameter of nanocomplexes was analyzed with Zetasizer nano Z (Malvern). The morphology of nanocomplexes was examined with Transmission Emission Microscope (JEOL: JEM 1400). The UV-Vis absorbance was analyzed with Shimadzu UV 2450. Fluorescence was measured with Synergy 2 Multi-Mode Reader (BioTek).

MB Design and Preparation. MBs were designed using IDT's OligoAnalyzer Tool. An 18-atom hexa-ethylene glycol spacer (iSP18) was used to hold together the loop sequence and thiolated group (Table 3.1) while the short 20 base sequence complexed with the Lrg1 mRNA exon sequence, with an expect value of 0.017 (human) and 0.008 (mouse). Oxidized (disulfide) MB (Integrated DNA Technologies) was chemically reduced by Dithiothreitol (DTT) to form thiolated MB. Briefly, 8 μ L of 100 μ M MB was mixed with 3.2 μ L of 0.1 M DTT in 400 μ L RNase and DNase-free

water for 1 h at room temperature. DTT was then removed using NAP-5 Columns (GE Healthcare Life Sciences).

Mouse Scramble MBs for gold nanorod conjugation	ThioMC6-D-iSp18-GAGCGGCACTACCAGAGCTAACTCACGCTC-3Cy3Sp
Mouse Lrg1 MBs for gold nanorod conjugation	ThioMC6-D-iSp18-GAGCGAAGAGTGAGAGGTGGAAGAGCGCTC-3Cy3Sp
Mouse Lrg1 MBs with dark quencher	Cy3-GAGCGGAAAGCCCATCGTGTGTTCTCGCTC-3BHQ_2
Human Lrg1 MBs for gold nanorod conjugation	ThioMC6-D-iSp18-GAGCGGAAAGCCCATCGTGTGTTCTCGCTC-3Cy3Sp
Human Scramble MBs for gold nanorod conjugation	ThioMC6-D-iSp18-CCACACCGACAAGCGCACCGATATGACGTGTGG-3Cy3Sp
Lrg1 complementary short sequence	AGAACACACGATGGGCTTTC
eGFP complementary short sequence	GAGCAAAGACCCCAACGAGAA
Human Lrg1 Primer (For)	GATCGTGCCACTGCACTCTA
Human Lrg1 Primer (Rev)	GAAAGCCCCATCGTGTGTTCT
Human GAPDH Primer (For)	GGTCTCCTCTGACTTCAACA
Human GAPDH Primer (Rev)	AGCCAAATTGTTGTCATAC
siRNA target sequence 1	GCUGCAAUUAGAACGGCUA
siRNA target sequence 2	GAACACUGCAACCCGCUUA
siRNA target sequence 3	UGGAAAGCCUCUCGCCGA
siRNA target sequence 4	CAAACUGGGACAUGCGGGA
Non-targeting siRNA (ctrl siRNA)	UGGUUUACAUGUCGACUAA

Table 3.1. Nucleotide sequences used in in vitro and ex vivo experiments (each sequence starts from 5' and ends at 3')

Complexation of Gold Nanorods and MBs. Citrate-capped gold nanorods (25 nm, absorbance 550 nm, Sigma-Aldrich) were filtered with 0.2 μ m syringe filter to remove any aggregate. Later, 3 μ L 1% Tween 20 (Bio-Rad) was added to the 0.3 mL aqueous solution containing 13.5 μ g gold nanorods and the whole solution was vortexed for 10 min. The solution was added to reduced thiolated MBs (2 μ M) and left for 1 h at room temperature. Next, the solution was salt-aged with 5 M NaCl in water to achieve concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 M over a period of 3 h. After every addition of salt solution, the MB-gold nanorods solution was sonicated

for 10 s. Finally, excess MBs were removed through centrifugation at 15,000rpm at room temperature for 30 min. The final complexes were suspended in 100 μ L PBS with 0.01% Tween-20.

Cell Labeling with Lrg1MB-Gold Nanorod Nanosensors. HaCaT cells (both untreated and Lrg1 silence) were labeled with 1.8 μ g of Lrg1MB-gold nanorods in 1 mL Opti-MEM medium. As a control, cells were also labeled with scramble MB-gold nanorods. The cells were then left in the incubator for 4 h, washed once with PBS to remove the excessive scramble MB-gold nanorods, followed by fixation in formalin solution (Sigma) for 10 min before fluorescence imaging. RT3 cells (carcinoma squamous cells) were also labelled in the similar way to serve as a control.

RT-PCR: 0.12×10^6 cells were collected after silencing with Lrg1 siRNA, and lysed with RNAzol (Molecular Research Center, Inc.) Then, cDNA conversion was performed with qScript cDNA Supermix (Quanta Biosciences). Primer sequences for Lrg1 are listed in Table 3.1. Then, CT values for gene of interest were obtained through real time PCR (Quant Studio® 6 Flex Real-Time PCR System). Thereafter, $2^{-\Delta CT}$ calculation was used to evaluate Lrg1 fold change (normalized against cells which were not silenced with Lrg1 siRNA), while $2^{-\Delta CT}$ represents the ratio of Lrg1 to GAPDH mRNA, for any sample group.

Fluorescence quenching and MB conjugation efficiency

experiments: The fluorescence quenching efficiency of gold nanorods was examined by mixing 0.5 μM MB with a series of 45 $\mu\text{g/ml}$ gold nanorod solution (0, 10, 20, 40, 60, 80, 100, 200 μL). Next, to determine the maximum amount of MB conjugation onto gold nanorods, 100 μl of 45 $\mu\text{g/ml}$ gold nanorods was mixed with different MB concentration (0, 0.3, 0.5, 0.7, 1, 1.5, 2 μM). To determine the amount of unconjugated MB, mixture was centrifuged at 6,000 rpm at 4°C for 10 minutes, and supernatant was tested for Cy3 fluorescence intensity.

Specificity of Lrg1 MB-gold nanorod nanosensors: To test the specificity of Lrg1 MBgold nanorod nanosensors towards human Lrg1 mRNA, a short sequence based on Lrg1 mRNA was purchased from IDT (Table 3.1) to determine if hybridization can be observed. As a control, an eGFP target sequence (Table 3.1) was used as a scramble strand. Briefly, 0.25 μM of Lrg1 short sequence or eGFP sequence was added to 3 μL of the nanosensors in 300 μL PBS. The mixture was then placed in an incubator for 0 min, 30 mins, 1 hr, and 17 hrs before their fluorescence was measured with multiplate reader. All experiments were repeated for at least three times.

Cellular Lrg1 silencing: HaCaT cells were plated in 6-well plates (0.12 x 10⁶ cells per well) in high glucose Dulbecco's Modified Eagle Medium

(DMEM) with 10% fetal bovine serum (FBS), and 1% penicillin streptomycin (Lonza). After 24 hours, cells were washed once with Opti-MEM Reduced Serum medium (Gibco) and transfected with Lrg1 siRNA (Table 3.1; Dharmacon™) using Lipofectamine 2000 (Life Technologies). After 24 hours, cells were washed once with PBS and replaced with complete DMEM. Thereafter, cells were left in the incubator for 24 hours before any further experiment.

Fluorescence Activated Cell Sorting (FACS): Cells of interest were labeled with 1 drop of NucBlue Live ReadyProbes (Life Technologies) for 10 mins before they were trypsinized and centrifuged to remove excess staining reagents. The cells were finally re-suspended in 1ml of PBS for FACS using BD LSR II flow cytometer.

Confocal Imaging: Cells of interest were trypsinized, washed with PBS, and labeled with 4 µL/mL DiO Cell-Labeling Solution (10 mins) and 1 drop of NucBlue Live ReadyProbes (Life Technologies, 10mins) in PBS on ice. Thereafter, cells were centrifuged at 1000 rpm to remove the excessive staining reagents. Later, labeled cells were placed in the culture plates pre-coated with MSC Attachment Solution (GE Healthcare) for 10 mins before being fixed with formalin and viewed under Zeiss LSM 780 confocal microscope.

Cell Scratch Assay. 0.6×10^6 HaCaT cells were seeded onto one well of a 6-well culture plate. One day later, a 200 μ L pipet tip was used to create a cross scratch in the well. Cells were then washed with complete DMEM once and examined for proliferation rate by observing cell closure at 0, 12, 24, 30, 35 h. For Lrg1MB-gold nanorods labeling, cells were treated at 12 h with 1.8 μ g of nanosensors for 4 h before fluorescence imaging. For immunofluorescence staining, HaCaT cells were fixed with 4% paraformaldehyde and blocked with PBS containing 0.5% BSA, 1% Tween 20, and 1.5% Triton X-100 for 1 h before being incubated with primary antibodies against Lrg1 (Proteintech Group, USA, 13224-1-AP) overnight at room temperature and identified with Alexa Fluor 488 and DAPI.

In vivo delivery of Lrg1 MB-gold nanorods for non-invasive detection. All procedures were performed in accordance to IACUC (#140963) of Nanyang Technological University. Briefly, Lrg1 MB-gold nanorods were designed based on mouse mRNA sequence (Table 3.1). The synthesized nanosensors were mixed with Aquaphor® in a 1:1 wt/v % (10mg with 10 μ l) prior to topical application to the mice. The mice were shaved using [MOSER® CHROMINI PRO] and followed by [Veet® Hair Removal Cream Sensitive]. This is to ensure the hair does not trap the cream. The topical cream of nanosensors were applied on the dorsal skin and secured with Tegaderm™ and Coban™ for overnight. Next, the mice were imaged

with In Vivo Imaging System (IVIS). Thereafter, mice were euthanized by CO₂ asphyxiation, and the skin was excised and fixed in 10% formalin solution for histology studies.

Histological sectioning and immunostaining. For fluorescence microscopical observation of dextran molecules and NPs into the skin, the excised skin samples were cryosectioned to preserve the fluorescence. Briefly, skin tissues were embedded in Optimum Cutting Temperature (O.C.T.) (Sakura® Finetek) first. Then they were sectioned at 10µm using Leica CM3050 S Cryostat and stained with DAPI and Lrg1 antibodies under standard protocols.

3.3. Results and Discussion

Design, Synthesis, and Characterization of Lrg1MB– Gold Nanorod Complexes. Sequence similarity searching with Basic Local Alignment Search Tool (BLAST) showed that the 1233–1252 bp of mouse Lrg1 mRNA sequence and the 1514–1533 bp of human Lrg1 mRNA sequence are highly specific to human and mouse Lrg1 mRNAs respectively (SI Figure S1). To create a loop structure, stem sequences were introduced (Table S1), and MBs were stable as depicted by IDT Oligo Analyzer, with close ΔG values of -2.82 kcal/mol (human) and -2.81 kcal/mol (mouse).

Mus musculus leucine-rich alpha-2-glycoprotein 1 (Lrg1), mRNA	Homo sapiens leucine rich alpha-2-glycoprotein 1 (LRG1), mRNA
<p>ORIGIN</p> <pre> 1 ggtagggact atataaagcc accttctcgg ggtcttgagg acagacatag agggagcagct 61 atggtctcttt ggcagcatca aggaagcctc caggatctca agacctgctt tcccagaacc 121 ctgtttcttc tggcctcttt gggcaggggtg tccagcctca aggaatgctt gatactgag 181 tcggctgagg gttagcaggt ctctgcatc ggtccacacg agtttccgag ctctctccct 241 gccgacactg tccatctgtc ggtggaattc tccaactga cgcagctgcc cgcgcgcc 301 ctgcaagggt gtccgggtct ggggaagctg cactctcga gcaatcgct cgcagcgctg 361 tctccgagt tgcggcgcc cgtgccccgg ctgcgcgcc tggatctgac cgcgaatgct 421 ctccgagcc tgcctccgg gttgttcagc acctcgcca acctgagcac ctgtgtctg 481 agagagaacc agctgaggga ggtgaagcga caatggcttc aggacctgga gccctgggc 541 cacttgagcc tggcggagaa ccagctgagt tgcgtccct cgggctctct cgcagcctc 601 ggcgctctg acacctcga ccttgggtac aactctgtg agtctgctgc ggaaggcttc 661 ctgcggggcc caaggcggt gcagcgctg cactggaag ggaacgact gcnagagctg 721 gggatagcc tgcctggcc caacgcttc ctgcgctc tgttccgaa tgcacaacg 781 ttggctgggg tggcgccgg ctctctcag ggcctacgc acctggatat gttgatctg 841 tcaataact ctgtctcag cagcctccg ggcctgtgg ctttctctgg gaggcgacc 901 cgcgacatc aggaagcgtt cgacatctc cacaacccct ggaatctgta caagaacctg 961 gcgagacctt gccgctgct ggtcgcaac cgaacaaga tgttctaca gaacgacacg 1021 cgtctgctgg ggcggcagc catgaagggt cagcgctgc tggacgtggc agagctgggg 1081 tcccttgaga gatgatgct ggggtgctg caaaggcgc cccgctgcc actgagccaa 1141 tgggtccca tgtcagctg cagcttctc attctctcag ccaggaatgc tatctcgga 1201 ctccctctc aggtctctc cactgcac acctcttca cctctcactc ttctgtgctc 1261 ggcgccagg ccactgtg tttatctgc ttgacacgt ctgttcagg ggtcaccaa 1321 cgagttaata aacgctctc tagctgact gaaaaaaaaa aaaaaaaa aa </pre> <p>//</p>	<p>ORIGIN</p> <pre> 1 gcagagctac catgtctct tggagcagac agcagcaaaa aagccaggg ggcattcaac 61 ccatgtttc tagaactctg ttctgtgac tgcgtgtgc agctcagcc tggggggctc 121 cccgtagccc caaagactgc caggtgttcc gctcagacca tggcagctcc atctctgtc 181 aaccacttgc cgaatcccc ggtcacttgc cagcagcac cgtcagctg gccctggaat 241 tctcaacct gaccacctg ccagccacc tctccaggg cgtctctag ctcaagaat 301 tgaaccttc cagaatggg ctggaagc tctgtcaga attctctgg ctagtgcg 361 agctgaaggc gctgactca accgaaacg cctgacccg gctgccccg agctctcttc 421 agctctgc cacttgac acctggtat tgaagaana cagctggag gctctggag 481 tctgtggt acagcctg aaactctgg gcatctgga ctgtctgg aaagccttc 541 ggaacitgc cccgggctg ctggcaact tcaacctct ggcacccct gacttgggg 601 agaacctg ggaactct cactgac tctggagg tctgtgca tgaagaagg 661 tacatctaga agcaacaaa ttgcaatgc tgggaanaa tctctctg cgcagccg 721 acctgctga cttctctg aacgcaaa agctggcag ggtgagcc agtgccttc 781 agggctctg cagctgac atgtggacc tctcaataa tcaactggc agctgccc 841 agggctctg ggcattctc ggcagccaa actgggcat cggggatgc ttgcacatc 901 cggcaacc ctgggtgt gacagacc tgaagact ctatgttg ttraggctc 961 aaaaagaa atgttttcc cagatgca cgcgtgac taggctena gccctgaag 1021 gccagaact cctgagtg gccagctcc agtagacca gggcttgg ttgagggtg 1081 ggggtctgt agaacctg aaccgctta acaataatc ctgctttg cgggtctg 1141 gggctcagc ctgtaatcc agcatttg gggccagg tggcgatgc agaggtcag 1201 gagatgaa caacttgc taactgta aaactctgc ctctcaaa atataaaaa 1261 tggcagac gttgtggg gcaacttg tccagcac tggggatg gggcgagg 1321 aatggctga actgggagc cggagctgc ggtgagcaa gatctgca ctgactta 1381 gcttggga cagagcaga ctgtcaaa aaataaaa ttaaatata aaacaaata 1441 tctgtcttt tacagtgaa actgggct gtcataag gctggagcc agtttctc 1501 atcatgct ccttgaacagagcttgc tacc agtccagg tggctctc 1561 gttcgatgt cgtctctg ttccagat ctctgaat ctgatttc cagccctc 1621 gttctctt ccagctgc cttgcca ctaggagct ctttctga ctctgttc 1681 ccaagtcag gggctctc ggtgcagg tctatgct agtccact agtgggg 1741 cagaccaa ggtttaata aataacct gaaactga </pre> <p>//</p>

Figure 3.2. Human and Mouse Lrg1 mRNA sequences. Red box indicates exon sequences

Gold nanorods were selected because of their NIR absorption which facilitates deep tissue imaging. These unmodified gold nanorods had a longitudinal length of 52.6 ± 0.8 nm and a transverse width of 34.2 ± 1.0 nm (Figure 3.3). The most critical question during the complexation step through gold–sulfur bond was whether gold nanorods were able to effectively quench the fluorescence of the dye (i.e., Cy3) on the MBs. As shown in Figure 3.4A, the absorbance of gold nanorods overlaps well with the Cy3 fluorescence emission. To examine the actual quenching efficiency, a range of gold nanorods solutions were modified with the Lrg1MB solution at a fixed MB concentration. As shown in Figure 3.4B (black line), the fluorescence was strongest when the ratio of MB against gold nanorods was above 8000. However, a gradual increase of the gold nanorods in the synthesis (i.e., a

gradual decrease of the MB/gold nanorods ratio) led to a decrease of fluorescence, with the maximal quenching of 95% fluorescence intensity observed at 1500 MBs per gold nanorod. Next, we fixed the concentration of gold nanorods while tuning the MB concentration in the synthesis. As shown in Figure 3.4B (red line), there was negligible fluorescence when the MB/gold nanorod ratio was below 1500 until a spike increase in fluorescence intensity showed up when the ratio was 1500, suggesting that excess unbound MB was present in the mixture. Therefore, we conclude that the average number of MBs per gold nanorod should be around 1500 which is comparable to the previous report¹²⁶. One direct consequence of this MB coating is the increase of hydrodynamic diameters of the particles (Figure 3.4C). The size increased from 52 to 65 nm in dynamic light scattering (DLS). Energy dispersive X-ray (EDX) analysis showed the phosphorus peak surrounding the gold nanorods, indicating the presence of oligonucleotides (Figure 3.4D). One thing to note is that the longitudinal and transverse peaks were not differentiated in DLS, which was due to the low aspect ratio of gold nanorods used here. In summary, the quenching of MB fluorescence, increase of nanorod hydrodynamic diameter, and EDX revealed that MBs were successfully conjugated onto gold nanorods.

Figure 3.3. Transition electron microscope image of unmodified gold nanorods.

Figure 3.4. Characterization of Lrg1MB-gold nanorod complexes. (A) UV-vis absorbance spectrum of gold nanorods (AuNRs) and fluorescence emission of Cy3. (B) Quenching effect during the complexation of MBs and gold nanorods. (C) Hydrodynamic diameters of gold nanorods before and after the complexation with MBs. (D) EDX analysis of Lrg1MB-gold nanorod

complex. The presence of phosphorus (a component of DNA) is as indicated with the black arrow.

Next, we determined the specificity of the complexes toward Lrg1 complementary short sequence. We mixed either Lrg1MB-gold nanorods (stated as nanosensors) or scramble MB-gold nanorods (stated as mismatched nanosensors) with Lrg1 complementary short sequence or the scrambled sequence. As shown in Figure 3.5A, there was ~50% increase of fluorescence in 30 min only when the Lrg1MB-gold nanorods were mixed with Lrg1 complementary short sequence. It is expected to be the result of distancing the dyes from the gold nanorods upon the binding of Lrg1 complementary short sequence and MBs. No fluorescence restoration was observed when scrambled sequence was added instead. In addition, scramble MB-conjugated gold nanorods could be activated by neither Lrg1 complementary short sequence nor the scrambled sequence (Figure 3.5B).

The sensitivity of this probe was examined with a range of Lrg1 complementary short sequence concentrations, in which 0.25 μM Lrg1 sequence was sufficient to trigger a 40% increase of fluorescence within 30 min (Figure 3.6A). Furthermore, a study was performed to study the detection limit of these nanosensors. A linear relationship was observed between 0.01 and 0.25 μM (Figure 3.7). Last but not least, despite saturating scramble

MB-gold nanorods with 2 μ M Lrg1 complementary short sequence, no detectable change in fluorescence intensity was observed (Figure 3.5B).

Figure 3.5. Specificity of Lrg1MB-gold nanorod nanosensors against Lrg1 complementary strands. Fluorescence restoration of (A) Lrg1MB-gold nanorods and (B) scramble MB-gold nanorods before and after the introduction of Lrg1 short sequence over a period of 17 h. All values are mean $\pm$ SD, $n = 3$.

Figure 3.6. Fluorescence intensity recovery in the presence of different Lrg1 short sequence concentration with (A) Lrg1 MB-gold nanorods and (B) Scramble MB-gold nanorods, over a period of 0 min, 30mins, 1 hour, and 17hours. All values are mean $\pm$ SD, n=3.

Figure 3.7. Limit of detection for Lrg1 MB-gold nanorods. A range of concentration of Lrg1 short sequence was used to study the lowest possible

detectable amount. A linear relationship was observed at lower concentration (red box: between 0.01 and 0.25 μM). Therefore, the lowest possible detectable concentration falls between 0.01 and 0.05 μM . All values are mean $\pm$ SD, $n=6$, $*p<0.05$, $***p<0.0001$.

In Vitro Testing of Lrg1MB–Gold Nanorod Cytotoxicity and Uptake in Mammalian Cells. The cytotoxicity of MB–gold nanorod complexes was examined through MTS cell proliferation assay with HaCaT keratinocyte cells. The concentration of the complexes was tuned between 0.45 $\mu\text{g}/\text{mL}$ and 2.7 $\mu\text{g}/\text{mL}$. As shown in Figure 3.8, any concentration below 1.8 $\mu\text{g}/\text{mL}$ did not significantly affect the cell viability. Interestingly, the unmodified gold nanorods had negatively influenced the cell viability under the same concentration. This difference could be caused by the cytotoxicity of aggregated unmodified gold nanorods in cells¹²⁷⁻¹²⁸. In the following in vitro experiments, the MB–gold nanorods' concentration was controlled below 1.8 $\mu\text{g}/\text{mL}$. The cellular uptake was studied by incubating HaCaT cells with 1.8 $\mu\text{g}/\text{mL}$ complexes for 4 h. As shown in Figure 3.9A, Cy3 fluorescence showed up in the cytoplasmic region around the nuclei (blue). 40% cells were successfully labeled (positive for Lrg1) in this process (Figure 3.10). Meanwhile only 7.52% cells (a negligible subpopulation) were found to exhibit Cy3 fluorescence, when labeled with scramble MB– gold nanorods. To confirm the location of the nanosensors, we labeled the cell membrane

with lipophilic fluorescent dye (DiO). As shown in Figure 3.9B, red signal from nanosensors was mainly present within the green membrane boundary. It is also notable to mention that red signal was negligibly found in the cell nucleus.

Figure 3.8. Cytotoxicity of Lrg1 MB-gold nanorods. All values are mean $\pm$ SD, n=3, *p<0.05

Figure 3.9. Cellular localization of Lrg1MB–gold nanorods. (A) Fluorescence images of HaCaT cells labeled with Lrg1MB–gold nanorods and DAPI. (B) Confocal images of a HaCaT cell. Red (Lrg1MB–gold nanorods), blue (nucleus), green (plasma membrane), and merged image (red + green + blue). (Scale bar represents 10 μm .)

Figure 3.10. Fluorescence-activated cell sorting of HaCaT labelled with Lrg1 MB-gold nanorods and scramble MB-gold nanorods

The cytotoxicity test confirmed the safety of the MB–gold nanorods while the cellular uptake experiments revealed their capability of entering cell cytoplasm through endocytosis and thereafter residing in cell cytoplasm for mRNA detection. Next, we evaluated the functionality of the Lrg1MB–gold nanorods in detecting the Lrg1 mRNA expression in cells.

Cells were split into three treatment groups. The first group were treated with Lrg1 MB-gold nanorods directly. The second group were treated with Lrg1 siRNA to silence Lrg1 gene before being labeled with Lrg1 MB-gold nanorods. The third group were treated with scramble MB-gold nanorods. After 4 h, fluorescence signal in group 2 was ~50% lower than group 1 (Figure 3.11A and B). Similarly, fluorescence signal in group 3 was ~75% lower than group 1. To confirm this observation, the cellular Lrg1 mRNA in three groups was quantified through RT-PCR (Figure 3.11C). Lrg1 mRNA expression was found to be ~80% lower in Lrg1 silenced cells when being compared to the untreated cells. Thus, Lrg1 mRNA expression was well-represented by Lrg1MB-gold nanorod signal.

Figure 3.11. Lrg1 MB-gold nanorods monitor gene regulation in keratinocytes. (A) Fluorescence images of normal and Lrg1-silenced HaCaT cells labeled with Lrg1MB-gold nanorods, and cells labeled with scramble MB-gold nanorods. (B) Fluorescence signal quantification from (A) using ImageJ analysis. (C) RT-PCR analysis of Lrg1 gene in untreated, Lrg1 silenced, and control silenced HaCaT cells, normalized against Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH) gene. All values are mean $\pm$ SD, $N \geq 3$. Scale bar represent 100 μ m. * $p < 0.05$.

Besides the differentiation of Lrg1 gene expression in HaCaT cells under normal and Lrg1-silenced conditions, Lrg1MB-gold nanorods were also able to differentiate Lrg1 gene expression across different cell lines. For example, RT3, a malignant keratinocyte cells have lower Lrg1 expression as compared to normal cells (HaCaT). Under the same labelling condition, higher fluorescence signal was observed in HaCaT cells (Figure 3.12A) and almost 9 times of that in RT3 cells (Figure 3.12B). RT-PCR analysis revealed the Lrg1 gene expression in RT3 cells was only 20% of that in HaCaT cells (Figure 3.12C). Therefore, our nanosensors allowed the differentiation of the normal keratinocytes (HaCaT cells), HaCaT cells silenced with Lrg1 siRNA, and RT3 (Figures 3.11 and Figure 3.12).

Figure 3.12. Lrg1 detection using nanoparticles in HaCaT vs RT3. (A) Representative fluorescence images of HaCaT cells and RT3 cells labelled with Lrg1 MB gold nanorods. (B) Fluorescence signal quantification from (A) using ImageJ analysis. (C) RT-PCR analysis of Lrg1 gene in HaCaT cells and RT3 cells, normalized against GAPDH. All values are mean $\pm$ SD, $N \geq 3$. Scale bar represent 100 μ m unless otherwise stated.

Monitoring Lrg1 mRNA Expression in the in Vitro Wound Healing Model with Lrg1 MB-Gold Nanorods. Cell scratch assay is a convenient in

vitro wound healing model. Here, Lrg1 MB-gold nanorods were used to examine the dynamic expression of Lrg1 in HaCaT cells in the healing model. To ensure the consistent imaging of the same area, scratch areas were marked with a scratch using a blade. As shown in Figure 3.13A, the initial scratched area at 0 h was displayed in light blue. Cell migration could be observed within 12 h and an almost complete closure was observed by 30 h. We labeled the cells with Lrg1 MB-gold nanorods for 12 h after inducing the scratch. To note, the presence of MB-gold nanorods did not inhibit cell migration and closure. As shown in Figure 3.13B, ~50% higher fluorescence could be observed in cells at the scratched area as compared to cells in unscratched area (Figure 3.14A). This is indicative of Lrg1 upregulation when keratinocytes underwent migration during wound healing process^{100, 129}. To confirm this observation, immunostaining of Lrg1 protein was performed on the cells (Figure 3.13C). A 2-fold increase in fluorescence was observed for HaCaT cells at the scratched area as compared to HaCaT cells at the unscratched area (Figure 3.14B).

Figure 3.13. Lrg1 upregulation in migrating HaCaT cells in the wound healing model. (A) The in vitro wound healing model (scratch assay); blue indicates the area without cells. (B) Fluorescence images of HaCaT cells at the scratched or unscratched areas after being labeled with Lrg1MB- gold nanorods (red). (C) Immunostaining of Lrg 1 proteins in HaCaT cells at the scratched or unscratched areas. Red (Lrg1MB-gold nanorods), blue (nucleus), green (immunostaining of Lrg 1 proteins). (Scale bar represents 100 μm .)

Figure 3.14. Quantification of fluorescence signal in cell scratch assay mode shows ~50% higher fluorescence signal for scratched HaCaT cells when labeled with Lrg1 MB-gold nanorods (A), and a 2-fold increase in fluorescence signal was observed for scratched HaCaT cells when labeled for Lrg1 using immunocytochemistry (B). All values are mean $\pm$ SD, $n \geq 3$.
* $p < 0.05$

In a separate in vivo experiment, this Lrg1MB-gold nanorod sensor demonstrated the ability to label and detect the keratinocyte cells in the skin epidermis layer of the mouse (Figure 3.15). This is on par with our in vitro findings (Figures 3.9 and 3.11), demonstrating high expression of Lrg1 mRNA in HaCaT cells using Lrg1-targeted nanosensors. On the other hand, skin applied with scramble MB-gold nanorods showed minimal signal in the epidermal layer.

Figure 3.15. IVIS imaging and histological analysis of the mice that were topically applied with Lrg1-MB gold nanorods and scramble MB-gold nanorods. (A) IVIS imaging; (B) Quantification of fluorescence signal in A; (C) Immunohistological chemistry of mouse skin for Lrg1 protein; (D) Immunofluorescence staining of the mice that were topically applied with Lrg1-MB gold nanorods and scramble MB-gold nanorods.

These studies confirm the ability of Lrg1MB-gold nanorods to transfect in vitro human skin keratinocytes and in vivo mice skin efficiently, with noninvasive monitoring of Lrg1 gene expression. In this thesis, the cellular uptake of ~65nm Lrg1 MB-AuNR was confirmed with fluorescence,

confocal microscopy, and flow cytometry (Fig 3.9 & 3.10). *Both clathrin- and caveolae- mediated endocytosis in particular, may be responsible for internalization into cells*¹³⁰⁻¹³¹. While the nanosensor showed great potentials in detecting Lrg1 mRNA in various models, there are questions left to answer.

First, the nanosensor's sensitivity remains a challenge. While the overlap of the gold nanorods' absorption spectrum and Cy3 fluorophore emission coincides (Figure 3.4A), slight background fluorescence remains. Distinct gold nanoparticle morphology could be adapted for improved quenching. And it is also possible to shorten the distance between gold nanorod and MBs, leading to stronger fluorescence quenching. Second, it is important to study the impact of cell culture media on the stability of Lrg1 MB-AuNR versus unmodified AuNR, by measuring the size of NPs with DLS to determine the degree of aggregation, and whether the addition of Lrg1 MB does improve nanoparticle stability. Third, it is of importance to study gold nanorod uptake in the different cell lines. Possible studies in the future include silver staining to quantify the MB-gold nanorods uptake¹³². Third, it remains a question whether the developed nanosensor's detection limit is able to achieve an early diagnosis of pathological angiogenesis. In the future, in vitro/in vivo studies can be performed to study the Lrg1 copy number in individual cells (both healthy and pathological state) and correlate it to the nanosensor's detection limit. Last but not least, it is worth studying if the Lrg1

MB-gold nanorods can penetrate further into the skin of the mouse. In this thesis, the penetration of these nanosensors are only limited to the epidermis as most Lrg1 expression are found in the epidermis layer. Biomarkers which are specifically found in the dermis layer, e.g. Collagen Type I (found in dermal fibroblasts) can be used as a model biomarker to study if the nanosensors can penetrate into the dermis layer.

3.4. Conclusion

This article reports the design, synthesis, characterization, and utilization of the Lrg1 mRNA nanosensors for the noninvasive, real-time monitoring, and verification of angiogenic biomarker Lrg1. This sensor is constructed by conjugating MBs onto the gold nanorods, which prequenches the MB fluorescence. As target mRNA hybridizes with the sensor, the straightening of MB structure separates the fluorophore from the gold nanorod and restores its fluorescence. The nanosensors were tested with target sequences, skin keratinocytes under various conditions, and in vivo mouse skin. In all circumstances, it showed consistent performance in revealing the dynamic changes of Lrg1 expression without the need of cell/tissue disruption. We believe this nanosensor will pave the way to examine and monitor the development of angiogenesis in a real time manner.

3.5. Acknowledgement

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Chapter 4. Transdermal delivery of small interfering RNAs with topically- applied mesoporous silica nanoparticles for facile skin cancer treatment

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4.1. Introduction

Skin and subcutaneous disorders were the 4th leading cause of nonfatal disease burden worldwide, revealed by the Global Burden of Disease (GBD) 2013 Study. Diagnosis and treatment of skin conditions represent a significant healthcare burden¹³³. Advances in molecular genetics have allowed us to identify pathogenic mutations for the diagnosis or classification of skin disorders like psoriasis, atopic dermatitis and skin cancer¹³⁴. It also permits the development of targeted and personalized therapeutics like RNA interference (RNAi) therapies.

RNAi therapies utilize small RNA such as small interfering RNAs (siRNAs) to interfere with the expression of specific genes related with the disorders.^{135,136} The first-ever siRNA product was approved in 2018 by FDA for the treatment of polyneuropathy in people with hereditary transthyretin-mediated amyloidosis¹³⁷, marking a significant milestone in the history of RNAi technology and paving the way for a new type of therapeutic. The first-in-human siRNA therapy for skin disease was done through intradermal injection for pachyonychia congenita, an inherited skin disorder. Encouraging results were reported, but the study also revealed the technical hurdles to overcome the skin's barrier in a patient-friendly and clinically-relevant manner.

Naked siRNA is a large size molecular with molecular weight ≈ 13.5 kDa and is negatively charged. These properties prevent them to bypass stratum corneum and stably cross cell membrane. Thus, a wide range of topical delivery strategies are under development for the efficient delivery of siRNA into the skin. These include dendrimer¹³⁸, nanoparticles⁹⁻¹⁰, iontophoresis¹³⁹, microneedles¹³⁵, “self-delivery” siRNAs¹⁴⁰ etc. Regardless of the strategies, the goal is the same, i.e. to protect and transport these siRNA to the site of action in the cells of target tissues. The field of skin topical application is currently dominated by lipid and dendrimer-based particles. However, these nanoparticles often face problem of low drug loading, burst release and stability issues¹⁴¹.

Mesoporous silica nanoparticles (MSNP) are a powerful general tool for packaging fragile pharmaceuticals and ensuring their safe passage through cell membranes, and eventually releasing their cargo at the point of destination. Attractive features of these nanoparticles include extremely large surface areas associated with large pore volumes leading to improved loading efficiencies¹⁴², long-term stability¹⁴³, and great flexibility regarding the introduction of molecular functionalities in the pores and on the external particle surface¹¹. Researchers have demonstrated that the regulation of pore structure, pore diameter, and particle size of MSNPs can lead to sustain drug release profile. Qu et al demonstrated that drug release behavior was

proportional to the pore channel length¹⁴⁴. Particle sizes ranging from 100 to 300 nm are deemed optimal for cellular uptake by endocytosis. Additionally, the internal particle surface can be adapted by implementing basic, acidic or hydrophobic residues that are compatible with the cargo of choice¹⁴⁵. MSNPs received vast attention as a drug delivery system. These include conjugating ligands for selective targeting in localized therapy¹⁴⁶⁻¹⁴⁷, and controlled dosage and smart behavior where MSNPs respond to internal stimuli to do away with the need for external device to trigger cargo release, e.g. pH¹⁴⁸, redox potential¹⁴⁹, and enzyme¹⁵⁰.

Tapping on these properties of silica NPs, several companies were initiated with the aim of bringing silica NPs as drug delivery tools through the clinical trials. These include (1) AuroLase, PEG-coated silica-gold nanoshells for NIR facilitated thermal ablation in lung tumors¹⁵¹⁻¹⁵² (NCT01679470); (2) Cornell Dots, silica NPs with NIR fluorophore, radiolabeled cRGDY targeting peptide and PEG coating for imaging of brain tumors¹⁵³⁻¹⁵⁴ (NCT01266096). Studies have been performed to utilize MSNPs in transdermal drug delivery, including the delivery of methotrexate to improve epidermal accumulation for psoriasis treatment¹⁵⁵, the insulin delivery in combination with microneedles¹⁵⁶, and the lidocaine delivery¹⁵⁷. Excited by these pioneering works, this project is to explore the potential role of MSNPs in the transdermal delivery of siRNA. The successful implementation would

enable high siRNA loading in MSNPs, controlled release of the siRNA, and good stability of MSNPs to prevent enzymatic degradation in the cells.

Taking the molecular beacon (MB) as the model drugs, we first optimized the preparation procedure of the MSNP-based formulation. Later cell experiments were employed to study the functionality of the formulation including the cellular uptake, the qualitative and quantitative detection of specific gene biomarkers. Finally, this system was used to topically deliver siRNA targeting TGF β R-1 (TGF β R-1) to the squamous cell carcinoma in a mouse xenograft model. In comparison to the PBS-treated control or MSNPs containing scrambled siRNA, MSNPs containing TGF β R-1 siRNA showed ~2-fold suppression of TGF β R-1 expression and corresponding ~2.5-fold suppression of tumor growth through the 18-day study.

4.2. Methods and Materials

All materials unless otherwise stated were purchased from Sigma-Aldrich. Hoechst, Calcein AM, and Propidium Iodide were purchased from Life Technologies. AlamarBlue reagent was purchased from Thermo Fisher Scientific. Dynamic Light Scattering (DLS) and zeta potential measurement were done on Malvern Nano-ZS. SEM was performed using JEOL JSM

6701F SEM. Fluorescence imaging was performed using Olympus LX71 inverted fluorescence microscope with an Infinity3-1 CCD camera (10x magnification), and confocal imaging was performed using Zeiss LSM 510 Confocal Microscope.

Synthesis of MSNP-oligonucleotide complexes: 200 nm MSNPs with 4 nm pore size (Sigma-Aldrich) were mixed with oligonucleotides in PBS and left to stir overnight. Thereafter, MSNP-oligonucleotide complexes were coated with PLL in a 1:1 ratio for 10 minutes. The excess PLL were removed by centrifuging the MSNPs-MB-PLL at 10,000rpm centrifugation for 15 mins, removal of excess PLL and resuspend in PBS.

Labeling cells with MSNPs for time course study and post treatment with Repsox: RT3 cells were seeded at a density of 0.25 million cells per well in a 6-well plate. The cells were treated with MSNPs-MB-PLL with a final mass of 20 μ g of MSNPs-MB-PLL. This treatment was performed with Optimem® media for overnight. Next, the excess NPs were washed off with PBS, and imaged using fluorescence microscopy to study the MSNPs-MB-PLL retention in the cells for 12, 36, and 108hrs. To the specificity and functionality of these MBs when released in the cell, RT3 cells were treated with a range of RepSox concentrations – 1 μ M, 5 μ M, 10 μ M, 25 μ M and 50 μ M, prepared using Opti-MEM. Cells were treated with 1ml of specified RepSox

concentration overnight, followed by labelling these cells with MSNPs-MB-PLL. The CTGF gene expression of these RT3 cells post Repsox treatment was quantified using RT-PCR, and compared to the fluorescence intensity by MSNPs-MB-PLL.

In vivo studies with xenograft tumor model: All animal studies were performed in compliance with the guidelines set by Institutional Animal Care and Use Committee (IACUC, Reference Number: A0391), NTU. SCID mice at the age of 6 weeks were subcutaneously injected with 2 million RT3 cells at dorsal site of the mice. 10 days later, the size of tumor was approximately 0.29cm³. Next, mice were shaved using [MOSER® CHROMINI PRO] and followed by [Veet® Hair Removal Cream Sensitive]. This is to ensure the hair does not trap the cream and for easy application of NPs and IVIS imaging. To study the penetration of MSNPs, Cy5 was loaded by mixing them overnight. Thereafter, the MSNPs were coated with PLL for 10 mins, followed by purification. A final amount of 0.03 mg of MSNPs-Cy5-PLL were mixed with Aquaphor in a 1:1 w/v ratio before applying onto the skin of the mice. Aquaphor® Healing Ointment is a commercial formulation for dry and cracked skin. It contains 41% Petrolatum, Panthenol and Glycerin to moisturize, nourish and protect the skin. This procedure was performed every 3 days (day 1, 4, 7, 11, 15, 18) for a period of 18 days. Thereafter, the mice were imaged under IVIS and the skin was harvested for histology study.

For the topical application of MSNPs-siRNA-PLL, preparation procedures is similar to that of Cy5, except TGF β R-1 siRNA was loaded. A final amount of 0.03 mg of MSNPs were mixed with Aquaphor in a 1:1 w/v ratio before applying onto the skin of the mice. The mice were repeatedly treated with this topical formulation every 3 days (day 1, 4, 7, 11, 15, 18). The tumor volume was monitored across the treatment period¹⁵⁸. Finally, the tumors were excised and processed for protein analysis using western blot.

Histological sectioning: For fluorescence microscopical observation of MSNPs-PLL w Cy5 into the skin, the excised skin samples were cryosectioned to preserve the fluorescence. Briefly, skin tissues were embedded in Optimum Cutting Temperature (O.C.T.) (Sakura® Finetek) first. Then they were sectioned at 10 μ m using Leica CM3050 S Cryostat and stained with DAPI under standard protocols.

Flow Cytometry: Cells were trypsinized and fixed with 4% Paraformaldehyde. After washing with 1 ml of PBS, cells were analyzed with Fortessa X20.

Western Blot: Total protein was extracted using RIPA buffer (0.0037mg/mL protease inhibitor (Roche), 1mM DTT (Sigma-Aldrich), 1mM PMSF (Sigma-Aldrich), and 100mM phosphatase inhibitors (Sigma-Aldrich). 200ug of protein from whole tumor tissues were then diluted in Laemmli 4X

buffer and heated at 100°C for 5 minutes before being loaded into 10% SDS-PAGE gels, and then transferred Immobilon-PSQ Membrane, PVDF (Merck Millipore). Blots were probed with TGFβR-1 antibody (Cell Signalling Technology) or GAPDH antibody (Santa Cruz Biotechnology).

RT-PCR: RNAzol®RT (Molecular Research Center, USA) was used for mRNA isolation and purification according to the manufacturer's instructions. RNA concentrations, 260/280 ratio and 260/230 ratio were determined using Nanodrop 2000C Spectrophotometer (Thermo Fisher Scientific, USA). 800-1000ng of RNA was reverse transcribed to cDNA using qScript cDNA Supermix (Quanta Biosciences, USA) according to manufacturer's instructions. PCR master mix containing relevant primers, nuclease free water and iTaq™ Universal Synergy Brands (SYBR®) Green Supermix (Bio-Rad, USA) was prepared. qRT-PCR was performed using Applied Biosystems StepOnePlus™ Real Time PCR System (Life Technologies, USA).

Primer sequences: GAPDH: Forward primer: 5'-AGGTCGGTGTGAACGGATTTG-3'
Reverse primer: 5'-TGTAGACCATGTAGTTGAGGTCA-3'; TGFβR-1:
Forward primer: 5'-TCAGCTCTGGTTGGTGTCTAG-3', Reverse primer: 5'-ATGTGA AGATGGGCAAGACC-3'; CTGF; Forward primer: 5'-

TTAAGAAGGGCAAAAAGTGC-3', Reverse primer: 5'-
CATACTCCACAGAATTTAGCTC-3'

Statistical Analysis: All experiments were repeated three times. To determine statistical differences, one-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference (HSD) post hoc test. Statistical significance was evident in cases of p-values corresponding to F-statistic of one-way ANOVA being less than 0.05 (*p < 0.05) or less than 0.01 (**p < 0.01).

4.3. Results

Synthesis of MSNP-oligonucleotide complexes

The MSNP-oligonucleotide complexes were built on MSNPs with 4 nm pore size. Oligonucleotides were loaded into the pores of MSNPs through simple incubation. The resulted complexes were further coated with a layer of poly-L-lysine (PLL) to facilitate the skin penetration (Figure 4.1A).

Taking molecular beacon (MB, single-stranded oligonucleotides that contain a hairpin structure) as the model oligonucleotide, we prepared the MSNPs-MB complex. The zeta potentials of MSNPs were -19 mV and -34 mV before and after complexation (Fig 4.2A). The further coating of PLL dramatically increased the zeta potential to +30 mV. The blank MSNPs had a diameter of ~200 nm (Fig 4.2B). The complexation with MBs didn't change the size significantly (~202nm). However, the coating of PLL increased the size to ~250nm.

The loading efficiency (wt %) of MBs was 1.42% in the final MSNPs-MB-PLL complexes (approximately 1.4 μg MB per mg of MSNPs-MB-PLL). The release profile of MB from the complexes was done in PBS for 48 hours at 37°C. The released MB was quantified through measuring the fluorescence of solution after the complexation with the complementary oligonucleotide strand. 65% of MBs were released from MSNPs-MB within

the first 6 hours and the rest were freed in the next 48 hours. Similar release profile was observed for MSNPs-MB-PLL (Figure 4.2C). Imaging with scanning electron microscope (SEM) revealed the rough surface of MSNPs, indicating its mesoporous structure¹⁵⁹ (Figure 4.1B - 4.1D). The loading of MBs and the coating of PLL didn't seem to change the morphology of the particles.

Figure 4.1. Preparation of MSNP-oligonucleotide complexes: (A) Illustration of the synthesis of MSNPs, MSNPs-MB, and MSNPs-MB-PLL. Scanning electron microscopy images of (B) MSNPs, (C) MSNPs-MB and (D) MSNPs-MB-PLL. Scale bar: 100 nm.

Figure 4.2. (A) Characterization of MSNPs derived formulations: (A) Zeta potential and (B) diameter of MSNPs, MSNPs-MB, MSNPs-MB-PLL (n =20). (C) Release profile of MBs from MSNPs, MSNPs-MB and MSNPs-MB-PLL in a period of 48 hours.

Cellular delivery of oligonucleotide with MSNPs

Next was to examine the delivery efficacy of oligonucleotides with MSNPs. Taking MBs targeting Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the model, we used MSNP-MB-PLL to label human squamous carcinoma cell line (RT3). Successful internalization of MSNP-MB-PLL and intracellular delivery of MB is proven through confocal imaging, following staining with Hoechst (nuclear stain; blue) and calcein AM (marking cell boundary; green) (Figure 4.6). Following which, we performed a longitudinal study to evaluate MB expression following MSNP-MB-PLL delivery. After 12-hours incubation, the intensity of cellular fluorescence in MSNPs-MB-PLL labeled cells was 1.7 times of that in MSNPs-MB labeled cells (Figure 4.3B).

This was attributed to the positive charge of MSNPs-MB-PLL which heightened the NP internalization into the cells¹⁶⁰. We also delivered MSNPs-PLL containing MBs for Green Fluorescent Protein (GFP) as a negative control. Since RT3 cells here didn't present GFP, no fluorescence was observed (Figure 4.4). At 36 hours, the fluorescence intensity from MSNPs-MB-PLL in RT3 cells dropped to 33% of that at 12 hours (Figure 4.3B). At the same time, the cellular fluorescence in MB labeled cells dropped to negligible value. 108 hours later, the fluorescence in MSNPs-MB-PLL and MSNPs-MB groups of cells continued to drop but the fluorescence difference in two groups kept at ~2 times. Minimal cytotoxicity of MSNPs was observed through the experiment (Figure 4.5).

Next, fluorescence-activated cell sorting (FACS) was carried to verify the observations in cell imaging experiment (Figure 4.3C). 97.9% of cells were fluorescent when they were labelled with MSNPs-MB-PLL. The numbers of fluorescent cells were 67.7% and 36.3% respectively when cells were treated with MSNPs-MB and MBs.

A confocal imaging was further performed to reveal the location of MSNPs-MB-PLL in the cell. RT3 cells were stained with DAPI (blue) for nucleus and calcein-AM (green) for marking cell boundary. Fluorescence signal matching the presence of MBs (pink) was observed throughout the cytosol of the cell except the nucleus. Last but not least, the combined z-

stack images showed that the Si-PLL NPs definitely reside in the cell (Figure 4.6).

Figure 4.3. Cellular delivery of oligonucleotide with MSNPs: (A) Representative fluorescence image of RT3 cells treated with MSNPs-MB-PLL, MSNPs-MB, and MB for a period of 120 hours. (B) Comparison of cellular fluorescence intensity in A. (C) Measurement of cellular fluorescence using flow cytometry. Scale bar: 100 μ m. n=3, all data are mean $\pm$ SD.

Figure 4.4. Representative fluorescent images of RT3 cells treated with MSNPs-GFP MB-PLL. Scale bar: 100 μ m.

Figure 4.5. Biocompatibility of MSNP-MB and MSNP-MB-PLL: (A) Representative fluorescence images of live/dead staining assay and (B) Alamar blue assay of RT3 cells treated with MSNPs-MB and MSNPs-MB-PLL 24 hours post treatment. Scale bar: 100 μ m. $n=3$, all data are mean $\pm$ SD.

Figure 4.6. Confocal Z-stacked image of RT3 cell treated with MSNPs-MB-PLL. Scale bar: 100 μm

Monitoring the dynamic change of cellular mRNA with MSNP delivered MBs

It is important to know whether MBs delivered with MSNPs were functional. Therefore, we manipulated the CTGF expression in RT3 cells with inhibitor (Repsox) before labeling the cells with MSNP-MB-PLL particles.¹⁶¹ As shown in Figures 4.7A & 4.7B, cells treated with higher concentrations of Repsox had shown less fluorescence from MB. This was confirmed through RT-PCR (coefficient of determination, $R^2 = 0.8659$) (Figure 4.7C).

Figure 4.7. Regulation of cellular mRNA with MSNP-oligonucleotide complexes: (A) Representative fluorescence images of RT3 cells labelled with MSNP-MB targeting CTGF mRNA and Hoechst. Scale bar, 100 μ m. (B) Quantification and normalization of the CTGF MB signal against the control. (C) Correlation between the normalized CTGF MB signal and normalized RT-PCR results.

Biodistribution of MSNP-PLL via topical delivery

A squamous cell carcinoma mouse xenograft model was created by injecting RT3 cells into the subcutaneous layer of the severe combined immunodeficient (SCID) mice. Initially, we studied the biodistribution of MSNP-PLL following topical application, through incorporation of Cy5 (MSNP-Cy5-PLL). As control, intratumor and intravenous injection was also performed. Topical application of MSNP-Cy5-PLL administrations (0.03mg dosage mixed with Aquaphor® in 1:1 wt/v ratio) were applied on the dorsal skin of the mice and left for 3 days. This procedure was repeated every 3 days for a period of 18 days (Figure 4.8A).

Once every week, mice were imaged with IVIS (Figure 4.9). As shown in Figure 4.8B & 4.8C, at the end of 18 days, the highest fluorescence was observed on mice with intratumor injection, followed by those with topical application. In addition, the tumor and the major organs were homogenized in PBS and the fluorescence was quantified using a fluorescence plate reader. The tumor of mice treated with intratumor injection showed the highest fluorescence followed by those with topical application of nanoparticles (Figure 4.8D). When nanoparticles were administered through topical application, their distribution in all major organs was observed to be low as compared to intratumor and intravenous injection.

The skin penetration of topically applied nanoparticles was revealed via imaging the histological samples of the skin and the tumor (Figure 4.8E). No fluorescence from nanoparticles was observed in the skin samples from mice treated with intratumor and intravenous injection, while significant amount of fluorescence was seen from the skin samples from mice treated with topical application of MSNP-PLL. Strong fluorescence was observed in the tumor from mice treated with both intratumor and intravenous injection. Unfortunately, there was little fluorescence from both skin and tumor samples treated with intravenous injection.

The further quantification of fluorescence profile (Figure 4.8F) confirms that the topical application allowed the nanoparticles to penetrate from skin epithelial layer to tumor tissue with consistent fluorescence distribution through the skin layers. The intratumor injection directly got the nanoparticles into the tumor with little signal from skin layers. The intravenous injection didn't provide similar nanoparticle distribution in the tumor as both topical application and intratumor injection, suggesting the nonspecific distribution of nanoparticles via intravenous injection.

Figure 4.8. Biodistribution of MSNP-PLL *in vivo* administered through three methods: (A) Schematic illustration of *in vivo* studies where SCID mice were inoculated with RT3 cells as a squamous cell carcinoma mice xenograft model. (B) Representative IVIS images of mice treated with MSNPs-PLL w Cy5 every 3 days for a total of 18 days. (C) Quantification of fluorescence in IVIS images in B. (D) Fluorescence intensity of isolated tumor and other major organs post 18-day treatment using a fluorescence plate reader. (E)

Fluorescence images of the skin and tumor tissues. (F) Fluorescence profile of MSNP-PLL through skin and tumor tissues in E.

Figure 4.9. Representative images of mice treated with MSNPs-PLL w Cy5 at Days 4 and 11.

Topical delivery of siRNA with MSNPs for the skin tumor treatment

MSNPs-siRNA-PLL were synthesized as MSNP-MB-PLL and contained 3.13 (wt%) of siRNA. The release profile of siRNA was also similar to that of MBs from MSNP-MB-PLL (Figure 4.10A). The gene silencing

capability of siRNA was well preserved in MSNP-siRNA-PLL complexes. As shown in Figure 4.11, the TGF β R-1 gene expression in RT3 cells was reduced by 60% 2 days after the treatment of MSNP-siRNA-PLL. Finally, MSNP-siRNA-PLL mixed with moisturizer were topically applied on the tumor in the squamous cell carcinoma mouse xenograft model (Figure 4.10B). The mice were treated every 3 days for 18 days, and the tumor volume was measured using the formula reported by Faustino et al¹⁵⁸. Quantification of tumor volume revealed there was minimal growth of tumor on mice treated with nanoparticles containing siRNA against TGF β R-1 (Figure 4.10C and D). On the other hand, if scramble siRNA was used, the tumor size was tripled in 18 days (Figure 4.10C and D). Western blot further showed the 50% reduction of TGF β R-1 expression in the MSNP-siRNA-PLL treated groups (Figure 4.10E).

Figure 4.10. Topical delivery of siRNA with MSNPs for the skin tumor treatment: (A) Release profile of siRNA from MSNPs-siRNA-PLL and MB from MSNPs-MB-PLL. (B) Schematic illustration of *in vivo* studies with the squamous cell carcinoma mice model. (C) Representative images of the mice during the topical treatment with MSNP-siRNA-PLL. (D) Normalized

tumor volume against that at day 1. (E) Expression of TGF β R-1 in the tumor tissue post the treatment.

Figure 4. 11. TGF β R-1 gene expression on RT3 cells post the treatment of MSNP-siRNA-PLL.

Figure 4.12. Fluorescence Image of mice skin treated with topical MSNPs-PLL w Cy5. White arrows indicate the hair follicles. Scale bar: 100µm.

4.4. Discussion

Skin disorders remain as one of the prevailing disease burdens worldwide. Misdiagnosis and lack of efficient treatment can lead to undesirable clinical outcome and this post a significant healthcare burden. In recent years, advances in genetics have allowed early detection of pathogenic mutations and promote the development of personalized therapeutics¹⁶²⁻¹⁶⁴ e.g. RNAi therapies. While the technology is promising for treatment of skin diseases, the current way of administering it is through intradermal injection¹⁶⁵, thus revealing the technical hurdles to overcome the skin's barrier in a patiently and clinically relevant manner. In light of the need for a non-invasive delivery method for nucleic acids treatment, this project utilizes nanoparticles as a vehicle for transdermal delivery of oligonucleotides. MSNPs have been extensively researched in recent years as a promising tool for packaging drugs and ensuring their delivery through cell membrane^{34, 155, 157}. Furthermore, the ease of controlling their size, pore volumes, and surface modification are attractive features for drug delivery. In the field of transdermal delivery, MSNPs have excellent biocompatibility with skin cells and studies have shown substantial amount of drugs delivered

transdermally with the use of MSNPs¹⁶⁶. Therefore, this study serves to explore the capability of MSNPs as a topical formulation in the transdermal delivery of siRNAs.

The initial studies on the synthesis of the NPs were done on MSNPs of size ~200nm with pore size 4nm (Figure 4.1B). MBs were used as the model oligonucleotides to be packed into the pores (Figure 4.1A). Since the skin barrier is negatively charged, the MSNPs-MB were coated with a layer of positively charged PLL for improve penetration. The successful PLL coating was confirmed through a zeta potential measurement of +30mV (Figure 4.2B). With the positive charged surface, improved penetration (~2-fold) was observed in vitro through fluorescence imaging (Figure 4.3A, B), FACS (Figure 4.3C), and confocal imaging (Figure 4.4) when the MSNPs-MB-PLL was delivered to RT3 cells to detect a house-keeping gene, GAPDH. After administration of MSNPs-MB-PLL into the cells, the release of MB from the pore of MSNPs might be regulated by diffusion of MB out of the pore, and degradation of MSNPs which lead to MB release^{11, 167}. Treated cells did not show any signs of toxicity 12 hrs after treatment with MSNPs-MB-PLL (Figure 4.5).

Next, it was worth studying if the MBs delivered inside the cell remain functional and specific. CTGF MB was used in this study to detect gene changes when the RT3 cells were subjected to different concentration of

Repsox treatment. Repsox has been studied to be an attenuator of CTGF gene expression and a similar trend was observed in this study (Figure 4.7B). With increasing Repsox concentration, the fluorescence of CTGF MB decreased. The study compared the fluorescence intensity to the gold standard PCR gene expression and a good correlation was observed ($R^2=0.8659$), confirming the specificity and functionality of MBs inside the cell.

The transdermal drug delivery using topically applied MSNPs was studied in a SCC xenograft model. MSNPs-Cy5-PLL were mixed with Aquaphor® to form a topical mixture (1:1 w/v ratio) and applied on the skin every 3 days for 18 days (Figure 4.8A). This study was benchmarked against intratumor injection. IVIS imaging revealed localized distribution of Cy5 in the mice tumor (Figure 4.8B), with comparable fluorescence intensity observed for both intratumor injection and topical application (Figure 4.8C). On the other hand, intravenous injection was observed to be ~6 times lower than that of intratumor injection and topical application (Figure 4.8C). In addition, a biodistribution study in mice showed a high amount of Cy5 signal in the kidneys of mice treated with intratumor injection (Figure 4.8D), suggesting the renal clearance of NPs, therefore resulting in a low amount of NPs reaching the tumor. Histology of the skin and tumor revealed excellent penetration of MSNPs-Cy5-PLL when applied topically (Figure 4.8E, F). The NPs were found in the epidermis, dermis, subcutaneous layer, and even in

the tumor (suggesting a penetration depth of ~1200 μ m. On the other hand, NPs were only found in the tumor for intratumor injection, and limited amount was observed in intravenous injection (Figure 4.8E, F). While the penetration of these NPs was observed to be promising, it was important to study the mechanism of entry of these NPs since the final complexes (i.e. MSNPs-MB-PLL) had size of ~250 nm. Previous research showed that NPs of size ~20 – 200nm can be delivered into the skin layers through penetration and retention in the hair follicles^{12, 168-169}. In this study, similar trans-follicular transportation of MSNPs-PLL w Cy5 was also observed (Figure 4.12). Fluorescence signals can be observed both in the upper parts and the roots of the hair follicles, similar to that reported by Su et al.¹⁶⁹ Therefore, the observations demonstrate that the NPs permeate into the deep sites of the skin through the follicle route, and thereafter diffuse into the surrounding dermal tissues¹⁶⁹.

With the promising results, MSNPs-PLL were used as the delivery tool for siRNA delivery. In particular, TGF β R-1 siRNA was delivered to suppress the gene expression in the tumor. In vitro results showed promising outcome of ~60% gene knockdown (Figure 4.11). This study was performed for 18 days with MSNPs-scramble siRNA-PLL as the control. Measurement of tumor volume revealed minimal growth of tumor when mice were treated with topically applied MSNPs-TGF- β R1 siRNA-PLL (Figure 4.10D). On the other

hand, the mice treated with MSNPs-scramble siRNA-PLL saw an increasing growth of tumor size. The knockdown efficiency was confirmed through Western Blot, where TGF β R-1 was found to be attenuated in MSNPs-TGF- β R-1 siRNA-PLL, approximately half of that in MSNPs-scramble siRNA-PLL and control (Figure 4.10E).

The mentioned nanotechnology in this study showed promising transdermal delivery of oligonucleotides. However, there are questions left to be answered. Firstly, there was lack of consistency and understanding for the drug loading efficiency. We achieved 1.42% loading for MBs and 3.13% for siRNA. This difference may be due to the different size of MB and siRNA or the presence of fluorophore molecules. Secondly, in clinical setting of SCC treatment, the suppression of TGF β R-1 may not be the best choice. A clinically-confirmed target is highly needed here to fully reveal the effectiveness of this platform in topical delivery of siRNA. Thirdly, the biodistribution of MSNPs-MB-PLL was only tested at Day 18. Moving forward, it would be crucial to study the pharmacokinetics (PK) of MSNPs-MB-PLL absorption, elimination dynamics in the major organs (typically 2 hours with 10-15 minutes interval) following topical application, intravenous injection, and intratumor injection. Furthermore, it would be worth studying the maximum concentration (C_{max}) of MSNPs-MB-PLL in the tumor by different administration routes across the time course.

4.5. Conclusion

This article reports the design, synthesis, characterization, and utilization of MSNPs-PLL for the noninvasive transdermal delivery of oligonucleotides. This NP is constructed by loading oligonucleotides into the pores of MSNPs and thereafter a layer of PLL coating to improve transdermal delivery. The MSNPs-PLL were tested with MB as the model oligonucleotide on human skin squamous carcinoma cells in vitro and showed good penetration efficiency and the MB when released inside the cells retained its functionality and specificity. The MSNPs-PLL also showed excellent penetration in a xenograft tumor model. Finally, MSNPs-PLL enabled the delivery of TGF- β R siRNA in regulating gene expression in the tumor xenograft model. We believe this MSNPs-PLL will pave the way to more nanotechnologies as a delivery tool for non-invasive transdermal drug delivery.

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Chapter 5. Temporal Pressure

Enhanced Topical Drug Delivery

through Micropore Formation

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5.1. Introduction

Transdermal drug delivery (TDD) offers a convenient and patient-friendly way for the treatment of both local disorders and diseases of other organs through systemic delivery. It allows drugs to bypass the first pass metabolism while providing sustained and controlled delivery ^{2, 170}. While traditional cream or gel formulations passively deliver small and lipophilic drugs through the stratum corneum (SC) *via* diffusion, macromolecules (>500 Da) with very low permeability cannot diffuse through the compact lipid-rich matrix of SC¹⁷¹. Chemical and biochemical enhancement techniques have been developed over the past decades to disrupt SC for the delivery of large and hydrophilic drugs. However, these enhancers are potent irritants at high concentrations and thus affect therapeutic compliance ^{1, 172}. Physical enhancement methods amiable for routine and effective delivery are also developed (e.g. electroporation, laser ablation, iontophoresis)^{2, 25, 81}. Nonetheless, these physical means are accomplished through utilization of additional, specialized equipment alongside trained users to modulate skin permeability, thus presenting challenges in terms of patient convenience and home-use ¹⁷³⁻¹⁷⁴. Another technology worth mentioning is the microneedle (MN), which has received vast attention recently ¹⁷⁵⁻¹⁷⁶. These MN can be fabricated with different needle length and width (150 – 1500 microns long, 50 – 250 microns wide), with few drug loading options, coated MN, dissolving

MN, and hollow MN¹⁷⁶. Furthermore, However, due to their small sizes, the amount of drugs could be delivered is usually within microgram range ^{23, 177}. Amidst the popular technologies mentioned above, rubbing serves as a potential method to deliver drugs transdermally. However, this method is poorly reported, and their mechanism is still unclear ¹⁷⁸⁻¹⁷⁹. The limited literature review on rubbing suggests that the locally-applied pressure can benefit TDD. Herein, we establish a protocol to examine this hypothesis (Figure 5.2). Specifically, the pressure (0.14, 0.28, and 0.4 MPa) is generated by applying two neodymium magnets to pinch skin. After a given time, the magnets are removed, and therapeutics in the moisturizer are topically applied on the pressure-processed area. Here we report the successful delivery of nanoparticles (up to 500 nm), dextran molecules (up to 20 kDa), and insulin across the mice skin. The improved penetration was observed through inter- and trans-cellular routes. We optimize this process and propose 0.28MPa and 1 and 5 mins of application as the ideal procedure to achieve an effective penetration without compromising the skin barrier. To demonstrate the clinical relevance of temporal pressure, a chronic disease which requires daily injection was chosen: diabetes. There are over 425 million diabetic patients worldwide. By 2050, 1 in 10 people will be diagnosed with diabetes in Singapore. These patients require 3-4 injections per day to maintain glucose level, which are painful, with the generation of dangerous

needle waste, and same site injection may lead to unsightly lumps and fatty deposits. The current technologies used in insulin delivery include (1) insulin syringes which are painful and complicated for patients to use¹⁸⁰; (2) insulin infusion pumps which are very expensive and the presence of needle in the body may hinder certain movement¹⁸¹; and (3) insulin jet injectors which are expensive and painful¹⁸²⁻¹⁸³. There are also published platforms for improved insulin delivery which include microneedles⁶⁷ and insulin inhalers¹⁸⁴. However, microneedles are limited by their size for loading of drugs, while the efficiency of inhaled insulin is low and drug is lost during the inhalation process. Considering these limitations of insulin delivery technologies, we hypothesize that temporal temporal pressure would be a potential technology to provide easy, effective, safe, and cheap insulin delivery for patients. In this thesis, we demonstrated effective delivery of insulin into the systemic circulation of both normal and diabetic mice and achieve 65% and 80% reduction in blood glucose levels respectively via this method.

Figure 5.1. Schematic illustration of Temporal Pressure enhanced Transdermal Drug Delivery.

Figure 5. 2. Schematic of the use of temporal pressure for transdermal drug delivery. Mice were treated with magnets for up to 1.5 hours, followed by the topical application of drugs for a period of 0-24 hours.

5.2. Methods and Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Neodymium magnets of different surface field (1795G, 3309G, and 4440G) were obtained from K&J Magnetics. FluoSpheres™ carboxylate-modified microspheres (20, 100, 200, 500nm) (catalog number: F8786, F8801, F8810, and F8812 respectively) with red fluorescent (excitation: 580nm; emission: 605nm) were acquired from ThermoFisher (Singapore). According to Thermofisher's certificate of analysis, these microspheres are suspended in a medium of distilled water with 2nM sodium azide, possess a charge density of 0.3557meq/g.

Rhodamine B-conjugated 3kDa dextran (catalog number: D3308) was acquired from ThermoFisher (Singapore), while dextran of sizes 5kDa, 10kDa, and 20kDa were acquired from NANOCS (USA) (catalog number: DX5-RB-1, DX10-RB-1, and DX20-RB-1 respectively). Briefly, water-soluble amino dextran was reacted with succinimidyl ester derivative of Rhodamine B. Following dye addition, unreacted amines on the dextran were capped to yield a zwitterionic dextran and purified through size chromatography to remove non-conjugated dye. All animal studies were performed in compliance with the guidelines set by Institutional Animal Care and Use Committee (IACUC, Reference Number: A18025), NTU. Normal bright-field and fluorescence imaging was performed with a LX1 inverted microscope

(Olympus) equipped with Retiga-2000R CCD camera. The settings (exposure time: 200 ms; gain: 8) were fixed. Confocal imaging was done using a Leica SP8 upright confocal microscope (German).

Transdermal delivery of nanoparticle (NP) and dextran with pressure treatment in mouse model: 6-weeks-old C57BL/6J male mice (InVivos Pte. Ltd., Singapore) were randomly grouped and anaesthetized using 2% isoflurane in oxygen. Next, the mice were shaved using [MOSER® CHROMINI PRO] and followed by [Veet® Hair Removal Cream Sensitive]. This is to ensure the hair does not trap the cream. The dorsal loose skin was then pinched (Figure S1), and magnets were placed carefully on each side to prevent injury to the mice. Magnets were placed for 1 min, 5 mins, 0.5 hour, and 1.5 hours to produce the intended pressure. The procedures hereafter is similar for both dextran molecules and NPs topical application. After pressure application, 0.05 mg of dextran molecules or 0.2 mg of carboxylate-modified NPs were mixed with Aquaphor® 1:1 ratio (w/v). Aquaphor® Healing Ointment is a commercial formulation for dry and cracked skin. It contains 41% Petrolatum, Panthenol and Glycerin to moisturize, nourish and protect the skin. Magnets were removed carefully by sliding them apart after stipulated time. The formulation containing NPs or dextran was then topically applied on the skin marks caused by the pressure, followed by Tegaderm™

and Coban™ application to secure the mixture overnight. The next day, mice were anaesthetized, and Tegaderm™ and Coban™ were removed. The excess topical mixture was removed by gently wiping with gauze and PBS. Mice were imaged with In Vivo Imaging System (IVIS). Thereafter, mice were euthanized by CO₂ asphyxiation, and the skin was excised and fixed in 10% formalin solution for histology studies.

Transdermal insulin delivery on normal mice: Experimental test group sizes were three mice per treatment, to ensure a balance between sufficient replication of results and a reduction in mice number. Mice was fasting for 12 hours prior to treatment. Then mice were shaved using [MOSER® CHROMINI PRO] and followed by [Veet® Hair Removal Cream Sensitive] under anesthesia. After pressure application, topical insulin (1U of insulin, Mixtard® mixed with Aquaphor®, 1:1 ratio, w/v) was applied onto pressure-marked skin. The topical application was secured using Tegaderm™ and Coban™. Blood glucose was measured using One Touch UltraMini® by snipping off a small portion of the tail. Blood glucose levels were measured at time 0, 0.5, 1, 2, 3, 4, 5 hours.

Transdermal insulin delivery on STZ-induced diabetic mice: Male C57BL/6J mice were weighed at week 7 and marked with an ear puncher.

They were fasted overnight (~16 hours), followed by measurement of fasting blood glucose (FBG) using a glucometer (Accu-Chek® Guide Wireless Blood Glucose Meter, Roche). Mice with FBG of >11.1 mmol/L were excluded from the injection. One day before STZ administration, mice were fasted overnight. STZ was prepared in a concentration of 0.02 g/ml of buffer containing 0.1 M citrate buffer. STZ was then administered at 50 mg/kg to the STZ-induced diabetic mice, while only citrate buffer was injected into mice for the control group. This procedure was repeated each day for 5 days, followed by 4 weeks of monitoring before mice were tested again for FBG. Mice with blood glucose greater than 21 mmol/L were considered successfully induced with diabetes. In the transdermal delivery experiments, diabetic mice were anaesthetized using 2% isoflurane in oxygen. Next, they were shaved using [MOSER® CHROMINI PRO] and followed by [Veet® Hair Removal Cream Sensitive]. The dorsal loose skin was then pinched (Figure S1), and magnets were placed carefully on each side to prevent injury to the mice. Magnets were placed for 5 mins to produce the intended pressure. Next, 10 µl of 10 U of insulin (Mixtard® 30 HM Penfill) was mixed with Aquaphor® 1:1, w/v ratio before application. After removing the magnets, the mixture of insulin was then topically applied on the skin marks caused by the pressure, followed by Tegaderm™ and Coban™ application to secure the mixture while monitoring the blood glucose at 0, 0.5, 1, and 2 hours.

To mimic a clinical setting, the above-mentioned study was performed for a period of 5 days. The diabetic mice were fasted for 4 hours before each day's experiment. As a comparison, topical application post pressure treatment was performed alongside subcutaneous injection of 2U insulin on the dorsal portion, topical application with 10U insulin, transdermal delivery with dissolvable MNs for 3 minutes, and control. In another group, drug-free solid poly (methyl methacrylate) (PMMA) MNs were thumb-pressed on dorsal skin for 3 minutes before the insulin-Aquaphor formulation was applied as mentioned above. Blood glucose was measured at 0, 1, 2, 4, 6 hours of first and second day, and 0, 2, 6 hours for subsequent days. Plasma insulin level was measured by drawing the blood using capillary blood collection tubes and insulin levels were measured using human insulin ELISA kit (Crystal Chem).

Fabrication of insulin-containing dissolvable microneedle: Insulin-containing dissolvable microneedles were made via micromolding. Poly(dimethylsiloxane) (PDMS) micromolds were firstly prepared by pouring PDMS mixture (SYLGARD® 184 SILICONE ELASTOMER KIT, Tat Lee Engineering Pte Ltd; 10:1 w/w ratio of prepolymer to curing agent) into a stainless - steel master (consisting of 10×10 arrays of sharp pyramids needles, with height of $\approx 1000 \mu\text{m}$, interspacing of $\approx 700 \mu\text{m}$, and base width

of $\approx 300\text{ }\mu\text{m}$; Micropoint Technologies, Singapore), followed by degassing with a vacuum oven and curing at $70\text{ }^{\circ}\text{C}$ for 3 hours. Subsequently, this micromold was filled with 0.5 g/ml HA solution (Sodium hyaluronic acid (HA, Mw 3 kDa), Freda Biochem Co.,Ltd. (China). $\geq 95.0\%$ purity) containing 10 U insulin through centrifugation method (4000 rpm , 3 mins). Following overnight drying at room temperature, a backing layer of bare - HA solution (0.5 g/ml) was added as supportive base layer. The final patch was peeled off from the micromold after overnight drying and stored at $4\text{ }^{\circ}\text{C}$ in a dehumidified condition.

Solid microneedles: Solid, non-dissolving PMMA MNs were obtained from Micropoint Technologies Pte Ltd, Singapore. Briefly, PMMA (Mw: 75 kDa) was mold-casted to have needle height of $1000\mu\text{m}$, in 10×10 array with a density of 400 needles/cm^2 . Individual microneedle has a square pyramid shape.

Transdermal delivery in the rabbit model: Pathogen-free New Zealand rabbits were purchased from and housed under standard laboratory conditions ($22 \pm 1^{\circ}\text{C}$; relative humidity $40\text{--}55\%$) at Prestige World Genetics (PWG) Laboratories Singapore. Rabbits were fed with normal rabbit chow and watered ad lib. The in vivo rabbit experiments were performed under the

regulations of Good Laboratory Practice Compliance Programme and Association for Assessment and Accreditation of Laboratory Animal Care International (IACUC reference number: BN16098). Rabbits were anaesthetized using ketamine (60mg/kg) and xylazine (5mg/kg). The dorsal hair was removed by shaving and depilation, followed by pressure application using magnets for 1 min or 5 mins, followed by 24 hours or 48 hours of topical drug application. Given the bigger dorsal skin surface area as compared to mice, 4 different sets of time periods were performed for each rabbit. Positions of each set of time periods were randomized on the rabbit dorsal skin. 0.2mg of carboxylate-modified NPs were mixed with Aquaphor® 1:1 w/v ratio before application. The mixture was then topically applied on the skin marks caused by the pressure, followed by Tegaderm™ and Coban™ application to secure the mixture for 24 or 48 hours.

Molecular study on the impact of temporal pressure on the skin barrier:

To study the molecular changes in the skin barrier enabling the penetration of molecules and nanoparticles, the mice were shaved using [MOSER® CHROMINI PRO] and followed by [Veet® Hair Removal Cream Sensitive]. Thereafter, the dorsal loose skin was pinched (Figure S1) with different strengths of magnets (0.14, 0.28, and 0.4MPa) for 5 mins, followed by application of a mixture of PBS in Aquaphor® in a 1:1 w/v ratio on the

pressurized skin to mimic the use of aqueous drug. Next, Tegaderm™ and Coban™ were applied to prevent the mice from removing the topical application. After an overnight (12 hrs) application, the mice were euthanized by CO₂ asphyxiation, and the skin was excised and fixed in 10% formalin solution for histology and immunostaining studies.

Histological sectioning and Haematoxylin and Eosin staining: For fluorescence microscopical observation of dextran molecules and NPs into the skin, the excised skin samples were cryosectioned to preserve the fluorescence. Briefly, skin tissues were embedded in Optimum Cutting Temperature (O.C.T.) (Sakura® Finetek) first. Then they were sectioned at 10µm using Leica CM3050 S Cryostat and stained with hematoxylin and eosin (H&E) under standard protocols.

To look for microscopic histological changes, samples were sectioned using paraffin-embedded tissue. Briefly, skin tissues were transferred into 70% ethanol after fixation and processed for paraffin embedding using an automated tissue processor (Leica Tissue Processor HistoCore Pearl). Paraffin processed tissues were immediately embedded into paraffin blocks and sectioned at 5µm. Haematoxylin (Cat no. 3801570, Leica) and eosin (Cat no. 3801615, Leica) histological stain was performed and mounted using Organo/Limonene mount™ (Cat no. O-8015, Sigma). Slides were

allowed to dry overnight and imaged using an Axioscan.Z1 slide scanner (Zeiss).

Immunostaining: Paraffin embedded skin sections were first deparaffinized and rehydrated using decreasing concentrations of ethanol (100%, 95%, 70%) followed by water and then recalibrated to 1xPBS. Tissues were stained overnight using rabbit anti-Cx43 primary antibody (C6219, SigmaAldrich) of 1:1000 dilution after being permeabilized and blocked. This was followed by an 1hour incubation of 1:500 goat anti-rabbit AF488 secondary antibody (A11008, ThermoFisher, Singapore). Tissue sections were counter stained with DAPI. To stain occludin, tissues were deparaffinized and processed with enzymatic antigen retrieval using proteinase K. Similar to Cx43 staining, tissues were stained overnight using rabbit anti-occludin primary antibody (71-1500, ThermoFisher, Singapore) of 1:100 dilution after being permeabilized and blocked. This was followed by one hour incubation of 1:500 goat anti-rabbit AF555 secondary antibody (A21428, ThermoFisher, Singapore) and subsequently counter stained with DAPI.

Fluorescence and Confocal microscopy, and Image analysis: For all figures (except for Figures 5 and S11), slides were imaged using an Olympus

IX71 inverted microscope. All images were taken using a 10x objective. For quantification of signal, 3 representative images of each sample were analysed using ImageJ. Data was plotted and statistically analysed using one-way ANOVA with post Tukey HSD (Honestly Significant Differences), unless otherwise stated. For Figures 5 and S11, slides were imaged using a Leica SP8 upright confocal microscope. All images were taken using a 40x objective. For quantification of signal, 3 z-stacked images of 3µm were taken in the relevant regions and analysed using ImageJ. Data was plotted and statistically analysed using Prism6 software. Kruskal-Wallis test followed Dunn's post hoc test was done, and statistical significance is achieved when $p < 0.05$, *.

Statistical analysis: Statistical comparisons between groups, unless specified, were determined by one-way ANOVA with post-hoc Tukey HSD (Honestly Significant Differences) using a web-based statistics calculator from: http://astatsa.com/OneWay_Anova_with_TukeyHSD/. For all tests, $p < 0.05$, * was considered statistically significant. For Figures 5 and S11, data was plotted and statistically analysed using Prism6 software. Kruskal-Wallis test followed Dunn's post hoc test was done, and statistical significance is achieved when $p < 0.05$, *.

5.3. Results

The effectiveness of this procedure was initially studied on mice by taking 200 nm fluorescent nanoparticles (NPs) as the model drugs and compared against microneedles (MN). The 0.14 MPa pressure was applied for 1.5 hours while MN was applied for 5 mins as previously reported¹⁸⁵⁻¹⁸⁶. NPs were applied right after the removal of magnet and MN and were left on the skin for 24 hours before being removed. The treated skin was subjected to further analysis. As shown in Figure 5.3A, fluorescent NPs were found to be localized only at areas where pressure and MN treatment was performed (red circle), without spreading to surrounding tissues. The amount of NP retained in the skin was minimal for the sample without pressure treatment (20% of the signal from that with pressure and MN treatment, Figure 5.3B). No difference in amount of NP in the skin was observed between pressure and MN treatment. Histology showed that NPs mainly stayed on the SC layer without pressure treatment but reached the dermis layer upon following pressure and MN treatment (Figure 5.3D). Further quantification revealed that pressure treatment increased the NP concentration in the dermis layer at least 5-fold (Figure 5.3C).

When there was no pressure, NPs entered the skin mainly through appendages (Figure 5.3D). The pressure treatment allowed NPs to get into

the skin layers through both transappendageal and non-transappendageal routes (trans- and inter- cellular routes). NPs tended to agglomerate when they entered through follicular and eccrine routes, but pressure treatment permitted NPs to enter through trans- and inter-cellular routes and distribute more evenly within the skin. On the other hand, MN penetration into the dermis allow the NPs to migrate into the dermal tissue (Figure 5.3D). Hematoxylin and Eosin (H&E) staining reveals thickening of the epidermis after 1.5 hours of 0.14 MPa pressure, and overnight application of NPs (Figure 5.3E), while no significant immune responses were made for w/o pressure and microneedles.

Figure 5. 3. Temporal pressure enhanced transdermal delivery. (A) Representative image of mice topically applied with fluorescent nanoparticles (NPs) after the pressure and MN treatment. (B) Quantification of the fluorescent signal in A. (C). Quantification of nanoparticles count in the dermis layer. ** $p < 0.01$. (D). Fluorescence imaging of histological skin samples in A (E) Left: H&E staining of skin samples, Right: the appearance

of mouse skin with or without the pressure treatment, and MN. Scale bar: 100 μm . $n=3$, all data are mean $\pm$ SD, * $p<0.05$; ** $p<0.01$.

Pressure is the key element in this methodology. We treated mice with 0.14, 0.28, and 0.4 MPa pressure respectively (Figure 5.4A). Regardless of pressure intensities, NPs were localized only at areas where pressure was applied (Figure 5.5B). However, greater pressure led to stronger NP signal in the skin. In comparison to the control, 0.14 MPa brought a ~5-fold increase of the NP signal, 0.28 and 0.4 MPa provided 7-fold and 9-fold improvement respectively (Figure 5.4C). A further histological analysis of the skin samples confirmed the improved penetration of NPs in the skin layers (Figure 5.5C). Similar to previous observations (Figure 5.3D), NPs entered the skin through both transappendageal and non-transappendageal routes. The increase of NP signal in dermal layer was less than we observed in *in vivo* imaging. For example, ~9-fold increase of NP signal was observed for 0.4MPa treatment with IVIS imaging (Figure 5.4C), while there was an only 2.25-fold increase in dermal layer (Figure 5.4E). The difference comes from the different signal sources. IVIS imaging is capable of collecting signals from both epidermal and dermal layers¹⁸⁷, and thus fluorescent signal in Figure 5.4C comes from both epidermal and dermal layers. In another words, there was also an increase in NP retention in epidermis layer post the pressure treatment.

While 0.4 MPa treatment provided the highest NP retention in the skin, we observed that the SC to stratum basale layers of the mouse skin was damaged under this pressure (Figure 5.5C). Treatment with 0.14 and 0.28 MPa increased the epidermal thickness to approximately two-fold (Figure 5.6B). The sudden decrease in the epidermal thickness of mice treated with 0.4 MPa confirmed our observation of the loss of SC to stratum basale layers. Under all pressures, the tissue in dermis layer became disoriented with an increase in hematoxylin stained nuclei (~1.5, ~1.9, and ~2.7-fold respectively) (Figure 5.6A), which suggests the inflammation at the treated areas. As 0.14 MPa treatment did not provide a significant increase ($p > 0.05$) in NP concentration in dermal layer when compared to the control (Figure 5.4C), 0.28MPa was selected for the subsequent experiments to maximize the penetration of the drugs and while minimizing the damage to the skin.

Besides the pressure, another factor in this pressure enhanced transdermal delivery is the duration of pressure application (Figure 5.4B). As shown in Figure 5.4D, longer treatment brought more NPs into the skin. Compared with the control, 0.5 and 1.5 hrs pressure treatment improved the NP fluorescence signal in the skin 6-7 folds. Interestingly, there was ~4 folds increase even with 1 and 5 mins treatment. Histological analysis confirmed a 70-90% increase of NPs in the dermis as well (Figure 5.4F). Earlier, we observed thickening of epidermis layer (Figure 5.5C and 5.6B) and increased

nuclei count with 0.28 MPa after 1.5 hrs of treatment (Figure 5.6A). When the treatment was reduced to 1, 5 mins and 0.5 hour, both the epidermal thickening (Figure 5.4G) and inflammation (Figure 5.7A & 5.7B) were comparable to control.

Figure 5. 4. (A) Overview of procedure performed to optimize pressure and application time. (B) Representative IVIS image of the mice treated with 0.28MPa for different application time. (C) Quantification of fluorescent signal in IVIS imaging of mice treated with different pressure (0.14, 0.28, 0.4 MPa).

(D) Quantification of fluorescent signal in IVIS imaging of mice treated with the same pressure (0.28 MPa) but with different time (1 min, 5 mins, 0.5 hours, and 1.5 hours). (E) Quantification of fluorescent signal in the dermis layer of mouse skin treated with different pressure (0.14, 0.28, 0.4 MPa). (F) Quantification of fluorescent signal in the dermis layer of mouse skin treated with the same pressure (0.28 MPa) but with different time (1 min, 5 mins, 0.5 hours, and 1.5 hours). (G) Fluorescence imaging and H&E staining of mouse skin treated with the same pressure (0.28MPa) but with different time (1 min, 5 mins, 0.5 hours, and 1.5 hours). Scale bar: 100 μ m. n=3, *p<0.05; **p<0.01.

Figure 5.5. Optimization of pressure force in the temporal pressure enhanced transdermal drug delivery. (A) Illustration of the protocol to study

the effect of pressure; (B) *In vivo* imaging of mice treated according to the protocols in (A). (C) Fluorescence imaging and H&E staining of histological skin samples treated with different pressures.

Figure 5. 6. Quantification of nuclei count from a fixed ImageJ perimeter (~2000 pixels) measurement in skin sections of mice subjected to different pressure forces for 1.5 hours and (B) the corresponding epidermis thickening. n=3, all data are mean $\pm$ SD, *p<0.05, **p<0.01.

Figure 5. 7. Quantification of nuclei count from a fixed ImageJ perimeter (~2000 pixels) in skin sections of mice subjected to the treatment of 0.28 MPa pressure after different time periods and (B) the corresponding epidermis thickening. n=3, all data are mean $\pm$ SD, *p<0.05, **p<0.01.

Next, we examined the suitability of this methodology for various types of drugs including polymer and NPs, The polymer models were dextran with sizes of 3k, 5k, 10k, and 20k Dalton (Figure 5.8A). They were topically applied on pressure-treated skin under the same mass concentration. We noticed that fluorescence intensities were different for these dextran molecules under the same mass concentration (Figure 5.9). The fluorescence intensity from 3 kDa dextran is 4 times of that from 10 kDa and

20 kDa dextran. Thus, results in the following experiments were normalized to account for this difference in fluorescence intensity.

In vivo imaging revealed that pressure treatment increased penetration of all sizes of dextran molecules in skin (Figure 5.8B) as compared to control. Data demonstrated ~2-fold increase for both 3kDa and 5kDa when treated with pressure. Furthermore, ~3.5-fold increase for 10kDa and ~1.5-fold increase for 20kDa was observed as compared to without treatment samples (Figure 5.8C). This increasing trend was consistent regardless of the treatment time (1 or 5 mins). By normalizing the fluorescence signals in dermis (Figure 5.8D) against the fluorescence intensity of each dextran (Figure 5.9), we found that there was higher amount of smaller dextran (i.e. 3 - 10kDa) in the skin than dextran with higher molecular weights (20kDa) (Figure 5.10). Histological analysis confirmed all dextran molecules reached the dermis with the pressure treatment (Figure 5.8D), but these molecules only accumulated on the SC layer with no pressure treatment.

Next, we examined the transdermal delivery of NPs ranging from 20 to 500 nm. Different from Dextran molecules, different sizes of NPs under the same mass concentration showed similar fluorescence intensity (Figure 5.11). Without the pressure treatment, the smaller the NPs, the stronger

fluorescence the mouse skin had (Figure 5.12). With the pressure treatment, drastic increase in the amount of NPs were observed across all particle sizes. Histological analysis showed that pressure treatment significantly improved the NP's concentration in the epidermis and inside the appendages (Figure 5.13A). More importantly, NPs were able to enter the dermis layer and distribute uniformly. Quantification of fluorescence particle count showed that mouse skin treated with 20nm NPs had the highest fluorescence intensity (Figure 5.13B), similar to that observed in IVIS (Figure 5.12). Mice treated with 100 and 200nm NPs post temporal pressure demonstrated an average of 2 times more NPs found in the dermis. When mice were treated with 500nm NPs with pressure, particles were found in the SC/epidermis only with negligible NPs in the dermis (Figure S13A). Clearly, 500nm is the cutoff size for NPs.

Figure 5. 8. Topical delivery of dextran molecules post the pressure treatment. (A) Schematic of experiments. (B) IVIS image of mice after topical delivery of dextran molecules post the pressure treatment (C→ no pressure, 1→ 1min pressure treatment, 5→ 5min pressure treatment). (C) Normalized quantification of dextran in treated skin area in B. (D) Fluorescence imaging of histological skin samples in B. Scale bar: 100 μ m. n=3, all data are mean $\pm$ SD, *p<0.05; **p<0.01.

Figure 5.9. Fluorescence intensities of solutions containing different molecular sizes of dextran under the same mass concentration.

Figure 5.10. Quantification of fluorescence from dextran of sizes 3, 5, 10, and 20kDa in the dermis layer. $n=3$, all data are mean $\pm$ SD, * $p<0.05$, ** $p<0.01$.

Figure 5.11. Fluorescence intensity detected from different sizes of nanoparticles under the same mass concentration.

Figure 5.12. IVIS quantification of mice skin treated with no NP (C), 20nm (20), 100nm (100), 200nm (200), and 500nm (500) NPs under without and with pressure conditions respectively (in log scale).

Figure 5.13. Temporal pressure aided transdermal delivery of nanoparticles. (A) Histological analysis of mouse skin after the topical application of nanoparticles with different sizes; (B) Quantification of nanoparticle fluorescence in dermis layer.

The rabbit is a widely-used animal model for skin research due to their relatively large size and docile nature, with comparable stratum corneum thickness to pigs and humans¹⁸⁸⁻¹⁹⁰. Using the above established protocol, we treated the rabbit skin with pressure and topically applied NPs in the moisturizer. *In vivo* imaging showed that the pressure treatment improved the NP fluorescence 3 folds (Figure 5.14A&C). Histology confirmed the

increase of NP concentration in dermis layer (Figure 5.15), and no obvious skin damage was observed in H&E stained sections (Figure 5.14B). Aside from the dorsal skin, temporal pressure could also be applied on the rabbit's ears, delivering drugs on popular *in vivo* rabbit ear wound models e.g. keloid scars ⁵³.

Figure 5. 14. Transdermal delivery of nanoparticles (NPs) on rabbit skin with the temporal pressure treatment. (A) Workflow schematics for the rabbit experiments. (B) IVIS fluorescence imaging of excised rabbit skin post the treatment of pressure and NPs. Circled areas represent the area of interest; (C) Fluorescence imaging and H&E staining of rabbit skin post the treatment; (D) Quantification of NP fluorescence signal in A. n=3, all data are mean $\pm$ SD, *p<0.05; **p<0.01.

Figure 5. 15. Quantification of nanoparticle fluorescence in the dermis layer of rabbit skin. n=3; all data are mean $\pm$ SD, *p<0.05, **p<0.01.

The above experiments confirm that temporary pressure can help polymer, and NPs to enter the dermis layer of the skin through non-appendage routes. Although the mice skin did not suffer from any physical

damage in this process visually, high-resolution imaging revealed some micro-physiological changes (Figure 5.16A & 5.17A). In mice skin, numerous micropores of area $\sim 3.21 \mu\text{m}^2$ (derived from 55 samples) were observed between keratinocytes in the epidermis (black arrows), while no micropores were observed in the untreated mice. A similar phenomenon was observed in rabbit skin, where numerous micropores of area $\sim 8.25 \mu\text{m}^2$ (derived from 92 samples) were observed in the epidermis (Figure 5.18).

We further studied the proteins preserving epidermal integrity, Connexin 43 (Cx43, gap junction protein) and Occludin (tight junction protein). An increase in Cx43 expression was observed in all mice treated with pressure with 0.4MPa pressure resulted the highest expression (Figure 5.16B, D & 5.17B). On the other hand, Occludin expression was down-regulated by pressure (Figure 5.16C, E, & 5.17C).

Figure 5.16. Micropores formation and altered junction protein expression in pressure-treated mouse skin. (A) H&E staining of the mouse skin post the pressure treatment. The staining was done 12 hours after the pressure treatment and histological sectioning. Confocal images of pressure treated skin stained with DAPI and Connexin 43 antibodies (B), and Occludin (C). ImageJ quantification of Connexin 43 (D) and Occludin (E) expression in the epidermis layer. Scale bar: 50 μ m. n=3, all data are mean $\pm$ SD, *p<0.05.

Figure 5. 17. Micropores formation and altered junction protein expression in pressure-treated mouse skin. (A) H&E staining of the mouse skin post the pressure treatment. Confocal images of pressure treated skin stained with DAPI, (B) Connexin 43 and (C) Occludin. Scale bar: 50 μm .

Figure 5. 18. (A) Representative H&E stained images of rabbit skin with and without temporal pressure treatment. Micropores of average size $8\mu\text{m}$ (B) were observed at the epidermal layer (black arrows, A), which could have potentially led to the penetration of drugs.

With the optimized condition (1 and 5 mins, 0.28 MPa), we tested whether this strategy could be used for the transdermal delivery of real therapeutics like insulin (5.8k Dalton). The protein (1U or 0.035 mg) was topically applied on mice that were fasted overnight (Figure 5.19) and treated with 0.28 MPa pressure for 1 or 5 mins. 30 mins later, a slight drop in blood glucose (10%) was observed in post 1 min treatment, which progressively dropped to $\sim 50\%$ after 3 hours (Figure 5.20). On the other hand, blood glucose was observed to drop to $\sim 35\text{-}40\%$ 30 mins post 5 mins pressure treatment, and remained at $\sim 20\%$ after 3 hours. We suspect this could be due to the slow diffusion of insulin molecules from skin to the circulation with only 1 min of pressure treatment. This set of experiments allowed us to

choose 0.28 MPa pressure and 5 mins treatment in the rest insulin delivery experiments.

Figure 5. 19. Illustration of the transdermal delivery of insulin with temporal pressure treatment.

Figure 5. 20. Change of blood glucose levels in mice after the transdermal delivery of insulin with temporal pressure treatment.

The blood glucose level of treated mice was continuously monitored for 5 hours, to study the effect of long-lasting insulin in the systemic circulation ¹⁹¹. As shown in Figure 5.21A, 5 mins temporal pressure treatment plus topical insulin application decreased the blood glucose in mice to ~35% of its initial physiological state in 30 mins, and this was maintained for almost 5 hrs. There was no hypodermic shock on the mice which were physiologically active and normal the next day. On the other hand, when mice were treated with topical insulin only, there was no change of blood glucose level that was similar to that of control mice, confirming that the protein (i.e. insulin) cannot enter the skin and subsequently into the bloodstream. As a positive control, the same amount of insulin (1U) was subcutaneously injected and resulted in a drastic decrease of blood glucose to ~15% of initial state in 30 mins. The blood glucose was undetectable (0%) at 2 hrs onwards, indicating a hypoglycemic state. In view of this, the corresponding hypoglycemia index (defined as the decline in blood glucose from the initial value to the nadir, divided by the time taken to reach nadir) was calculated to measure quantitatively the extent to which insulin elicited hypoglycemia. Topical insulin delivery with temporal pressure showed a remarkably reduced hypoglycemic index compared with insulin injection (Figure 5.22C), suggesting that insulin treatment via temporal pressure has a much lower

chance of experiencing hypoglycemic shock as compared to insulin injection

67.

Figure 5. 21. Blood glucose control in normal mice with topically delivered insulin post the pressure treatment. (A) Description of 4 conditions. (B) The change of blood glucose in mouse circulation with topically delivered insulin post the pressure treatment over a period of 5 hours. (C) Quantification of insulin in the blood circulation post the pressure treatment and topical delivery of insulin over a period of 5 hours. n=3, all data are mean $\pm$ SD, *p<0.05, **p<0.01.

We further performed ELISA immunoassay to quantify insulin which entered the circulation (Figure 5.21B). There was ~15 mU/L of insulin in the blood at the first and second hour, which was sufficient to cause a drop in blood glucose ¹⁹². The concentration of plasma insulin increased to ~160 mU/L at the fifth hour. The total insulin in circulation accounted to 13.4% of

the insulin topically applied on the skin (Figure 5.22D). On the other hand, negligible insulin was detected in the blood without pressure treatment. To confirm that the drop of blood glucose across 5 hours in Figure 5.21B was due to the topically applied insulin, we tail-vein injected the same amount of insulin (e.g. 160 mU/L). As shown in Figure 5.22A, the blood glucose dropped to the similar level as in the mice treated with pressure and topically applied insulin in the first 30 mins. However, blood glucose rose after 1 hour, which should be due to the enzymatic digestion of insulin. This observation is in par with the quantification of plasma insulin using ELISA (Figure 5.22B). Insulin level increased in the first hour but gradually decreased thereafter to negligible level in 4 hours. The integrity of skin barrier was studied through H&E staining, and no inflammation was observed (Figure 5.22E).

Figure 5. 22. (A) 5 hours monitoring of blood glucose in mice injected with 160mU/L of human insulin (this amount was found to entered the blood circulation via temporal pressure in Figure 6C). As compared to insulin delivered via temporal pressure (red line), drop in blood glucose was observed within the first 30 minutes which then started to increase after the first hour (blue line). This observation is in par with the amount of plasma insulin detected in (B), where plasma insulin levels start to decrease after the first hour (blue line in insert). (C) Quantification of hypoglycemia index, which was defined as the decline in blood glucose from the initial value to the nadir, divided by the time taken to reach nadir. (D) Quantification of the amount of human insulin in blood circulation / amount of insulin applied on skin (%). (E)

H&E staining of mouse skin after insulin delivery. Scale bar: 100 μ m. n=3, all data are mean $\pm$ SD, *p<0.05, **p<0.01.

The clinical potential of this methodology was further explored with the diabetic mouse models. Normal C57BL/6J mice were made diabetic by daily Streptozotocin (STZ) injection of 50 mg/kg over a 5-day period¹⁹³. Increased urination was observed from the diabetic mice in approximately 4 weeks after STZ injection (Figure 5.23A). In addition, diabetic mice rapidly lost weight (Figure 5.23B) and their blood glucose was measured at ~25 – 30 mmol/L while normal mice exhibited blood glucose of ~7.5 mmol/L (Figure 5.23C).

Initially, we used the same condition (1U of insulin with 5-min temporal pressure) as Figure 5.21 for the diabetic mice. Unfortunately, there was no significant drop in blood glucose over a 2 hours period (Figure 5.24A). Plasma insulin was measured at ~20 mU/L at the first hour, similar to the control (Figure 5.23D). When the insulin amount was improved 10 times, there was a significant drop in blood glucose from ~25 to ~7 mmol/L over 2 hours (Figure 5.24A).

Figure 5. 23. (A) Increased urination visible weight loss in diabetic mice (B) Body weight measurement of diabetic and normal mice. (C) Blood glucose measurement of diabetic mice. (D) Plasma insulin quantification in diabetic mice during the two hours of treatment. (E) H&E staining of the mouse skin after the periodic treatment. Scale bar: 100 μ m.

We benchmarked this methodology with other three insulin delivery methods including subcutaneous injection ¹⁹⁴, dissolvable microneedles (MNs) ⁶⁷, and topical application post the MN treatment (Figure 5.25). Same amount of insulin was either topically applied or encapsulated in MNs. Within 5 days, the positive control (i.e. subcutaneous injection of insulin) consistently controlled the blood glucose level that dropped from 25.9 mmol/L to 4.3 and 1.0 mmol/L within the first and sixth hour respectively

(Figure 5.25B). The insulin levels in blood were 16.1, 3169, and 2002 mU/L accordingly (Figure 5.24B). With the pressure treatment and topical application of insulin, the blood glucose dropped from 28.6 to 9.2 mmol/L at the first hour and then to 1.7 mmol/L in the sixth hour. Plasma insulin increased from 13 to 350 mU/L and 1076 mU/L at the first and sixth hour (Figure 5.25F). If there was no pressure treatment, topically applied insulin won't cause any change of blood glucose (Figure 5.25A & C) and there was negligible insulin in the plasma as well (Figure 5.24B). When diabetic mice were treated with solid MN (made of poly(methyl methacrylate) (PMMA)) and topical application of insulin⁶⁷, blood glucose quickly dropped from 23.2 to 9.6 within the first hour (Figure 5.25D) and then to 3.4 mmol/L at the 6th hour. The insulin levels were 46 and 115 mU/L of plasma insulin accordingly (Figure 5.24B). If insulin was delivered with dissolvable MNs, a gradual drop in blood glucose was observed from 27.4 to 22.3 mmol/L at the first hour and then to 10.8 mmol/L at 6th hour (Figure 5.25E), with 18.6 mU/L of plasma insulin detected and later increased to 40.5 mU/L at the sixth hour (Figure 5.24B). We also notice that the skin barrier was compromised in this period (Figure 5.23E). The continuous pressure treatment thickened the epidermis and the cell-cell bond between stratum basal and peripheral dermal layer being compromised.

Figure 5. 24. (A) Blood Glucose of diabetic mice treated with different conditions for 2 hrs. (B) ELISA quantification of insulin in the circulation of the diabetic mice for first 6 hrs, to reinforce the blood glucose drop in Figure 7.

Figure 5. 25. Blood glucose control in diabetic mice for a period of 5 days with (A) control, (B) subcutaneous injection of insulin, (C) topical application of insulin, (D) topical application of insulin post MN treatment, (E) transdermal delivery of insulin with dissolvable MNs, and (F) topical application of insulin post pressure treatment. $n=3$, all data are mean $\pm$ SD.

5.4. Discussion

This project develops a new methodology that utilizes the temporal pressure to enhance the penetration of all kinds of drugs ranging from small molecules to protein and NPs (up to 500 nm). Although magnets were used here, any pressure-generating device like mechanical actuator is suitable as well. It started from the delivery of 200 nm NPs with 0.14 MPa pressure (Figure 5.3). NP concentration in dermis layer was improved 2 times, where penetration occurred through non-transappendageal routes with even distribution within the skin (Figure 5.3D). The further screening allowed us to optimize the pressure and treatment time. Higher pressure induced better skin penetration (4-fold, 7-fold, and 9-fold increment respectively for 0.14, 0.28, and 0.4 MPa, Figure 5.4A). A longer pressure treatment also provided a better penetration. Considering the balance between efficiency and efficacy, we identified 1 min and 5 mins of 0.28MPa treatments for the delivery of dextran (3k, 5k, 10k, and 20k Dalton) and NPs (20, 100, 200, and 500 nm) in the mouse models. The results were encouraging, in which both 1 and 5-min pressure treatment improved the concentrations of all four-size dextrans (Figure 5.8C & D) and NPs smaller than 500 nm (Figure 5.13) in the dermis layer. This pressure-aided transdermal delivery is due to the formation of micropores ($3.21\ \mu\text{m}^2$) in the skin epidermal layer (Figure 5.16).

In addition, there was an increase in the gap junction and a decrease in tight junctions between cells.

The clinical indication of this methodology was explored with the insulin delivery model (Figure 5.19). By applying temporal pressure for 1 min, mediocre effect was observed over 3 hours period (Figure 5.20). On the other hand, a simple extension of the pressure treatment to 5 mins significantly improved the delivery efficacy. The blood glucose was further monitored for 5 hours (Figure 5.21). Mice treated with 5 mins of 0.28MPa showed drop of blood glucose to ~35% of initial state for 5 hours (Figure 5.21B), without any hypoglycemic shock (Figure 5.22C). After 5 hrs, 160 mU/L of insulin was found in circulation (Figure 5.21C), accounting to 13.4% of topically applied insulin (Figure 5.22D). Unfortunately, similar regulation of blood glucose was not observed in diabetic mice if 1U of insulin was topically applied post 5-min pressure treatment (Figure 5.24A). The limited therapeutic effect of 1U insulin in diabetic mice may be due to the limited blood vessels in the skin, causing limited diffusion of insulin into the circulation to take effect¹⁹⁵⁻¹⁹⁶. To address this issue, topically applied insulin concentration was raised 10-folds to increase the amount of insulin entering the blood vessels. The insulin was found to be 1,100 mU/L at the 2nd hour, approximately 10-fold of the amount of insulin in mice topically applied with 1U of insulin (Figure 5.23D). It effectively decreased the blood glucose in diabetic mice (Figure 5.24A)

We further benchmarked our methodology with the conventional injection and MN-aided topical deliveries (Figure 5.25 & Figure 5.24B) ¹⁹⁷⁻¹⁹⁸. Looking at the glucose regulation, pressure-aided delivery was as effective as the MN pre-treatment plus topical application and subcutaneous injection did. All three strategies reduced the blood glucose level to single-digit number in 1 hour. For dissolvable MN-aided delivery, the glucose level decreased in a slower speed and reached the single-digit level around 6 hours. When we looked into the insulin level in the blood, subcutaneous injection and pressure-based delivery significantly increased the insulin level from two-digit number to three- and four-digit number in the 1st and 6th hours respectively. MN pretreatment plus topical application brought the insulin level to two-digit number in the 1st hour and above 100 mU/L in 6 hours. These suggest that the timely regulation of blood glucose was due to the effective delivery of insulin into the circulation in the injection, pressure- and MN-aided delivery. Although dissolvable MNs have been shown to be efficient in controlling the blood glucose^{67, 199}, its effect was slower here, which might be due to the entrapment by the HA matrix⁶⁷, or aggregation and degradation of insulin monomers during the MN fabrication process ²⁰⁰⁻²⁰². Finally, the subcutaneous injection and pressure-aided delivery provided the highest insulin concentrations in circulation. This suggests that pressure-

aided delivery is more effective than MNs to bring topically applied molecules into the circulation.

Comparison with other technologies from a capability's perspective

This study has demonstrated the efficacy of Temporal Pressure in delivering molecules and nanoparticles across the skin barrier in a non-invasive manner. In this section, a comparison will be done against other similar technologies. Jet injectors have been used since 1990 to deliver medications⁷¹. Despite its accomplishment, it is not widely accepted in routine practices due to risk of infection, pain, injury of operator's finger, etc. Furthermore, technical difficulties such as clogging of the injector, splash and splatter are among other factors that discouraged healthcare professionals from using them. In terms of the amount of pressure asserted on the skin to achieve effective transdermal drug delivery, currently in the market, Dermojet produces 1,420psi (equivalent to 9.8 MPa), and Madajet produces 1,800psi (equivalent to 12.4 MPa). Several studies have reported it to be painful²⁰³ with high chance of causing harm to the skin²⁰⁴. On the other hand, this study demonstrated that 0.28 MPa was sufficient to deliver molecules and nanoparticles across the skin and into the systemic circulation, with no pain and major damage observed in the skin.

Another similar technology is the use of pressure wave generated by intense laser radiation to permeabilize the SC and cell membrane²⁰⁵. Lee et al. performed similar study to deliver insulin transdermally using pressure wave²⁰⁶. The amount of pressure produced was 730bar (equivalent to 73 MPa) for 100ns to 1 μ s to achieve blood glucose drop for 3 hrs. While this technology is promising, the cost of the tool and replacement of its energetic materials used to produce pressure wave is high. On the other hand, this study utilizes cheap magnets to produce the pressure for effective delivery. To enhance the user's experience, we aim to develop a wearable device, incorporated with microelectronics to produce temporal pressure.

Risk and Challenges

Impairing skin barrier properties risks potential infection when bacteria enter the body through a break in the skin⁷¹. This can be prevalent when the transdermal delivery system induces micropores as part of disrupting the skin barrier for enhancing drug delivery. Technologies of such strategy includes microneedles and thermal ablation. Study shows that microneedles of length 770 μ m can create micropores of diameter $\sim$ 71 μ m with depth of $\sim$ 153 μ m²⁰⁷. Thermal ablation creates pores of up to 50 microns in depth, reaching into the epidermis²⁰⁸. Since our technique similarly induce the formation of micropores, there is slight chance of infection. However, we

have to emphasize that temporal pressure creates pore size of $\sim 3.2 \mu\text{m}^2$. The formation of small pores can minimize the likelihood of bacteria in the skin. This is supported by the absence of immune adaptive response in the histology study. Furthermore, this pore formation is reversible, and the induced damage is far less than that from needle or microneedle injection. Furthermore, subsequent topical cream can be formulated to contain antibacterial substances for additional safety.

We are excited by the above discovery, but also realize that there are few questions to be answered before its clinical application. First of all, human skin thickness differs individually and in different parts of the body, thus it is necessary to explore the effectiveness of various pressure strength and time of application on different thickness of human skin and different parts of the body ²⁰⁹⁻²¹⁰. Secondly, the temporal pressure did effectively deliver insulin into diabetic mice, but there was the hyperthickening of the epidermis layer after 5 days of continuous application (Figure 5.23E). This might be minimized by switching the application sites regularly. Finally, daily application of temporal pressure via the magnet might be inconvenient and also pose a problem to the sensitive skin.

5.5. Conclusion

This report introduces a pressure-based transdermal delivery methodology. We discovered that the pre-treatment of the skin with pressure would generate micro-sized pores on the epidermis layer, which allowed the molecules, NPs, and proteins to diffuse into the deep layers of the skin and circulation. The pressure and duration were optimized to minimize the damage to the skin, while achieving an effective delivery. Using dextran molecules and NPs, we demonstrated effective transdermal delivery of up to 20 kDa molecules and 500 nm NPs. Furthermore, using diabetic mice as a preclinical model, we showed the effective insulin delivery for 5 days, without the need for needle penetration. ~13.4% of topically applied insulin entered the circulation. This concept and its methodology can be further extended to topical application of any formulation post a min-long pressure treatment, and such application might be acceptable for any patient without the concern of safety and cost.

5.6. Acknowledgement

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Chapter 6. Summary and Future Outlook

6.1. Summary

Transdermal drug delivery (TDD) is an exciting field with the global market reaching over \$8 billion in 2016. Compared to oral delivery, TDD avoids the first-pass effect of the liver where most of the administered drugs get trapped and digested, leading to unwanted side effects at the major organs. Clinically, hypodermic injections are painful procedures which generate dangerous biohazard and environmental waste and pose risk of disease transmission if needles were to be reused.

TDD is complicated and difficult to achieve because the skin serves as a natural barrier. Described as a brick wall, the corneocytes and the lamellar lipids prevents the invasion of foreign particles ($> 500\text{Da}$).

Over the years, TDD has evolved through three generations which can be classified under two main categories, passive and active strategies. Passive strategies include today's gels, patches for clinical use for delivery of small, lipophilic, low-dose drugs, skin penetration enhancers and incorporation of nanotechnology for topical application. On other hand, active strategies include complex technologies (electroporation, cavitation ultrasound) to produce external forces to disrupt the stratum corneum for improve penetration of a variety of drugs including macromolecules (DNA, proteins) and virus-based vaccines.

In the field of passive strategy, considerable efforts have been made on the delivery of drugs using nanoparticles (NPs), which include lipid-based formulations, dendrimers and micellar NPs, polymeric NPs, and metallic NPs. In recent years, much focus has been placed on metallic NPs and polymeric for their biocompatibility and tunable properties for enhance TDD. Motivated by pioneering works, this thesis aims to further explore the use of metallic and polymeric NPs as a carrier for oligonucleotides, for detection and regulation of biomarkers.

Firstly, gold nanorods (AuNR) was used as the metallic carrier for oligonucleotides (as described in Chapter 3). In brief, molecular beacon probes (MB), hairpin-like DNA oligonucleotides with specific recognition towards LRG1 mRNA was developed to detect changes in gene expression during pathological angiogenesis. These MBs were conjugated onto the AuNR via gold-thiol bonds to form Lrg1 MB-AuNR. The size and conjugation efficiencies were firstly evaluated. Importantly, the integrity of MB probes following conjugation onto AuNR was also addressed. Following which, the Lrg1 MB-AuNR were studied for cellular internalization and cytocompatibility in human skin keratinocytes. Next, similar trends between the signal generation and gold standards of RT-PCR and Western Blotting were observed when the cells were subjected to chemical and mechanical treatments to alter Lrg1 gene expression. Finally, Lrg1 MB-AuNR were

topically applied on the mice skin for Lrg1 gene detection *in vivo*. Fluorescence histological studies reveal Lrg1 gene expression in the epidermis, similar to that observed in *in vitro* skin keratinocytes cells.

Secondly, mesoporous silica nanoparticles (MSNPs) was used as a polymeric carrier for MBs (as described in Chapter 4). Different from AuNR, the MBs were encapsulated into the pores of MSNPs, and were modified with positively charged poly-L-lysine to form MSNPs-MB-PLL. Similar to Chapter 3, the encapsulation efficiency of MBs, size and surface charges, as well release profile of MBs from MSNPs-MB-PLL were firstly evaluated. Following which, MSNPs-MB-PLL was studied for actual cellular application and cytocompatibility with housekeeping gene target, GAPDH. Next, by choosing MBs targeted towards gene that is regulated with chemicals, reduction in signals was correlated with RT-PCR. Subsequently, the ability for MSNPs-PLL to penetrate the skin was tested in a xenograft squamous cell carcinoma (SCC) model with Cy5 fluorescence dye. Following successful and promising demonstration that MSNPs-PLL can effectively deliver oligonucleotides across the skin, we then proceeded to deliver TGF β R-1 siRNA. An 18 days study was performed in a xenograft SCC model where the topical application of MSNPs-siRNA-PLL was performed every 3 days. Finally, the tumors were excised and studied for TGF β R-1 regulation.

Despite the use of biocompatible nanomaterials with enhanced modification for improve TDD, the penetration is still limited by the size of NPs. Trans follicular route plays a major role in TDD, where NPs of size ~100-200nm enters the skin. However, the follicular route may lead to unspecific delivery of drugs. In light of this, active strategies are widely used, based on the application of external physical forces to increase skin penetration. Current technologies such as iontophoresis, ultrasound, electrophoresis require expensive, bulky, and complicated instruments operated only by healthcare professionals. Microneedles (MN) are intensively studied as a potential alternative to injection. However, its drug loading is limited by the size of it. Therefore, development of a cheap, easy to use, and effective technology for TDD will benefit patients and healthcare institutions.

In this thesis, for the first time, temporal pressure is described and developed as a technology for TDD. Pressure is achieved with the use of magnets of specific forces. The optimization of Temporal pressure was described in this chapter to achieve a balance between effective TDD, while protecting the skin integrity. Following which, model drugs namely dextran molecules and nanoparticles of different sizes were delivered in vivo and studied with IVIS and histology. Importantly, the mechanism behind temporal pressure was also addressed. To prove its translatability, insulin was used

as proof of concept in normal and diabetic mice for a 5 days treatment. The effectiveness of temporal pressure in insulin penetration was measured by the drop in glucose level, and amount of insulin in blood plasma. Finally, the efficacy of temporal pressure was also compared against hypodermic injection and microneedles.

In conclusion, proposed NPs application in passive TDD have been demonstrated to be an effective carrier for genetic probes and regulators in various skin conditions (pathological angiogenesis and squamous cell carcinoma) and confirmed using gold standard PCR method and Western Blot. Following which, temporal pressure in active TDD have been demonstrated to be a promising alternative to conventional hypodermic injection as well as the recent microneedles. Importantly, both the passive and active strategies described did not result in significant damage to the skin integrity. On this note, nanotechnology remains as a potential carrier for drugs and temporal pressure has great potential in the future to assist in the field of transdermal drug delivery, as will be elaborated in the following section.

6.2. Future Directions

As hypothesized and briefly demonstrated herein, capability of nanotechnology and temporal pressure in enhancing TDD non-invasively and in a patient-friendly manner. Such technology can benefit the field of TDD in various ways, both for in vivo optimization well as allowing more comprehensive pre-clinical studies to be performed. Several ways in which both the described technologies could be adapted are listed below.

Optimization of MB-AuNR assembly and maximize delivery depth for detection of dermal tissues biomarkers.

As mentioned previously, MB-AuNR's sensitivity remains a challenge to be solved. While the AuNR's absorption spectrum may overlap with the fluorophore emission, slight background fluorescence is inevitable. Distinct gold nanoparticle morphology could be adapted for improved quenching, such as the distance between AuNR and fluorophore can be shortened for stronger fluorescence quenching. Furthermore, the ability of MB-AuNR to detect early diagnosis of pathological angiogenesis remains a question. In the future, in vitro/in vivo studies can be performed to study the Lrg1 copy number in individual cells (both healthy and pathological state) and correlate it to the nanosensor's detection limit.

Extending beyond just *in vitro* studies, it is of importance to further validate the penetration of these MB-AuNR in skin. Possible studies include silver staining to quantify the MB-AuNR in the skin. Last but not least, this thesis was limited to studying Lrg1 expression which was expressed highly in the mice skin epidermis. To study the penetration depth and the efficacy of these NPs in the dermal tissues, biomarkers such as Collagen Type I (found in dermal fibroblasts) can be used as the model biomarker to validate.

Development of MSNPs to achieve improved targeted TDD.

The current version of MSNPs-PLL only utilize positively-charged poly-L-lysine coating to non-specifically enhance nanosensors interaction with cell membrane and thus its internalization efficiency. For the purpose of delivering drugs to a single cell type in *in vitro* settings, such formulation is facile, versatile and yet meets the criteria. Nonetheless, skin is a more complex culture system consisting of multiple cell types and MSNPs internalization by specific cells of interest would be beneficial.

Such selectivity can be achieved by conjugating the surface of MSNPs with targeting moieties, including but not limited to antibodies and aptamers (oligonucleotides with specific hybridization towards proteins- in this case cell surface marker). While it may potentially alter release profile of

MB probes encapsulated within the pores, conjugation with these targeting moieties may also facilitate greater cellular internalization efficiency.

Technology development for temporal pressure

The efficacy of temporal pressure was demonstrated, and it is a promising alternative to hypodermic injection. It is also worth studying if temporal pressure could enhance the transdermal delivery of gold nanorods and mesoporous silica nanoparticles in Chapter 3 and 4 respectively. However, the current state of technology development for temporal pressure is at its preliminary with the use of magnets to produce the pressure. Before consideration can be made for clinical application, several concerns need to be address. Firstly, human skin thickness differs individually and in different parts of the body, thus it is necessary to explore the effectiveness of various pressure strength and time of application on different thickness of human skin and different parts of the body²⁰⁹⁻²¹⁰. Secondly, pressure application using magnets is inconvenient and dangerous for people with metal implants. Therefore, a device can be designed similar to a wearable watch, incorporating microelectronics e.g. inverse piezo electronic to produce pressure. Last but not least, only insulin was studied as the model drug in this thesis. A wide range of possibilities remain to be explored, which include vaccines, nanoparticles, hormones, etc.

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