**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**MICRO AND NANOTECHNOLOGY FOR
REGENERATIVE MEDICINE: CELL
TRACKING AND ENGINEERING**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2017

MICRO AND NANOTECHNOLOGY FOR REGENERATIVE MEDICINE: CELL TRACKING AND ENGINEERING

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

2017

ACKNOWLEDGEMENTS

At the beginning of the thesis, I would like to appreciate my two supervisors Prof Yuejun Kang and Chenjie Xu most sincerely. They are always very patient, considerate and enthusiastic to provide help whenever I have some problems and difficulties. Thank them for teaching me and sharing with me their precious experiences for research project design, problem solving skills, scientific writing and presentation, as well as collaboration with others.

Besides my supervisor, I also want to thank my collaborators including Prof Bin Cao, Prof Milan Mrksich, Dr. Zhigang Xu, Dr. Yingdan Zhang, Dr. Sreejith Sivaramapanicker for their help and support during my researches.

Special thanks to my group members in the two groups, including Dr. Chuah Yon Jin, Dr. Nishanth V Menon, Li Min Tay, Raditya Anggara, Peng Xue, Yafeng Wu, Yingnan Wu, Dang Tran Chien, Dr. David Yeo, Dr. Keming Xu, Dr. Min Wang, Dr. Hao Chang, Christian Wiraja, Mingyue Cui, Yiyuan Han, Mengjia Zheng, Duong Phan Khanh, Daniel Lio, Huseyin Enis Karahan, Zayim Razina S/O Seei Syed, and so on.

Finally, I want to thank my parents, sister and husband for their endless support and contribution and my friends for their encouragement and comfort.

BIOGRAPHY

Shiying Liu graduated from Xiamen University with double Bachelor Degree in Biochemical Engineering and Mathematics & Applied Mathematics in 2010 in China. Then she was recommended to Institute of Process Engineering, University of Chinese Academy of Sciences without examination and obtained a Master Degree in Biochemical Engineering in 2013. She joined as a PhD student in School of Chemical and Biomedical Engineering, Nanyang Technological University in 2013. She is very interested in addressing biological problems by micro- and nanotechnology, including four directions. The first one was to track mesenchymal stem cell differentiation into adipocytes, osteocytes, and chondrocytes for a long term by photostable polymeric fluorescent nanoparticles. Secondly, pH and glutathione responsive drug delivery systems and multi-adjuvant whole cell tumor vaccine were developed for anti-cancer chemotherapeutic and immunotherapy. In addition, she also developed a facile and gentle strategy to load hydrophilic molecules into polymeric microneedles by swelling for abnormal scar treatment. Last but not least, microcontact printing technique was used for patterning single cells with desired sizes and shapes to investigate nanoparticle labeling efficiency and transfer between cells.

LIST OF PUBLICATIONS

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1. [Shi-Ying Liu](#)[†], Wei Wei[†], Hua Yue, De-Zhi Ni, Zhan-Guo Yue, Shuang Wang, Qiang Fu, Yue-Qi Wang, Guang-Hui Ma*, Zhi-Guo Su*. Nanoparticles-based multi-adjuvant whole cell tumor vaccine for cancer immunotherapy. **Biomaterials**, 2013, 34, 8291-8300. (IF= 8.978)
2. Shuo Huang[†], [Shiying Liu](#)[†], Kai Wang, Cangjie Yang, Yimin Luo, Yingdan Zhang, Bin Cao, Yuejun Kang*, Mingfeng Wang*. Highly fluorescent and bioresorbable polymeric nanoparticles with enhanced photostability for cell imaging. **Nanoscale**, 2015, 7, 889-895. (IF= 7.915)
3. [Shiying Liu](#)[†], Li Min Tay[†], Raditya Anggara, Yon Jin Chuah, Yuejun Kang*. Long-term tracking mesenchymal stem cell differentiation with photostable fluorescent nanoparticles. **ACS Applied Materials & Interfaces**, 2016, 8, 11925-11933. (IF= 7.332)
4. [Shiying Liu](#), David C Yeo, Christian Wiraja, Hong Liang Tey, Milan Mrksich, Chenjie Xu*. Peptide delivery with poly(ethylene glycol) diacrylate microneedles through swelling effect. **Bioengineering & Translational Medicine**, 2017, 2, 258-267.
5. [Shiying Liu](#), Pradeep Bugga, Milan Mrksich, Chenjie Xu*. Nanoparticle labelling transfer between single cells. (in preparation)
6. [Shiying Liu](#), Nishanth V.M., Sreejith S., Kang Yuejun*. Alleneamide-thiol site specific labeling of CD44 vector for selective cancer bioimaging. (in preparation)
7. Zhigang Xu[†], [Shiying Liu](#)[†], Yuejun Kang*, Mingfeng Wang*. Glutathione- and pH-responsive nonporous silica prodrug nanoparticles for controlled release and cancer therapy. **Nanoscale**, 2015, 7, 5859-5868. (IF= 7.915)
8. Zhigang Xu[†], [Shiying Liu](#)[†], Yuejun Kang*, Mingfeng Wang*. Glutathione-responsive polymeric micelles formed by a biodegradable amphiphilic triblock copolymer for anticancer drug delivery and controlled release. **ACS Biomaterials Science & Engineering**, 2015, 1, 585-592.

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10. Zhigang Xu, [Shiying Liu](#), Hui Liu, Cangjie Yang, Yuejun Kang, Mingfeng Wang*. Unimolecular micelles of amphiphilic cyclodextrin-core star-like block copolymers for anticancer drug delivery. **Chemical Communications**, 2015, 51, 15768-15771. (IF= 6.628)
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12. Lifeng Wang, Keming Xu, Xiaochun Hou, Yiyuan Han, [Shiying Liu](#), Christian Wiraja, Cangjie Yang, Jun Yang, Mingfeng Wang, Xiaochen Dong*, Wei Huang*, and Chenjie Xu*. Fluorescent Poly (glycerol-co-sebacate) acrylate nanoparticles for stem cell labeling and longitudinal tracking. **ACS Applied Materials & Interfaces**, 2017, 9, 9528-9538. (IF= 7.332)
13. Sreejith S., Nishanth V.M., Yue Wang, Hrishikesh Joshi, [Shiying Liu](#), Kok Chan Chong, Yuejun Kang, Handong Sun and Mihaela C. Stuparu*. All-organic luminescent nanodots from corannulene and cyclodextrin nano-assembly: continuous-flow synthesis, non-linear optical properties, and bio-imaging applications. **Materials Chemistry Frontiers**. (Cover)
14. Zhigang Xu, Peng Xue, Yong-E Gao, [Shiying Liu](#), Xiaoxiao Shi, Meili Hou, Yuejun Kang*. pH-responsive polymeric micelles based on poly (ethyleneglycol)-b-poly (2-(diisopropylamino) ethyl methacrylate) block copolymer for enhanced intracellular release of anticancer drugs. **Journal of Colloid and Interface Science**, 2017, 490, 511-519. (IF= 3.758)

CONFERENCE PROCEEDINGS

1. Poster presentation. Highly fluorescent and bioresorbable polymeric nanoparticles with enhanced photostability for cell imaging. **Biomedical Engineering Society 8th Scientific Meeting, Singapore, 17th May, 2014.**
2. Oral presentation. Long-term tracking mesenchymal stem cell differentiation with photostable fluorescent nanoparticles. **6th International Conference on Mechanics of Biomaterials and Tissues, Hawaii, USA, 6-10 Dec, 2015.**
3. Poster presentation. Peptide delivery with poly(ethylene glycol) diacrylate microneedles through swelling effect. **School of Chemical and Biomedical Engineering PhD Symposium, Singapore, 21-22 Mar, 2017.**
4. Oral presentation. Peptide delivery with poly(ethylene glycol) diacrylate microneedles through swelling effect. **9th International Conference on Materials for Advanced Technologies, Singapore, 18-23 Jun, 2017.**

AWARDS

1. National Encouragement Scholarship (2007)
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5. Third prize in National Challenge Cup Shanghai Expo Special Contest (2009)
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8. Awarded “Excellent Graduate” (2010)
9. NTU Research Scholarship (2013-2017)

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SUMMARY

Regenerative medicine is providing a promising treatment method for degenerative diseases, and has been extensively investigated in recent decades for bone, skin, cartilage, neurons, blood vessels, skin regeneration, and so on. One of the most important branches is to exploit multi-potent stem cells to differentiate into targeted tissues and organs before transplantation. Mesenchymal stem cells (MSCs) have been identified and widely explored as a promising and main source for regenerative medicine, with great differentiation potentials into osteocytes, adipocytes, and chondrocytes but less concern of ethical issues. However, a tough problem is to track the cell location, migration, fate and distribution of these transplanted cells, with clearer understanding about these will help instruct the optimum cell dosage, transplantation time, and evaluate efficacy. It is imperative to develop a photostable probe with excellent biocompatibility to track the MSCs for a long-term without affecting the cell proliferation and potency.

Another issue which is expected to be solved in this thesis is to develop an effective and conveniently used drug delivery system for treatment of keloid scars to achieve skin regeneration. Keloid scars, as a complex abnormal scar, cause not only aesthetic disfigure to the patients, but also have led to pruritus, infection, and much distress. Traditional treatments include surgery removal, silicone or pressure dressings, hypodermic injections of drugs like corticosteroid, fluorouracil (5-FU), and so on. However, these methods may result in pain, infections, and adverse effects as well as less compliance to patients. Transdermal delivery route by microneedles can provide a non-invasive method for delivering drugs with low cost and conveniences to patients.

This thesis targets to solve the above two problems by applying micro and nano technology. Specifically, enhancing the photostability of organic fluorophores will be achieved for long-term stem cell tracking in Chapter 2 and 3 by covalent

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In order to solve the second problem for keloid scar treatment, Chapter 4 will exhibit the application of biocompatible polymeric microneedles made from (polyethylene glycol) diacrylate (PEGDA) for loading of connexin mimetic peptide-Gap 26 by the swelling effect, which is expected to protect therapeutic biomolecules (including peptides) from enzymatic digestion that occurs in the oral route. Then the peptide-loaded microneedles will be applied to a keloid scar model and the effect of peptide on gap junction based intercellular communication and collagen I expression will be investigated *in vitro* and in an *ex vivo* model.

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
MSCs	Mesenchymal stem cells
ESCs	Embryonic stem cells
FDA	Food and drug administration
UCB	Umbilical cord blood
iPSCs	Induced pluripotent stem cells
MRI	Magnetic resonance imaging
NPs	Nanoparticles
GFP	Green fluorescent protein
QDs	Quantum dots
Cx	Connexin
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
PBS	Phosphate buffer saline
EDTA	Ethylenediaminetetraacetic acid
DPP	Di(thiophene-2-yl)-diketopyrrolopyrrole
PCL	Polycaprolactone
PPAR- γ	Peroxisome proliferator-activated receptors
Col I	Collagen type 1

Col II	Collagen type 2
Agg	Aggrecan
OCN	Osteocalcin
ALP	Alkaline phosphatase
RT-PCR	Real time Polymerase chain reaction
mRNA	Messenger ribonucleic acid
cDNA	Complementary DNA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
CT	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
MN	Microneedle
PEGDA	Poly(ethylene glycol) diacrylate
FRAP	Fluorescence recovery after bleach
PDMS	Polydimethylsiloxane
UV	Ultraviolet
ANOVA	Analysis of variance

Chapter 1: Introduction

1.1 Regenerative medicine

Human tissue and organs may be damaged by age, disease, or trauma. The limited source for transplantations and consequent immune problems require the development of another kind of novel therapy, which is able to regenerate tissues and decrease the reliance on transplantations. Regenerative medicine emerged as a promising field which targets to promote tissue regeneration by exploiting the multi-disciplinary expertise, including engineering and life science principles, which is potentially able to restore diseased and injured tissues and even the whole organs. Specifically, regenerative medicine aims to repair cells, tissues, and organs by exploiting the therapeutic value of body's own growth factors and stem cells[1-4]. It can utilize autologous or allogeneic stem cells to enhance the intrinsic healing processes and supplement repair deficiencies. By replacing, engineering, or regenerating human cells, tissues or organs can be regenerated to restore or establish normal function.

Since the appearance of the regenerative field several decades ago, it has received increasing attention and achieved much progress. A number of therapies especially for wound healing and orthopaedics applications, have obtained the approval from Food and Drug Administration (FDA) and are currently commercially available[1].

Among the strategies in regenerative medicine, many of them deliver autologous or allogeneic therapeutic and typically differentiated cells, which still have proliferation capacity[1]. For example, Carticel utilizes autologous chondrocytes, which are collected from articular cartilage, expanded *ex vivo*, and then implanted into the site of injury, to treat focal articular cartilage defects and has been approved by FDA in the orthopaedic field. This product leads to effective recovery and the result is comparable with that observed by microfracture and mosaicplasty techniques. There are also other examples like

laViv, targeting to improve the nasolabial fold wrinkles by injection of the autologous fibroblasts, and Epicel for severe burn wounds by autologous keratinocytes. These autologous cells are collected from a patient's tissue, and often require a long time before treatment for the cell expansion *in vitro*. Another promising candidate is the allogeneic cell source with low antigenicity, which can allow off-the-shelf tissues with low risk of immune reaction. Other FDA-approved examples include GINTUIT and Apligraf, which exploit allogeneic human foreskin fibroblasts during the fabrication of wound-healing grafts.

1.2 Stem cell platform

With the direct contribution to innate healing, stem cells have been widely recognized to provide a promising source for regenerative medicine. In fact, there are many types of stem cells either extracted or bioengineered by molecular programming to provide enormous progenitor cells, which are endowed with multi-potency and can be applied for various regenerative applications. Stem cells from natural sources include embryonic stem cells, perinatal stem cells (such as stem cells derived from umbilical cord), as well as adult stem cells (such as stem cells derived from bone marrow including mesenchymal stem cells (MSCs)). In addition, iPSCs technology pioneered by Shinya Yamanaka's lab in Kyoto, Japan, also attracts much attention which can program adult cells and induce them to become pluripotent stem cells by gene encoding.

1.2.1 Embryonic Stem Cells

Embryonic stem cells (ESCs), derived from the inner cell mass of a preimplantation blastocyst, have the pluripotent capacity and can differentiate into various cell types, including neuron-like cells, dendritic cells, pancreatic islet-like cells, osteocytes, chondrocytes, adipocytes, skin, and so on. This amazing potency makes ESCs be able to produce a large number of transplantable cells for therapeutic use. In order to characterize pluripotency of

human ESC, several marker expressions can be detected, such as alkaline phosphatase, POU transcription factor Oct3/4, Nanog, Cripto/TDGF1, proteoglycans TRA-1-60/81, GCTM-2, and embryonic antigens SSEA-3 and SSEA-4. Early lineage commitment of the three germinal layers has been monitored by the expression of markers such as Sox1 for neuroectoderm, Pdx-1 for endoderm, and Flk-1 for mesoderm.

Currently, there are many clinical trials using ESCs-derived cells, including retinal pigment epithelium[5], pancreatic endoderm derivatives (for type 1 diabetes)[6], and cardiac progenitors (for severe heart failure)[7]. However, there is an unavoidable concern for the regenerative application of ESCs, that is the risk of teratoma formation after transplantation due to their unlimited growth capacity. However, further clarifications about embryonic developmental pathways to induce ESC into tissue-specific lineages can potentially solve this problem. On the other hand, proper regulation of ESC differentiation before and after transplantation is also very important for the safety in the clinical application.

1.2.2 Perinatal Stem Cells

In addition to ESCs, an increasing evidence has proved the differentiation capacity of perinatal stem cells, such as the ones from umbilical cord blood (UCB). These cells also exhibit stemness properties and are normally collected at the birth. UCB-derived stem cells transplantation with less immune tolerance, can lead to high degree of engraftment and less serious graft-versus-host disease[2]. And these UCB-derived stem cells can also be expanded, continuously cultured *in vitro*, and also differentiate into three embryonic germinal layers, including the endoderm (like mature hepatocytes, etc), mesoderm (like adipocytes, chondrocytes, osteoblasts, etc), and ectoderm (like neurons, etc)[2]. UCB transplantations have been successfully performed in more than 10,000 patients which have genetic, hematological or immunological disorders[8].

A main limitation of UCB transplantation is the low cell dose of UCB derived stem cells, thus resulting in an increasing risk of graft failure[9]. Besides, it's necessary to have a bigger investment in the public cord blood tanks considering the importance of HLA-matching and cell dose on outcomes after UCB transplantation. And there should be accredited cord blood banks and bone marrow donor registries especially for patients who don't have an HLA-matched sibling to benefit hematopoietic stem cell transplantation[8].

1.2.3 Engineered stem cells

The advanced development of biotechnology allows to perform genetic reprogramming for adult cells and render them with the embryonic differentiation abilities. These novel technologies including nuclear reprogramming can obtain the pluripotent stem cells without the need of embryo extraction. In 2006, Shinya Yamanaka's group in Japan achieved a great breakthrough in which they reprogrammed mouse skin fibroblasts into stem cells by overexpressing the stemness regulators[10], this marked the appearance of induced pluripotent stem cells (iPSCs). Thereafter, they and other groups in the world, such as James Thomson and George Daley also successfully converted human fibroblasts or somatic cells into human iPSCs[11-13]. Ever since, iPSCs attract increasing attention because they can avoid the ethical issues from using human embryos and the genetic programming can match to the donor[14-16].

Up to now, the clinical applications about iPSCs have not achieved a lot, with one which used autologous patient-specific iPSC derivatives for the treatment of age-related macular degeneration[17]. Starting from September 2014 to treat the first patient in Japan[18], the clinical trial was recently paused since the genetic mutations were found in the iPSCs from a second patient.

1.2.4 Mesenchymal stem cells

Adult stem cells are able to provide an abundant sources for progenitors from bone marrow, adipose tissue, as well as resident stem cell pools, compared with

the above stem cells[19]. Considering the easy accessibility, autologous status, and proliferative potential, these adult stem cells become the main source in the regenerative medicine clinically[20].

Although these stem cells may also be extracted from adipose tissue or the resident stem cell pool, currently the main source is from bone marrow. Mesenchymal stem cells (MSCs) with different potentials can be produced from bone marrow with high efficiency from the supporting architecture of the adult marrow, attracting an increasing attention for the regenerative medicine applications.

Mesenchymal stem cells can demonstrate multipotency to regenerate the bone, cartilage, muscle, and so on (Figure 1)[21]. They are also discovered to regenerate liver and pancreatic islet cell, as well as play a role in the protection of kidney, heart, or lung injury. In addition, by secreting various bioactive molecules, the regenerative microenvironment generated by mesenchymal stem cells can restrict the damage area and initiate a self-adjusted regenerative response.

Figure 1. Differentiation potentials of adult human MSCs. (Adapted with permission from[22]. Vasileios Karantalis, Joshua M. Hare. Use of Mesenchymal Stem Cells for Therapy of Cardiac Disease. Circulation Research. 2015; 116 (8): 1413-1430. Copyright (2015) Wolters Kluwer Health, Inc. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4429294/>)

Current clinical trials include mesenchymal stem cells from both endogenous and exogenous tissues, in the applications for treatment of osteogenesis imperfecta, Crohn's disease, and graft-versus-host disease[23]. It's favorable to apply the allogeneic mesenchymal stem cell to treat some acute diseases, such as acute myocardial infarction, and so on, since preparing allogeneic cells can efficiently save time without need to extract and expand autologous cells from the recipient. However, if the mesenchymal stem cells are not needed urgently for some patients, it will be better to extract and expand patients' cells before implantation as personalized cell-based therapy[24].

1.3 Mesenchymal stem cell tracking

The past decades also witnessed the increasing researches and achievements in stem cell field, which have broadened the treatment for many diseases, such as diabetes, neurodegenerative or cardiovascular diseases. Human stem cells, especially mesenchymal stem cells (MSCs), owning the self-renew ability and high differentiation potency, can provide an abundant source for the wide application in regenerative medicine, which is very promising to revolutionize the treatment of various diseases[22, 25, 26]. However, although many results *in vitro* or *in vivo* are promising, there are still many issues which are needed to be solved before human use. One of the problems is the shortage of methods to understand the fate, distribution, and the function of transplanted stem cells in the local microenvironment to make sure these transplanted cells have arrived in the sites of injury[27]. Previously, monitoring the fate of transplanted stem cells has to depend on the histological studies at the desired time points after the transplantation. Therefore, it's very necessary to develop a non-invasive imaging methods to real-time monitor the transplanted stem cells, which can benefit the optimization conditions for cell dosage, delivery route, and time

points of transplantations, as well as prediction of the therapy efficacy[28, 29]. The long-term tracking technique will help researchers have a better understanding about the distribution, motility, location, and roles of stem cells in the transplanted scaffold or tissue engineered organs, and further analyze their interaction with the patient's cells and body[22, 25, 26, 30, 31].

In order to achieve stem cells tracking, magnetic resonance imaging (MRI) is commonly used[32-34]. Many researchers developed magnetic contrast agents to try to achieve MRI *in vivo* by superparamagnetic iron oxide nanoparticles (SPIONs). For example, by coating the dextran or other polymers on the nanoparticles' surface, their stability and solubility can be increased and this way also reduces aggregation, which provides a useful tracking method for stem cells in MRI and is much better than the traditional gadolinium-based contrast agents. However, using this dextran coated SPIONs also causes problems. It is reported that stem cells are unfavorable to internalize these SPIONs by endocytosis; therefore, high labeling efficiency has to be achieved by using higher concentrations of the nanoparticles (NPs) and additional transfection agents. On the other hand, iron oxide NPs have been reported to promote the free hydroxyl radicals and reactive oxygen molecules after dissolving within cells, which may lead to some toxic effects to induce apoptosis of cell and alter the cell metabolism. What's more, Fe^{2+} irons dissolved may result in potential side effects on cells[32].

Other than MRI, fluorescence imaging can also be used as a technique for tracking MSCs in tissue engineering since it offers superb temporal and spatial resolution, high sensitivity and is easily applicable. Fluorescence imaging allows insights into living organisms without the need for dissection as a sensitive, cost-effective and non-invasive method[31, 35-39].

In order to achieve fluorescence imaging, a lot of fluorophores have been widely used owing to their high fluorescence quantum yield, relatively low toxicity, and structural versatility that enables facile tuning of the optical properties and conjugation with biomolecules[40-44]. For example, Alexa Fluor[®] family of

fluorescent molecules has been commercially available and used as cell and tissue labels in fluorescence microscopy and cell biology. Another example is 4',6-diamidino-2-phenylindole, denoted as DAPI, which has been a commonly used fluorescent probe for selectively labeling of DNA in cell nuclei.

Despite these advantages, most organic fluorophores suffer from their poor photostability, i.e. the fluorescence intensity decays quickly (tens of seconds) under continuous exposure to light. This phenomenon, also called photobleaching, limits the ability of employing fluorescence microscopy or spectroscopy for long-term tracking of biological activities.

Apart from available organic dyes, researchers also use genetic transfection to introduce the gene which can express fluorescent substances to achieve fluorescence labeling, such as green fluorescent protein (GFP). Unfortunately, the commonly used traditional organic or genetically encoded fluorophores, often have broad excitation and emission spectra, susceptible to photobleaching which restrict their applications in long-term and multiplexing studies. Also, genetic transfection is time-consuming which results in low labeling efficiency. Moreover, it has been reported that fluorescent protein transfection even influences the normal cell function, thus hindering the applications of GFP-based probes[45].

1.4 Nanoparticle based probes

To overcome the limitations of organic dyes and genetic proteins, various nanoparticles based fluorescent probes have been developed for stem cell tracking, especially semiconductor nanoparticles represented by quantum dots, silica nanoparticles, polymeric nanoparticles, and so on.

1.4.1 Quantum dots

In the past decades, new fluorescent probes with high quantum yields and wide absorption/emission spectra have been developed, which are called Quantum dots (QDs). As novel semiconductor NPs, they have attracted numerous

attention in various applications with superb optical properties[46-50]. They have large effective Stokes shifts. The quantum yield and molar extinction coefficients are high (~10-100 times of that of organic dyes). The molar extinction coefficient of QD emitted at 510 nm even if at the lowest absorption wavelength, 450 nm, is greater than that of rhodamine red at its maximum absorption[46] (150,000 versus 129,000 M⁻¹ cm⁻¹) (Figure 2A and B). Most importantly, they can be excited using one single excitation light source but have narrow and symmetric fluorescence spectra ranging from UV to near-infrared light, allowing to excite QDs with various emissions at the same time[51]. Therefore, QDs can eliminate autofluorescence through selecting a proper excitation wavelength[52, 53]. Furthermore, their fluorescent emission can be tunable with core size (Figure 2C), making it possible to control the probe color by adjusting the size and separate different probes without spectral overlap[46, 52]. Traditional organic fluorescences often encounter with the serious problem of photobleaching, thus failing to provide long-term stability and achieve multiplex labeling. QDs can tackle these issues by virtue of their good photostability[53]. All the distinctive properties make QDs popularly investigated in biological sensing and imaging.

In addition, QDs can also be conjugated to some biomolecules (proteins, DNAs) or antibodies to enhance biocompatibility. It was reported that human MSCs can be labeled with QDs which have been conjugated with arginine-glycine-aspartic acid peptide and differentiated into chondrogenic, androgenic, and adipogenic cells for a long-term tracing probe[30]. Xingyong Wu et al developed some specific immunofluorescent probes by surface modification and conjugation QDs with IgGs[53]. In the single cell level, they detected the dynamic fluorescence change and compared QD-608 to Alexa-488[53]. The fluorescence of Alexa 488 reduced fast and became dim within 2 min, nevertheless QD 608 kept their brightness for the entire time period (3 min) (Figure 3).

QDs have exhibited superb photostability and brightness in biological applications, however, the current popularly applied QDs such as CdSe and CdTe have heavy metal elements, which result in the toxicity in oxidative conditions. What's more, some reports indicate QDs may aggregate within cells and exhibit unstable fluorescence signals. These shortcomings compromise the QDs' application in the clinic[30, 34].

Figure 2. (A) Rhodamine red and genetically encoded DsRed2 protein's absorption and emission. (B) Six QDs' absorption and emission spectra with the black line indicating the absorption of QDs emitted at 510 nm. (C) Six QDs exhibit size-tunable fluorescence properties. Reprinted by permission from Macmillan Publishers Ltd: [Nature Materials][54], copyright (2005).

Figure 3. Photostability of QDs compared with Alexa 488 within single cell. (A) In the up row, QD-630-streptavidin (red) stained the nuclear antigens, while Alexa 488 (green) labeled microtubules in a 3T3 cell. In the below row, QD-630-streptavidin (red) stained microtubules, while Alexa 488 (green) labeled the nuclear antigens. During successive exposure to light from a usual mercury lamp (100 W) for 3 min, the samples were taken images at indicated time points. Scale bar, 10 μ m. (B) Normalized fluorescence intensity change curves of QD-608-streptavidin and Alexa 488 which have stained microtubules and nuclear antigens respectively. Reprinted by permission from Macmillan Publishers Ltd: [Nature Biotechnology][53], copyright (2003).

1.4.2 Silica nanoparticles

In addition to quantum dots, researchers also designed many other nanoparticles based on silica and polymers containing fluorescent dyes to improve the photostability. The biocompatible silica shell can protect the concentrated fluorescent dyes from oxidation or decomposition, thus generating strong fluorescence. Especially, the mesoporous silica NPs have attracted much attention as a novel biomaterial since they are biocompatible and biodegradable[55], can provide an adequate surface area and abundant pore volumes[56]. In addition, the silanol groups on the surface can be used for chemical modification for specific function[57].

There are two methods sol-gel or reverse microemulsion to fabricate fluorescent silica nanoparticles[58]. The famous Cornell dots or C-dots have been regulated by FDA for application in tumor and entering phase-I clinical trials. In this example, fluorescent organic dye was conjugated with a silica precursor to fabricate the dye-containing core, followed by co-condensation with the silica sol-gel monomers in a specific order. The fabricated C-dots exhibited strong brightness, photo-stability, and multi-functions on the surface[59].

In the applications of silica nanoparticles on stem cells, Chung et al found that the mesoporous silica nanoparticles containing fluorescein isothiocyanate can be internalized in human bone marrow mesenchymal stem cells, and they did not influence stem cells' viability and proliferation[60]. However, importing the mesoporous silica nanoparticles increased actin polymerization but induced not enough osteogenic signals in human mesenchymal stem cells[61].

1.4.3 Polymeric nanoparticles

Similar as silica nanoparticles, polymer-based platform can also provide biocompatibility for the stem cell tracking. In order to achieve fluorescence, there are two methods to contain fluorescent organic dyes, including physical encapsulation in the polymer solution before the fabrication of nanoparticles, and covalent conjugation with the polymer chain.

Currently, polystyrene (PS) NPs are popular fluorescent polymeric nanoparticles, which mainly encapsulate fluorescent dyes physically in the polymer solution. PS NPs can label stem cells by intracellular uptake by endocytosis and different surface functional groups have influences on cellular uptake. For example, Jiang et al. studied the uptake of two types of polystyrene NPs in MSCs by confocal fluorescence microscopy and flow cytometry, including plain PS NPs and amino-functionalized PS NPs (NPS)[62]. They found NPS nanoparticles could be rapidly internalized and accumulated to a much higher level in MSCs than plain PS nanoparticles, which was predominated by the clathrin-mediated endocytosis[62].

After intracellular internalization, PS NPs can be used as a tracer to track MSCs differentiation. In a further study, Andrea Tautzenberger et al applied phosphonate-functionalised polystyrene nanoparticles fabricated by miniemulsion polymerisation in the differentiation of human MSCs[63]. They have successfully intracellularly incorporated the nanoparticles into the MSCs without using any transfection agents[63]. They demonstrated that these polystyrene nanoparticles didn't affect cell viability and their potential for osteogenic, adipogenic and chondrogenic differentiation[63].

In addition to MSCs, PS NPs can also be applied to ESCs. It was found the ESCs exhibited columnar and embryoid body shape in low concentration (0.4 million/mL) polystyrene nanoparticles[64]. However, ESCs cultured in high concentration (12.2 million/mL) nanoparticles became fibroblast-like and less squamous types[64]. After 24 h, the viability of ESCs was reduced by 40% and 30% in the low and high concentration of nanoparticles, respectively, which implies the negative effect of polystyrene nanoparticles on ESCs[64].

In spite of the wide applications, PS NPs have some limitations which include low dye encapsulation and inadequate protection for the dye, leading to easy leaching, quenching and photobleaching[65]. In addition to PS, there are other polymers for the similar applications including poly(thiophene-3-yl-acetic acid),

poly(arylenediethylenes), polyacetylene, and so on[65]. However, current available polymer NPs mostly encapsulate dyes in the polymer solutions, while there are few polymeric NP products which are by covalent conjugation. Actually, extinction coefficients (typically $10^6 - 10^7 \text{ M}^{-1} \text{ cm}^{-1}$) and quantum yield (up to 80%) are generally high in the conjugated polymers, since π^* excited states can make excitons to easily diffuse within a polymer chain, which enhances the fluorescence responses.

1.5 Keloid scar treatment by transdermal delivery

In addition to stem cell tracking, there is another tough problem in the regenerative medicine field, which is abnormal scar treatment, including keloid scars. These scars are formed due to exuberant healing response[66]. The abnormal healing responses include three stages, inflammation at the first 10 days, proliferation from 10 to 14 days and maturation or remodeling from two weeks to years[67]. Keloids as elevated fibrous scars, often progress and exceed the borders of the original wound. Surgery removal has limitations as the keloid scars usually recur.

Keloids are easier formed in dark skin with a 15- to 20-fold increased risk probably due to abnormal melanocyte stimulating hormone. People with black or yellow skins have more possibility to form keloids than white skins[68]. In addition, formation of keloids is related with age, and people younger than 30 years old have higher risk, especially teenagers between 10 to 20 years old[69]. Positions in the body including sternal skin, cheeks, earlobes, and shoulders have more tendency to develop keloids[70].

Family genetic reason is recognized to influence the formation of keloid scar[71]. Besides, some accidents including trauma, delayed healing longer than three weeks, and burn, increase keloid development. Also, certain reasons like vaccinations especially bacille Calmette-Guérin vaccination, acne, ear piercing, lacerations, and biopsy procedures can cause abnormal scarring. Acne keloids

are pretty popular among them. Keloids can cause a lot of trouble to the patients, including not only cosmetic disfigurement and serious emotional distress but also pruritus and pain.

1.5.1 Conventional treatment for keloid scars

As mentioned, keloid scars can cause much trouble to the patients. Unfortunately, although various methods were developed to help management of keloid scars, there is still not an ideal therapy which can provide the best treatment for keloids. The main reason for the challenging clinical problem is the lesion often recur at a high rate even after almost any type of available treatment. Currently, the clinical doctors can only check family history or previous problems of scarring and estimate the risk of developing a scar before any surgical procedures. In some cases, doctors have to perform the surgery to remove the scars. Then the doctors have to inject immediately corticosteroid and pressure dressings to enhance wound healing and reduce the skin tension, which is helpful to reduce the risk of scarring[72, 73]. These common measures may cause pain, infection, and high cost. In addition, these available clinical measures require exemplary adherence and follow-up.

One popular method for keloid management, such as corticosteroid injection can suppress inflammation/mitosis as well as increase vasoconstriction of the scar. In a common practice, 10-40 mg/mL of triamcinolone acetonide suspension (Kenalog) is injected intralesionally. This manner can finally have effects on 50 to 100% of keloids, however, it has a high recurrence rate (9 to 50%) and causes a lot of pain[70]. In order to achieve better effects, corticosteroid injections are often combined with surgery to fresher keloids, which are more sensitive to treatments. However, it may result in atrophy, telangiectasias, and hypopigmentation as side effects. In addition to corticosteroid, intralesional injections of verapamil, fluorouracil, and bleomycin are also investigated for keloid scar treatment. For example, fluorouracil (50 mg/ mL, 2 to 3 times per week) injections intralesionally exhibited shrinking keloids safely and didn't

cause the side effects about tissue atrophy and telangiectasia, which are commonly caused in corticosteroid injections[74].

In addition to intralesional injections of drugs, the second method for the prevention and treatment of keloids is silicone sheeting noninvasively. The mechanism is because silicone sheets are able to soften and flatten the scars through altering the temperature, hydration, as well as the oxygen tension in the obstructed scar[69]. However, this method can only be used for the closed skins not for any open wounds. Although more than 60 related products are commercial, most of them are expensive. And these silicone sheets must be put on the scar regions for 12 to 24 hours every day and last for 2 to 3 months in order to achieve effectiveness, which causes a lot of comfortlessness[67]. Most importantly, a recent review indicated that most researches regarding the promising results about this method have limited quality and subjective bias[75].

In addition, combination of several methods can be applied to achieve a better treatment. In order to increase the reduction of recurrence, the surgical removal of scar is better complete, or only a little left on the wound margin. An effective result was achieved in one study by a combination comprising of surgery, intrakeloidal steroid injection and cream, and silicone gel sheeting. This triple therapy treated 180 patients and took 2 to 4 years for the follow-up. The final results from 120 patients demonstrated the recurrence rate was only 12.5% after 13 months[76], with 15 out of 120 patients still have keloid formation. The result is promising, but this triple therapy is time consuming and tedious. At first, 180 patients were involved in this study, however, sixty of them discontinued for the follow-up and only 120 patients came for review finally. Thus, this frequent follow-up caused inconveniences to patients, and it will be better there is a method which patients can apply by themselves and don't need to go to hospitals frequently.

1.5.2 Keloid fibroblasts engineering by transdermal delivery

Figure 4. Non-genetic cell engineering. (Reprinted from [77], copyright (2015) with permission from Elsevier)

Figure 5. Non-genetic cell engineering strategies including cell surface modification and delivering free drugs or encapsulated drugs by nanocarriers. (Reprinted from [77], copyright (2015) with permission from Elsevier)

In recent years, non-genetic cell engineering (Figure 4) have been extensively explored to achieve new therapeutic function by utilizing physical, chemical and material methods[77]. Compared with genetic engineering methods, these

non-genetic ways can overcome the concern of safety due to the lack of control over the genomic location of the inserted genes and the risk of insertional mutagenesis. Figure 5 shows the currently available major non-genetic cell engineering strategies. For example, through using drug delivery systems, free drugs can be delivered to targeted tissues. Using non-genetic engineering strategy can deliver a broad range of drugs, including synthetic drugs that cannot be produced by cells through genetic engineering.

In order to effectively inhibit keloid scarring and improve treatment, various drugs as mentioned in Section 1.5.1 have been injected into the keloid lesion by traditional needles. This traditional route causes a lot of pain to the patients, and may have the risk of infection. In addition, it also generates dangerous medical waste and needle re-use especially in developing countries may result in risk of disease transmission. Besides, as these treatments require frequent follow-up in the hospitals, which caused inconveniences to patients.

A novel technology to deliver drugs transdermally by microneedles has achieved great progress in recent years[78, 79]. Transdermal delivery has many advantages compared with the traditional hypodermic route and oral route. It's non-invasive and can be self-administered by patients, and also it can control the release of drugs for a long period of time (up to one week), which could improve patient compliance[78]. In addition, the microneedles with controllable depths can penetrate the skin stratum corneum and avoid the blood and nerve supply inside the skin, which don't cause any pain and infections to the patients, so this delivery method has better compliance. Finally, the transdermal delivery system is generally inexpensive. Currently, this transdermal delivery route has successfully been applied for various drugs, especially delivery of vaccines[78].

The common drugs as mentioned in Section 1.5.1 including for prevention and treatment of keloids, such as corticosteroid have adverse effects. In recent years, more researches clarifying the relationship between intercellular communication mediated by gap junctions and scarring provide new insights

for improvement for scar reduction. Gap junctions are formed by intercellular channels composed of connexin proteins, which are known to impact inflammatory response, wound closure, and scar formation[80-84]. Suppressing connexins activity by antisense oligodeoxynucleotide was found to reduce the spread of tissue damage, accelerate wound closure, and reduce scarring[85].

In order to inhibit connexins activity, various inhibitors were developed including halothane, octanol, 18 α -glycyrrhetic acid, carbenoxolone, and flufenamic acid. However, they have limitations with radical changes in membrane fluidity and influence cell's metabolic/signaling cascades[86]. And all of them exhibited poor specificity towards connexin (Cx) channels[86]. It's urgently required to have a tool that specifically target Cx channels by a rapid and easily reversible mode of action.

Connexin mimetic peptide, can mimic specific amino acid sequences of two well-conserved extracellular loops of connexins and interfere with their functions[87]. Gap 26 and 27 are the most popular mimetic peptide, with Gap 26 has better efficacy at the same concentration, and have been used extensively to suppress gap-junctional communication constructed of Cx43 mainly, but also Cx32, 40 and 37 in a wide range of cells and tissues[87].

1.6 Objectives of this dissertation

Tracking the cell movement, location, migration, and distribution of transplanted stem cells can help instruct the optimum cell dosage, transplantation time, and evaluate efficacy. Currently available tracking probes suffer from poor photostability or potential toxicity. It is very necessary to develop a photostable probe with excellent biocompatibility to track the MSCs for a long-term without affecting the cell viability, proliferation, and differentiation potency.

In another issue for treatment of keloid scars to achieve skin regeneration, traditional treatments including surgery removal, silicone sheet dressings,

hypodermic injections of drugs like corticosteroid, fluorouracil (5-FU), may result in pain, infections, and adverse effects as well as less patients' compliance. It's imperative to deliver drugs in a cheap, non-invasive and convenient way to patients without causing pain and infections.

This thesis aims to solve the above issues by developing and applying biocompatible and photostable fluorescent nanoparticles for long-term stem cell tracking and engineering keloid fibroblasts by transdermal delivery of hydrophilic peptide for keloid scar treatment.

More specifically, this thesis targets to achieve the following objectives:

- 1) To enhance the photostability of organic fluorophores, for example, di(thiophene-2-yl)-diketopyrrolopyrrole (DPP), it was covalently linked in the middle of a biocompatible and bioresorbable polymer chain, polycaprolactone (PCL). The polymer chain protection and steric effect are expected to suppress the aggregation of DPP to increase photostability. Subsequently, the polymer will be used to fabricate nanoparticles and applied for mesenchymal stem cell imaging. The nanoparticles' photostability will be compared in Chapter 2 with commercial organic dyes, well-recognized photostable quantum dots, and physical blends of dye/polymer complexes.
- 2) In order to efficiently track mesenchymal stem cells differentiation, the fluorescent probes should not only have strong fluorescence and are highly photostable, but also cause little negative effects on stem cell viability, proliferation behaviors, as well as differentiation performance. The second goal is to investigate the applications of the developed PCL-DPP-PCL nanoparticles in long-term tracking of MSCs differentiation. The effects on cell viability, proliferation, and multipotency of the MSCs in the three lineages *in vitro* including osteogenic, adipogenic and chondrogenic differentiations will be explored in Chapter 3.

- 3) In another target to develop a transdermal delivery system for keloid scar treatment, (polyethylene glycol) diacrylate (PEGDA)-based microneedles with good biocompatibility will be fabricated through photo-polymerization in Chapter 4. A model hydrophilic peptide, gap junction inhibitor (Gap 26), will be loaded into PEGDA microneedles through the swelling effect in the aqueous solution. Then the effects of peptide on gap junction based intercellular communication will be investigated and the delivery system will be applied to a keloid scar model and detected its therapeutic effects.

Chapter 2: Mesenchymal Stem Cell Imaging by Fluorescent and Bioresorbable Polymeric Nanoparticles with Enhanced Photostability

2.1 Introduction

Fluorescence imaging, due to its high specificity, sensitivity and imaging contrast, has proven to be an indispensable tool in modern biotechnology for advancing our understanding of various biological systems[44, 88]. Among a variety of probes for fluorescence imaging, small organic fluorophores have been most widely used owing to their high fluorescence quantum yield, relatively low toxicity, and structural versatility that enables facile tuning of the optical properties and conjugation with biomolecules[40-44]. For example, Alexa Fluor[®] family of fluorescent molecules has been commercially available and used as cell and tissue labels in fluorescence microscopy and cell biology. Another example is 4',6-diamidino-2-phenylindole, denoted as DAPI, which has been a commonly used fluorescent probe for selectively labeling of DNA in cell nucleus.

Despite these advantages, most organic fluorophores suffer from their poor photostability, i.e. the fluorescence intensity decays quickly (tens of seconds) under continuous exposure to light. This phenomenon, also called photobleaching, limits the ability of employing fluorescence microscopy or spectroscopy for long-term tracking of biological activities. Previous studies indicate that the formation of non-fluorescent triplet states (denoted as T_1), following the photonic excitation process, and the presence of molecular oxygen play a major role in the photobleaching of organic fluorophores. In air-saturated solutions, reactions between molecular oxygen and T_1 lead to substantial generation of reactive oxygen species (ROS), including $HO\bullet$, $HO_2\bullet$, and H_2O_2 , and rapid photobleaching.

Several strategies such as oxygen depletion and addition of protective agents as triplet-state quenchers, although being able to reduce photobleaching to some extent, have achieved limited success in fluorescence-based bioimaging[44]. Recently, Blanchard and coworkers[32] have reported a strategy of covalently linking protective moieties (e.g. cyclooctatetraene, 4-nitrobenzyl alcohol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) to cyanine fluorophores to enhance the photostability. Single-molecule fluorescence imaging showed remarkable enhancement of photostability in these modified fluorophores compared to the native ones without protective agents. Nevertheless, the improvement of photostability varies with different members of the cyanine class, suggesting the need for a deeper understanding of how the protective agents provide photostabilization[32]. Relatively high photostability has been observed in inorganic semiconductor quantum dots[50, 89-92], organic dye-silica core/shell NPs[38, 93], some organic and polymeric fluorescent dots[39, 94], and some optimized fluorescence proteins through mutagenesis[95]. For example, enhanced photostability has been recently reported in nanoparticles composed of organic fluorophores with aggregation induced emission (AIE) effect[94, 96-98], but such improvement has been only observed in limited groups of organic dyes and polymers. Despite these recent advances, a general strategy of obtaining highly luminescent, photostable and non-phototoxic fluorophores for long-term bioimaging is still in great demand[44].

Herein we report a facile strategy of enhancing the photostability by covalently linking an organic fluorophore, di(thiophene-2-yl)-diketopyrrolopyrrole (DPP), in the middle of a biocompatible and bioresorbable polymer chain, polycaprolactone (PCL) (Fig. 1a). This polymer, denoted as PCL-DPP-PCL, has a number-average molecular weight (M_n) of 38,300 Da and a polydispersity index (M_w/M_n) of 1.98. In contrast to small-molecule DPP derivatives which often show dramatic fluorescence quenching in solid states due to significant intermolecular π - π aggregation, we found that PCL-DPP-PCL tethered with

long PCL chains shows remarkably bright fluorescence in solid states, suggesting that the aggregation of DPP moieties is suppressed by the covalently attached PCL chains. In addition, PCL as a biocompatible and bioresorbable polyester has attracted much attention for use as an implantable biomaterial in tissue engineering and drug delivery[99]. Biodegradation of PCL is slow compared to other polymers, thus making it suitable for long-term delivery extending over a period of more than one year.

We expected that the integration of strong fluorescence of DPP with the biophysical properties of PCL could make PCL-DPP-PCL an ideal fluorescence probe for bioimaging. Considering the hydrophobic nature of both DPP and PCL in this polymer, we used Pluronic[®] 127 as a surfactant and followed a method of nanoprecipitation to disperse PCL-DPP-PCL as nanoparticles (NPs) in aqueous media (Figure 6a). The resulting NPs were highly fluorescent and showed remarkably enhanced photostability compared to commercial organic fluorophores in imaging of both human liver cancer cell line (HuH-7) and porcine mesenchymal stem cells (MSCs). Moreover, these polymeric NPs showed brighter and more robust fluorescence properties compared to commercially available imaging probes such as quantum dots (Qtracker[®]) in long-term tracing of cells.

2.2 Materials and Methods

2.2.1 Materials and reagents

Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), penicillin/streptomycin antibiotics, Alexa Fluor[®] 633 phalloidin, TrypLE[™] Express Enzyme (1×), PrestoBlue cell viability reagent, fetal bovine serum (FBS), and Qtracker[®] 705 Cell Labeling Kit were ordered from Life Technologies (Singapore). Other reagents including Bovine Serum Albumin (BSA), formalin solution, 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (Singapore). The synthetic details of DPPHT,

PCL-DPP-PCL, PCL-QA-PCL and the polymer NPs are described in Supporting Information.

2.2.2 General materials characterization

Scanning electron microscopy (SEM) was performed on a field emission scanning electron microscopy JSM 6701F (JEOL, Japan) operated at 10 kV. Transmission electron microscope (TEM) measurements were carried out on a JEM-3010 (JEOL, Japan) electron microscope operating. UV-vis transmission spectrum was recorded on a UV-2450 (SHIMADZU, Japan) spectrophotometer. Photoluminescence spectra were measured with LS 55 (PerkinElmer, USA) fluorescence spectrometer. The particle size was measured with a dynamic light scattering (DLS) instrument, Zetasizer Nano ZS (Malvern, UK).

2.2.3 Cell culture

HuH-7 Cell Line (Hepato cellular carcinoma cells) were obtained from National Cancer Center (Singapore) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in the incubator with 37 °C with 5% CO₂. Mesenchymal stem cells (MSCs) were isolated from porcine bone marrow aspirates following institutional guidelines, which demonstrated good purity and stability[100]. MSCs were also cultured in DMEM with 10% FBS and 1% penicillin/streptomycin in the incubator with 37 °C with 5% CO₂.

2.2.4 *In vitro* cellular uptake

In order to explore the cellular uptake of PCL-DPP-PCL NPs and physically blended PCL/DPP NPs, HuH-7 or MSCs were seeded on Petri dish and maintained in the incubator at 37 °C with 5% CO₂. Then, NPs were diluted in the medium (0.2 mg/mL) and incubated with cells at 37 °C with 5% CO₂ after 12 h, followed by washing excess NPs with 1×PBS for three times after 24 h. Then formalin solution was used to fix cells for 20 min followed extensive washing by PBS for three times. Then 0.1% Triton X-100 was used to permeabilize cells for 5 minutes at room temperature, followed by washing

twice by PBS. 5% BSA was used to block unspecific positions for 30 min. Then filamentous actin (F-actin) cytoskeleton was stained by Alexa Fluor[®] 633 phalloidin for 1 h at room temperature. Finally, nucleus was stained by DAPI for 1 min after washing. 405, 561, and 633 nm lasers were used as excitations for DAPI, NPs, and Alexa Fluor[®] 633 phalloidin, respectively. Their emissions were collected through a confocal microscope (LSM 780) with 421-481 nm, 569-621 nm, and 650-758 nm for three individual channels (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). In order to observe cell internalization of PCL-QA-PCL NPs, similar procedures were followed except that 405, 488, and 633 nm lasers were applied to stimulate DAPI, PCL-QA-PCL nanoparticles, and Alexa Fluor[®] 633 phalloidin, respectively. Their emissions were collected through a confocal microscope (LSM 780) with 421-481 nm, 508-534 nm, and 650-758 nm for the three channels (Objective: LD Plan-Neofluar 20x/0.4 Korr M27).

2.2.5 Photobleach assay

In order to perform the photobleach test of PCL-DPP-PCL NPs and physically blended PCL/DPP NPs, HuH-7 or MSCs were plated on a petri dish and maintained in the incubator with 37 °C and 5% CO₂. NPs were added to the medium after 12 h and cultured with cells at 37 °C with 5% CO₂ for 24 h. Subsequently, excess NPs were removed by PBS. Thereafter, cells were fixed and stained with Alexa Fluor[®] 633 phalloidin and DAPI as mentioned above. After being multiply-stained with a cocktail of three dyes, both MSCs and HuH-7 were continuously exposed to 405 nm, 561 nm, and 633 nm laser irradiation with a higher intensity than that in cellular uptake assays and the fluorescence emissions from each dye were recorded at specific time points (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). The nominal laser powers were calculated by multiplying the maximum laser power by the percentage of intensity output during photobleaching. In Figure 9 and 12, nominal laser powers of 405 nm, 561 nm, and 633 nm were 20 mW, 20 mW, and 5 mW, respectively. For the photobleach test of PCL-QA-PCL NPs, 405 nm, 488 nm, and 633 nm lasers

were used (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). In Figure 13, nominal laser powers of 405 nm, 488 nm, and 633 nm were 25 mW, 25 mW, and 5 mW, respectively. Images were obtained in every scans and bleaches were repeated after every scans. The time in second indicated on images was the moments for scanning.

2.2.6 Long-term cell tracing

HuH-7 tumor cells and MSC were plated in a 6-well plate and maintained in the incubator with 37 °C and 5% CO₂. Two nM PCL-DPP-PCL NPs and Qtracker[®] 705 were added after 12 h. After incubation for 48 h, excess NPs were removed away by PBS. Then the cells were trypsinized by TrypLE Express and subcultured into another 6-well plate. After a number of days (Day 1, 3, 5, 7 for HuH-7 and Day 1, 7, 14 for MSC), cells were imaged by fluorescent microscopy (Olympus IX71, excited at 530-550 nm, emission filter 575IF). Finally, the cells were washed by PBS and detached by TrypLE Express. After centrifugation (500 g, 5 min), supernatant were discarded and cell pellets were collected and resuspended in PBS for flow cytometry (LSRII, BD Biosciences). The fluorescence from PCL-DPP-PCL NPs and Qtracker[®] 705 were analyzed by PE (excitation/emission: 561 nm/(567 nm-597 nm)) and PE-cy5 (excitation/emission: 561 nm/(660 nm-760 nm)) channels, respectively. Blank cells without treatment with NPs were set as control. Finally, FlowJo software was used to analyze the data.

2.2.7 *In vitro* cytotoxicity

Cell viability effect of PCL-DPP-PCL NPs with different concentrations in HuH-7 and MSCs was evaluated by PrestoBlue assay. These cells were plated on a 96-well plate with 10000 cells/well and maintained in the incubator with 37 °C and 5% CO₂ for 12 h. Then, PCL-DPP-PCL nanoparticles with different concentrations were diluted in the medium and incubated for 24 and 72 h. Subsequently, excess NPs were removed away with PBS. Then, PrestoBlue reagent in DMEM were added. Simultaneously, PrestoBlue reagents in DMEM

only were also put to blank wells as control. Supernatant in each well were transferred into another new 96-well plate in order to reduce the absorbance background of NPs after 1 h. Then, the absorbance at 571 nm with reference wavelength at 600 nm was measured by Plate Reader (Tecan Infinite M200 series Pro) and the percentage reduction in each sample was calculated based on the following formula as suggested by the manufacturer.

% Reduction of PrestoBlue Reagent = $[(117216 \times A_1) - (80586 \times A_2)] / [(155677 \times N_2) - (14652 \times N_1)] \times 100$, where A_1 is absorbance of test wells at 570 nm, A_2 is absorbance of test wells at 600 nm, N_1 is absorbance of media only wells at 570 nm, and N_2 is absorbance of media only wells at 600 nm. Blank cells were regarded as control and its cell viability was regarded as 100%. Cell viability treated with a specific concentration of NPs = (% reduction of PrestoBlue reagent at this concentration) / (% reduction of PrestoBlue reagent of cells not treated by NPs). Statistics analysis of different groups was conducted by the paired Student's t-test using online calculator (<http://www.physics.csbsju.edu/stats/t-test.html>). All experiments were repeated in three times. p value of <0.05 was considered to be statistically significant.

2.2.8 2,5-bis(6-hydroxyhexyl)-3,6-di(thiophen-2-yl)pyrrolo[3,4-c]pyrrole 1,4 (2H,5H)-dione (DPPHT)

Potassium carbonate (8.28 g, 60 mmol) and DPP-monomers (*3,6-di(thiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione*) (6 g, 20 mmol) were mixed in 200 mL of dimethylformamide under stirring in a 250 mL three-neck round bottom flask under nitrogen protection. The mixture was heated up to 140 °C followed by addition of 6-bromo-1-hexanol (10.88 g, 60 mmol) dissolved in 25 mL of dimethylformamide dropwise over 1 hour. The reaction mixture was stirred at 140 °C overnight. Then the flask was allowed to cool to room temperature and rinsed with chloroform. The product was washed by saturated brine, and extracted by dichloromethane twice. The organic phase was dried with MgSO₄. The crude product was further purified by silica chromatography

(dichloromethane: tetrahydrofuran = 1:1) to obtain dark purple solid DPPHT (1.02 g, 10%)

2.2.9 Synthesis of PCL-DPP-PCL and PCL-QA-PCL

The DPP or QA initiator were added into a sealed Schlenk tube with nitrogen protection, and dissolved in anhydrous toluene under magnetic stirring. Stannous octoate as the catalyst was injected through the rubber seal with a syringe. ϵ -Caprolactone was injected into the reaction mixture. The mixture was degassed with N₂-bubbling before the Schlenk tube was submerged in an oil bath and the reaction mixture was heated up to 110 °C and kept at this temperature overnight. Crude product was purified by dissolved in tetrahydrofuran and precipitated in cold methanol twice.

2.2.10 General procedure for the synthesis of the PCL-DPP-PCL nanoparticles

In a typical procedure, 6 mL of PCL-DPP-PCL (3 mg/mL in THF) was injected into 30 mL of vigorously stirred solution of Pluronic[®] 127 (8.3 g/L in water) at room temperature for 3 mins. Then, the sample was left for stabilization for about 2 hours. Then the sample was dialyzed against DI-water using a 4 kDa membrane. The resulting products were collected by centrifugation at 11,500 g for 15 mins, and the sediments were redispersed in di-water before characterization or dispersed in 1×PBS buffer for cell experiments.

2.3 Results and discussion

2.3.1 Fabrication and characterization of NPs.

Figure 6 indicates the structure of PCL-DPP-PCL and a schematic demonstrating the fabrication of the NPs through a nanoprecipitation process[101-104]. In this process, an aliquot of PCL-DPP-PCL dissolved in tetrahydrofuran (THF) (3 mg/mL) was added to a vigorously stirred solution of Pluronic[®] 127 in water. Hydrophobic interaction of PCL-DPP-PCL drove the self-assembly and the polypropylene oxide (PPO) block of Pluronic[®] 127 which

collapsed to form the core of the NPs. The shell of the NPs consists of PEG chains originated from Pluronic[®] 127 that extrude to the aqueous media to provide the colloidal stability.

A representative scanning electron microscopy (SEM) image (Figure 6b) shows that the resulting particles appear spherical and uniform in size and shape. A statistical analysis of the particle size gives an average diameter of 51 ± 4 nm. Assuming a density of $1.14 \text{ g}\cdot\text{cm}^{-3}$ for PCL at room temperature and neglecting the contribution of Pluronic[®] 127 to the diameter of each NP, the estimated average mass of each NP ($d = 51$ nm) was 4.77×10^4 KDa, and that in average 1,240 polymer chains of PCL-DPP-PCL ($M_n = 38,300$ Da) existed in the core of each NP.

While we observed that the size of the NPs could be tuned by changing the concentration of the polymer in THF, the following results and discussion will only focus on NPs with an average diameter of 51 nm, which has been recently demonstrated to be within the optimal size range in monodisperse drug-silica nanoconjugates for deep tissue penetration and high retention in tumors[105]. Figure 6c shows that the internal structure of the PCL-DPP-PCL NPs appeared amorphous as measured with transmission electron microscopy (TEM). In addition, the size and shape of the NPs measured with TEM were consistent with those measured with SEM. Figure 6d shows the result of dynamic laser light scattering (DLS) measurement, which gives an average diameter of 110 nm for the NPs dispersed in water. The size difference observed using electron microscopy and DLS, which has also been observed for other soft-matter particles, reflects the fact that the polymer NPs existed as a colloidal and swollen state in aqueous media, while they collapse and shrink to smaller sizes after being dried in air and imaged under the high-vacuum condition of electron microscopy.

Figure 6. (a) Schematic showing the fabrication process of PCL–DPP–PCL nanoparticles by nanoprecipitation with Pluronic® 127 as the stabilizer. (b–e) The morphology characterization and optical properties of PCL–DPP–PCL nanoparticles: (b) SEM image showing the average particle size of 51 ± 4 nm. (c) TEM image of PCL– DPP–PCL nanoparticles from a water dispersion. (d) The dynamic light scattering test shows that the hydrated NPs have an average diameter of 110 nm in water dispersion. (e) The fluorescence emission spectra of PCL–DPP–PCL nanoparticles (orange) and DPPHT in THF (blue) under 488 nm excitation. (This figure is reprinted with copyright from[106])

a **b**

Figure 7. (a) Absorption spectra of PCL-DPP-PCL NPs and DPPHT in THF and (b) linear plots for standard samples and PCL-DPP-PCL NPs. (This figure is reprinted with copyright from[106])

Both the UV-vis absorption (Figure 7) and emission spectra (Figure 6e) of the PCL-DPP-PCL NPs appear similar to those of a small-molecule DPP derivative, [2,5-bis(6-hydroxyhexyl)-3,6-di(thiophen-2-yl)pyrrolo[3,4-*c*]pyrrole-1,4(2*H*,5*H*)-dione] (DPPHT) in dilute solution of THF. The NP dispersion in water shows characteristic absorption peaks of DPP at 505 and 550 nm, respectively, despite some light-scattering effect (Figure 7). The emission spectrum (Figure 6e) of the PCL-DPP-PCL NP dispersion shows a major

emission peak located at 560 nm, accompanied with a shoulder at 600 nm. There is a slight red shift (3 nm) of the maximum emission peak in the NP dispersion compared to that of DPPHT in THF.

The fluorescence quantum yield of the PCL-DPP-PCL NPs in water is as high as 0.29, based on Rhodamine 6G (quantum yield = 0.95, in ethanol)[107] as the standard. These results imply that the intermolecular π - π aggregation among DPP units is minimal in the core of the PCL-DPP-PCL NPs.

2.3.2 *In vitro* cellular uptake of NPs

To explore the potential of these NPs in bioimaging application, we first studied the cellular uptake *in vitro*. Two types of cell lines were chosen to this end. HuH-7 is a hepatocyte derived carcinoma cell line that has been used as a popular model for cancer research and some transfection study on hepatological infectious diseases.[108-110]

MSCs are multipotent stromal cells that are able to differentiate into a variety of important cell lineages, and have become a promising cell source for regenerative medicine and tissue engineering.[25] Therefore we believe these two types of cells are both representative and biomedically relevant to characterize the prepared PCL-DPP-PCL NPs for cell imaging. Figure 8 shows the fluorescence images of these two types of cells observed from three channels corresponding to different fluorophores: blue (DAPI), yellow (PCL-DPP-PCL NPs), and red (Alexa Fluor[®] 633 phalloidin).

Figure 8. Cellular uptake of PCL-DPP-PCL nanoparticles (NPs) in HuH-7 tumor cells (a-d and i) and MSCs (e-h and j) imaged by confocal laser scanning microscopy. The fluorescence of DAPI, Alexa Fluor[®] 633 phalloidin, and NPs are represented with blue (**a**, **e**), red (**b**, **f**), and yellow (**c**, **g**), respectively. Merged images of HuH-7 cells and MSCs from three channels are shown in (**d**) and (**h**), respectively. (**i**) and (**j**) are ortho-view images of z-stack, showing nanoparticles inside the cells. Scale bars: 10 μm (HuH-7) and 20 μm (MSC). For a, b, c, d, and i, pixel dwell time: 0.39 μs ; frame size: 1024 $\times$ 1024; pixel size: 0.086 μm . For e, f, g, h, and j, pixel dwell time: 1.00 μs ; frame size: 512 $\times$ 512; pixel size: 0.45 μm . (This figure is reprinted with copyright from[106])

The merged images from all three channels and the ortho-view of z-stack confirm that the NPs could be internalized by both HuH-7 tumor cells (Figure 8 a-d, and i) and MSCs (Figure 8 e-h, and j). Interestingly, the images in Figure

8 (a-d, and i) suggest that the HuH-7 tumor cell nearly finished the mitotic (M) phase or cytokinesis when the mother cell split into two containing roughly equal shares of cellular components. As shown in Figure 8, the internalized NPs are mainly located in the perinuclear cytoplasm, while not exhibiting obvious affinity to any specific intracellular organelle. For further applications targeting at specific subcellular compartments, it is possible that the polymeric shell of these NPs could be functionalized with various ligands, such as nuclear localization signals (NLS) to facilitate NP passage through nuclear pore complex (NPC) for gene delivery[111], or mitochondrial localization signals to enhance specific drug delivery to mitochondria and improve the therapeutic efficacy.[112]

2.3.3 Photostability of NPs in cellular imaging

Photobleaching is always a major concern in bioimaging applications of organic fluorophores. To characterize the photostability of PCL-DPP-PCL NPs, we compared their performance with two popular commercial fluorophores, DAPI and Alexa Fluor[®] 633 phalloidin, by a simultaneous photobleaching test for multiply-stained biological cells. After being stained with a cocktail of three dyes, MSCs were continuously exposed to 405, 561, and 633 nm laser irradiation and the fluorescence emission of each fluorophore was recorded at time points in 36.8-second intervals for a total duration of about 12 min. A band-pass filter combination was applied with bandwidths tuned to record the emission in three channels: blue (421-481 nm), yellow (569-621 nm), and red (650-758 nm). Figure 9a show the merged images of the same multiply-stained MSC and the chronological decay of fluorescence intensity. It can be observed that all three fluorophores showed relatively high fluorescence intensity at the beginning of the test, while the subsequent photobleaching of three fluorophores were not synchronous. The fluorescence intensity of Alexa Fluor[®] 633 phalloidin decayed most rapidly and the F-actin cytoskeleton was barely detectable after 110 s. The fluorescence intensity of DAPI and NPs decreased much more slowly. Notably, the NPs were able to maintain strong fluorescence

intensity during the test and maintain almost 90% of initial intensity even after 11 min.

A quantitative analysis of the relative intensity change (Figure 9b) by curve fitting using exponential functions provided more insight of the decay kinetics of three different fluorophores. The PCL-DPP-PCL NPs exhibited mono-exponential decay expressed by $[y=A_1*\exp(-t/\tau_1)+y_0]$, where (A_1+y_0) represents the initial relative intensity, y_0 is the relative residual fluorescence intensity, and τ_1 is the decay rate constant called *mean lifetime*. The *half-life* was calculated as $\ln(2)*\tau_1$ (Table 1). DAPI also showed mono-exponential decay (Table 2) as reported in some previous studies[113, 114]. Although there was no prior study reporting the decay kinetics of Alexa Fluor[®] 633 phalloidin, the fitting results showed that it could be described by a bi-exponential model $[y=A_1*\exp(-t/\tau_1)+A_2*\exp(-t/\tau_2)+y_0]$ (Table 3) in MSC. In this bi-exponential model, $(A_1+A_2+y_0)$ represent the initial relative intensity; the half-life was computed based on the longer *mean lifetime* (Table 3). A comparison of the half-life values of three fluorophores in MSC (Tables 1-3) clearly showed that NPs (230 s) exhibited significantly higher photostability compared to DAPI (132 s) and Alexa Fluor[®] 633 phalloidin (58.6 s).

As discussed previously, small-molecule DPP derivatives often show fluorescence quenching in solid states due to significant intermolecular π - π aggregation. The reason why PCL-DPP-PCL NPs exhibit much greater photostability could be due to the covalent bonds between DPP and PCL chains, which efficiently suppressed the aggregation of DPP molecules. In order to verify this hypothesis, we prepared PCL/DPPHT nanoparticles (Figure 10 a-c) by simple physical blending (i.e. without any covalent bond formation between these two components) of DPPHT (8.0×10^{-4} mmol) and PCL ($M_n = 22,000$, PDI=1.3) with a weight ratio similar to that of PCL-DPP-PCL. These physically blended PCL/DPP NPs could also be internalized by MSCs and accumulated in perinuclear region (Figure 11 a-d, and i). However, photobleach test revealed

that these physically blended NPs exhibited much weaker photostability (Figure 12) as compared to the covalently bonded PCL-DPP-PCL NPs (Figure 9). After 125 s of laser irradiation (561 nm), physically blended NPs could only retain about 13% of initial fluorescence intensity.

In addition to DPP-based fluorophores, this strategy to enhance the photostability could also be applied to other fluorophores with different excitation and emission wavelengths. As an example, we chose a quinacridone (QA) derivative (Figure 10g) as an initiator and synthesized PCL-QA-PCL NPs using a method similar to the preparation of PCL-DPP-PCL NPs. The SEM image (Figure 10d) showed that the resulting spherical particles had an average diameter of 117 ± 21 nm, which was consistent with the DLS (Figure 10f) and TEM (Figure 10e) results. Fluorescence microscopy (Figure 11e-h, and j) showed that they could be uptaken by MSCs and accumulated in the perinuclear region. The photobleach test suggested that the photostability of PCL-QA-PCL NPs (Figure 13) was relatively weaker than that of PCL-DPP-PCL NPs (Figure 9), which could be due to different properties of QA and DPP.

Figure 9. Photostability of fluorescent PCL-DPP-PCL nanoparticles (NPs) compared to Alexa Fluor® 633 phalloidin and DAPI in MSC. The fluorescences from DAPI, nanoparticles, and Alexa Fluor® 633 phalloidin are represented by blue, yellow and red, respectively. (a) Chronological decay of fluorescence intensity of a multiply-stained MSC at different time points imaged by confocal laser scanning microscopy. The whole image field was photobleached. Pixel dwell time: 0.50 μ s; frame size: 1024 $\times$ 1024; pixel size: 0.28 μ m. Scale bar: 20 μ m. (b) Relative intensity (instantaneous intensity / initial intensity) change of three fluorophores in the photobleached region. Curves are fitted using mono-exponential (PCL-DPP-PCL NPs and DAPI) or bi-exponential functions (Alexa Fluor® 633 phalloidin). The nominal powers of 405, 561, and 633 nm lasers are 20 mW, 20 mW, and 5 mW, respectively. (This figure is reprinted with copyright from[106])

Table 1. Parameters for fitting relative intensity change of PCL-DPP-PCL NPs in MSC (Figure 9b) using $y=A_1*\exp(-t/\tau_1) + y_0$ (Note: In this bi-exponential model, the half-life ($t_{1/2}$) was calculated by $\ln 2*\tau_1$.) (This table is reprinted with copyright from[106])

	Value	Standard Error
y_0	84.99	0.32
A_1	14.97	0.28
Mean lifetime τ_1(s)	331.82	17.28
Adj. R-square	0.9967	
Half-life ($t_{1/2}=\ln 2*\tau_1$, s)	230.00	

Table 2. Parameters for fitting relative intensity change of DAPI in MSC (Figure 9b) using $y = A_1 \cdot \exp(-t/\tau_1) + y_0$ (Note: In this bi-exponential model, the half-life ($t_{1/2}^*$) was calculated by $\ln 2 \cdot \tau_1$.) (This table is reprinted with copyright from[106])

	Value	Standard Error
y_0	79.64	0.80
A_1	17.17	1.09
Mean lifetime τ_1 (s)	190.62	32.56
Adj. R-square	0.9288	
Half-life ($t_{1/2}^* = \ln 2 \cdot \tau_1$, s)	132.13	

Table 3. Parameters for fitting relative intensity change of Alex Fluor[®] 633 in MSC (Figure 9b) using $y = A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + y_0$ (Note: In this bi-exponential model, the half-life ($t_{1/2}^*$) was calculated by $\ln 2 \cdot \tau_2$.) (This table is reprinted with copyright from[106])

	Value	Standard Error
y_0	2.80	0.06
A_1	56.34	1.27
Mean lifetime τ_1 (s)	14.28	0.68
A_2	40.86	1.23
Mean lifetime τ_2 (s)	84.56	2.02
Adj. R-square	0.9999	
Half-life ($t_{1/2}^* = \ln 2 \cdot \tau_2$, s)	58.61 s

Figure 10. SEM (a, d), TEM (b, e) and DLS (c, f) characterization of physically blended DPPHT/PCL NPs (a-c) and PCL-QA-PCL NPs (d-f). (g) The chemical structure of PCL-QA-PCL. (This figure is reprinted with copyright from[106])

Figure 11. Cellular uptake of physically blended PCL/DPPHT NPs (a-d and i) and PCL-QA-PCL nanoparticles (e-h and j) in MSCs imaged by confocal laser scanning microscopy. The fluorescence of DAPI, Alexa Fluor® 633 phalloidin, physically blended PCL/DPPHT NPs, and PCL-QA-PCL nanoparticles are pseudo-labeled with blue (a, e), red (b, f), yellow (c), and green (g), respectively. Merged images of MSCs from different channels are shown in (d) and (h), respectively. (i) and (j) are ortho-view images of z-stack, showing they could be uptaken by the cells. Scale bars: 20 μm . (For a-d and i, pixel dwell: 1.27 μs , frame size: 512 $\times$ 512; for e-h, and j, pixel dwell: 0.63 μs , frame size: 1040 $\times$ 1040). (This figure is reprinted with copyright from[106])

Figure 12. Photostability of physically blended PCL/DPPHT NPs compared to Alexa Fluor® 633 phalloidin and DAPI in a MSC. The fluorescence of DAPI, physically blended PCL/DPPHT NPs, and Alexa Fluor® 633 phalloidin are represented with blue, yellow and red, respectively. (a) Chronological decay of fluorescence intensity of a multiply-stained MSC at different time points imaged by confocal laser scanning microscopy. The green polygon indicates the region photobleached. Pixel dwell time: 1.27 μ s; frame size: 512 $\times$ 542; pixel size: 0.37 μ m. Scale bar: 20 μ m. (b) Relative intensity (instantaneous intensity / initial intensity) change of three fluorophores in the photobleached region. Curves are fitted using mono-exponential (DAPI) or bi-exponential functions (physically blended PCL/DPPHT nanoparticles and Alexa Fluor® 633 phalloidin). (This figure is reprinted with copyright from[106])

Figure 13. Photostability of PCL-QA-PCL NPs compared to Alexa Fluor[®] 633 phalloidin and DAPI in a MSC. The fluorescences from DAPI, PCL-QA-PCL nanoparticles, and Alexa Fluor[®] 633 phalloidin are represented with blue, green, and red, respectively. **(a)** Chronological decay of fluorescence intensity of a multiply-stained MSC at different time points imaged by confocal laser scanning microscopy. The whole image field was photobleached. Pixel dwell time: 0.64 μ s; frame size: 1024 $\times$ 1024; pixel size: 0.42 μ m. Scale bar: 20 μ m. **(b)** Relative intensity (instantaneous intensity / initial intensity) change of three fluorophores in the photobleached region. Curves are fitted using mono-exponential (DAPI) or bi-exponential functions (PCL-QA-PCL NPs and Alexa Fluor[®] 633 phalloidin). (This figure is reprinted with copyright from[106])

2.3.4 Long-term cell tracing using PCL-DPP-PCL NPs vs. Qtracker®

In addition to the photostability of fluorophore upon exposure to light, prior studies have suggested that the decrease of fluorescence intensity of stained cells in long-term study could be also due to loss of fluorophores during cell division and exocytosis[115]. Therefore we investigated the application of PCL-DPP-PCL NPs for long-term cell tracing by fluorescence imaging, and compared their performance with a commercial counterpart, the Qtracker® cell labeling kit consisting of highly fluorescent quantum dot nanocrystals (nanoscale atom clusters comprising a core, a shell and surface coating).

HuH-7 tumor cells were firstly incubated with NPs and Qtracker® for 48 h at concentration of 2 nM, respectively. Then the excess fluorophores were removed and washed by 1×PBS. The cells were then subcultured into a 6-well plate and cultured for up to 7 days. The fluorescence change of the labeled cells was monitored by fluorescence microscopy (Figure 15) and quantitatively analyzed at specific time points by flow cytometry for about 10,000 cell events in each test (Figure 14). The fluorescence of HuH-7 cells labeled with either NPs or Qtracker® gradually decayed and shifted from the high intensity spectrum (Day 1) toward the weak intensity regime (Day 7). It was noted that the NP-labeled tumor cells exhibited much higher fluorescence stability with 49.9% of the population remaining to be strongly fluorescent at Day 7; whereas only 8.68% of the Qtracker®-labeled cells maintained their fluorescence at Day 7. Similar comparative study was also performed with MSC culture for up to 14 days (Figure 16). The flow cytometry analysis and fluorescence microscopy again demonstrated the impressive long-term photostability of this PCL-DPP-PCL NP system, with 46.7% of the NP-stained MSCs retaining strong fluorescence after 2 weeks. These results indicate that the PCL-DPP-PCL NPs are highly advantageous as a stable fluorophore particularly for applications related to long-term cell tracing.

2.3.5 Cytotoxicity of PCL-DPP-PCL NPs.

Finally, we evaluated the cytotoxicity of the PCL-DPP-PCL NPs using PrestoBlue assay. PrestoBlue[®] reagent, Resazurin ($\lambda_{\text{max.abs}} = 600 \text{ nm}$) is a nonfluorescent blue compound, can be reduced in live cells to red in color and highly fluorescent resorufin ($\lambda_{\text{max.abs}} = 571 \text{ nm}$). Since the number of metabolically active cells is proportionally related with the reduction level, the data from absorbance readings can be represented as the percentage reduction of the PrestoBlue reagent, indicating the relative cell viability.

HuH-7 tumor cells and MSCs were incubated with NPs of different concentrations (up to 4 mg/mL) for 24 h and 72 h. Percentage reduction in each sample was measured with regard to that in cell culture without NP staining (the control).

Figure 17 shows that the cell viability of MSCs and HuH-7 cells was not significantly affected for up to 72 h, indicating minimum cytotoxicity of the PCL-DPP-PCL NPs. Statistical analysis by paired student t-test indicated that there was no significant difference between groups treated with different concentrations of NPs and the control for both HuH-7 and MSCs ($p > 0.5$).

Figure 14. Long-term tracing of HuH-7 tumor cells using (a, c) PCL-DPP-PCL nanoparticles and (b, d) Qtracker[®]. The fluorescence intensity change of the labeled cells was measured by flow cytometry analyses at different time points. The control represents the unlabeled cells. The percentages of cells with positive signals in (c, d) were identified based on the gating thresholds shown in (a, b) for NP and Qtracker[®], respectively. (This figure is reprinted with copyright from[106])

Figure 15. Long-term tracing of HuH-7 tumor cells using PCL-DPP-PCL NPs and Qtracker®. The fluorescence intensity change of the labeled cells is characterized by optical contrast and fluorescence microscopy measured at different time points. (This figure is reprinted with copyright from[106])

Figure 16. Long-term tracing of MSCs using PCL-DPP-PCL NPs and Qtracker[®]. The fluorescence intensity change of the labeled cells is characterized by (a) flow cytometry histograms with PCL-DPP-PCL NPs, (b) flow cytometry histograms with Qtracker, (c) flow cytometry dot plots with PCL-DPP-PCL NPs, (d) flow cytometry dot plots with Qtracker, (e) optical contrast and fluorescence microscopy measured at different time points. (This figure is reprinted with copyright from[106])

Figure 17. Cell viabilities of HuH-7 and MSCs after incubating with different concentrations of PCL-DPP-PCL NPs for (a) 24 h and (b) 72 h tested by PrestoBlue assay. Blank cells without any nanoparticles treatment were regarded as control. Values were expressed as means $\pm$ SD (n=3). Paired student t-test was used for statistical analysis between groups treated with different concentrations of NPs and control ($p>0.5$). (This figure is reprinted with copyright from[106])

2.4 Conclusions

In summary, we have presented a new type of highly fluorescent and bioresorbable polymeric NPs prepared through nanoprecipitation of a well-defined polymer, PCL-DPP-PCL, in the presence of Pluronic[®] 127 as the stabilizer. A key feature of our molecular design lies in the covalent insertion of DPP as a highly fluorescent moiety into the middle of each PCL chain. Such covalent bonding at the interface of DPP and PCL as well as the steric effect endowed by long PCL chains effectively suppress the π - π aggregation of DPP in the PCL matrix. As a consequence, PCL-DPP-PCL with M_n of 38,300 Da showed bright fluorescence in solid states. The NPs prepared through a nanoprecipitation process were highly fluorescent and could be internalized by both HuH-7 tumor cells and MSCs with little cytotoxicity to these cells. Most importantly, the PCL-DPP-PCL NPs exhibited significantly enhanced photostability compared to several commercial organic fluorophores, the physically blended DPPHT/PCL NPs and Qtracker[®] for imaging of both tumor cells and stem cells. These unique properties make the PCL-DPP-PCL NPs promising imaging probes to track a broad range of biological systems. Compared to previously reported fluorescent organic or polymeric NPs[39, 94], the 1.3% weight fraction of fluorescent components in these NPs is significantly lower and the polymer matrix (i.e. PCL) has proved to be biocompatible and bioresorbable. Therefore, the potential toxicity, if any, caused by the encapsulated fluorophores is minimized. Moreover, the fluorescence excitation and emission wavelengths of these synthetic NPs can be customized and finely tuned during synthesis for specific applications. Currently we are extending this strategy to polymers with strong fluorescence in near infrared range that is important for *in-vivo* imaging.

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Chapter 3. Long-term Tracking Mesenchymal Stem Cell Differentiation with Photostable Fluorescent Nanoparticles

3.1. Introduction

Stem cell-based therapies, aiming to treat diseases using the self-renewal and multilineage differentiation capacities of the transplanted stem cells, have achieved great progress in the past decade [116-119]. Among the various types of stem cells, mesenchymal stem cells (MSCs) are much less prone to the ethical issues and the risk of developing teratoma [120]. MSCs are able to differentiate into multiple types of mesoderm lineages, such as chondrocytes, osteocytes, adipocytes and some ectodermic and endodermic cells [120]. An increasing number of MSC-based therapies, especially for tissue repair or immune disorder diseases, have been attempted since 2004 [121]. However, there are many major issues remaining to be resolved before successful clinical applications on real patients [120, 122]. One of the critical challenges is the difficulty to track the fate, distribution and migration of the transplanted stem cells in the local microenvironment due to the limited tracking methods. Compared to traditional but highly invasive histological analysis, non-invasive imaging techniques are urgently demanded to track the transplanted stem cells for monitoring the therapeutic progress. The distribution and migration of the transplanted stem cells can provide a reliable evaluation for the cell-based therapeutic effect and elucidate the optimal conditions during surgery, such as cell dosage, route of delivery and the timing of transplantation [123, 124]. Additionally, in the applications of stem cell based tissue and organ regeneration, it is essential to delineate the specific contributions of transplanted cells and host cells to the damaged tissues or organs [125].

Fluorescence imaging has been widely applied in various biological fields as a non-invasive technique because of its unique advantages, such as high

sensitivity, rapid acquisition, lack of radiation, low cost and easy access [124-129]. The most popular labelling markers for fluorescence imaging are organic dyes, such as DAPI (4',6-diamidino-2-phenylindole) and Alexa Fluor family fluorophores. However, most organic dyes can only be used for short-term imaging because of photobleaching effect [125]. Another emerging type of fluorescent labels are quantum dots (QD), which emit strong fluorescence due to quantum confinement effect and the excitation and emission spectra are highly tunable based on their unique sizes [125, 130]. However, the typical semiconductor and heavy metal-based quantum dots could release metal ions particularly under UV illumination, which are highly toxic to the cells both *in vitro* and *in vivo* [131-133]. QDs comprising a cadmium selenide (CdSe) nanocrystal core and a zinc sulfide (ZnS) shell were developed to label human MSCs during osteogenic differentiation by liposome-mediated transportation [134]. It was found that although the labelled MSCs showed normal proliferation, the gene expressions of specific osteogenic markers including osteopontin, osteocalcin and alkaline phosphatase were significantly inhibited in the presence of QDs [134]. In another prior study, MSCs labelled with commercial quantum dots (QTracker 800, Thermo Fisher Scientific) were delivered to nude rats with segmental bone defects to track the cell distributions during the bone healing process after transplantation [131]. Although clear fluorescence could be observed *in vivo* from the treated rats, the QD-labelling reduced the function of transplanted cell and thereby the bone healing responses. To address this major issue, QD probes need to be modified with biomolecules or biocompatible polymers to reduce their cytotoxicity before applications. For example, CGGGRGD (Cys-Gly-Gly-Gly-Arg-Gly-Asp) was covalently bonded with CdSe/ZnS QDs to label human MSCs for induced differentiations into adipogenic, chondrogenic, and osteogenic lineages [125]. These bioconjugated QDs were shown to be able to maintain the viability as well as the multipotency of the labelled human MSCs (hMSCs). In addition to quantum dots, other nanoprobe, such as phosphonate-functionalised

polystyrene nanoparticles were also used for long-term tracing of hMSC differentiation after they were labelled with the fluorescent dye N-(2,6-diisopropylphenyl)-perylene-3,4-dicarboximide [135].

Previously, Huang and co-workers have prepared a well-defined synthetic polymer platform based on di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) covalently linked in the middle of biocompatible and bioresorbable polycaprolactone (PCL) polymer, which is abbreviated as PCL-DPP-PCL [106]. These nanoparticles exhibited bright fluorescence and strong photostability for cancer and stem cell imaging. Although the basic material properties, photostability and cytotoxicity to a few tumor cells and stem cells have been elaborated in details [106], however the potential influence of this polymeric nanoprobe on MSC multipotency and differentiation remains unclear. Herein we bring this study from cellular level to tissue level by long-term tracing MSCs labelled with PCL-DPP-PCL and elucidating the potential effect of these nanoparticles on the MSC multipotency. Specifically, we labelled hMSCs with PCL-DPP-PCL nanoparticles and then induced these labelled hMSCs into different cell lineages including adipocytes, chondrocytes, and osteocytes respectively, which were characterized by gene expression and histological staining. Additionally, the residue fluorescence within the differentiated labelled cells were measured by flow cytometry and confocal laser scanning microscopy. We believe that this work could be interesting and inspiring to researchers in the thriving fields of stem cell based therapies, tissue engineering and regenerative medicine.

3.2. Materials and Method

3.2.1 Preparation of PCL-DPP-PCL NPs

The detailed procedure to prepare and characterize the PCL-DPP-PCL nanoparticles (NPs) can be found in the previous report [106]. The prepared NPs were dispersed in sterile 1× PBS before use.

3.2.2 Cell culture

Human bone marrow derived mesenchymal stem cells (hMSCs) were obtained from consented patients in the National University Hospital (Singapore) following a protocol approved by Institution Review Board (IRB) [136]. The isolated cells were expanded in expansion medium, consisting of low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with sodium pyruvate and GlutaMax™ (Life Technologies, Singapore), 10% Fetal Bovine Serum (FBS) (Life Technologies, Singapore) and 100 units/ml penicillin-streptomycin (Life Technologies, Singapore), and incubated under 37°C with 5% CO₂ and humidified atmosphere. Non-adherent cells were removed after 48 h. The adherent cells remained in culture flasks for further expansion. The hMSCs in passages 3 and 4 were used for this study.

3.2.3 Cell labeling and flow cytometry

The hMSCs were seeded in 6-well plates in normal DMEM medium with 10% FBS and 1% penicillin-streptomycin. After cell attachment overnight, the original medium was replaced by fresh medium with dispersed PCL-DPP-PCL NPs at concentration of 0.2 mg/mL. Then the cells were grown at 37°C with 5% CO₂ for 3 and 7 days before the excess NPs were rinsed with 1× PBS.

For confocal laser scanning microscopy, the cells were fixed by formalin and then permeabilized by 0.1% Triton-X. Then the samples were blocked by 1% BSA and stained with Alexa Fluor® 633 phalloidin for F-actin and DAPI for nucleus. The 3-color laser source (405 nm, 561 nm and 633 nm) in a confocal microscope (Carl Zeiss, LSM 710) provided excitation for the fluorophores in DAPI, PCL-DPP-PCL and Alexa Fluor® 633 phalloidin with corresponding emission windows at 410-549 nm, 566-622 nm and 638-747 nm, respectively.

For quantitative analysis by flow cytometry, the cells were trypsinized by 1×TrypLE™ Express Enzyme (Life Technologies, Singapore) and then centrifuged. 1× PBS solution was added into the cell pellet to prepare

monodispersed cell suspension (500 μ l) for flow cytometry (LSR II, BD). The PE channel (excitation: 561 nm; emission: 567-597 nm) was used to detect the fluorescence signals from PCL-DPP-PCL NPs. Regular cells without NP treatment were used as negative control. Approximately 1×10^4 cell events were recorded in each test and the data were analyzed by FlowJo software to obtain the mean fluorescence intensity (MFI) of cell populations. Then the MFI of each labelled group was normalized by the MFI of corresponding control groups, which was able to quantitatively indicate the change of fluorescence intensity before and after cell differentiations.

3.2.4 Cell proliferation

The influence of PCL-DPP-PCL NPs on hMSC proliferation was evaluated using PrestoBlue assay. Firstly, both labelled and unlabeled hMSCs were seeded on a 24-well plate at a density of 2000 cells/well and cultured at 37°C with 5% CO₂ for 14 days. The medium was changed every 2-3 days. The absorbance was measured at day 3, 7, 10, and 14. Percentage (%) reduction that indicate the cell proliferation rate was calculated based on the formula provided by the manufacturer.

3.2.5 Pre-differentiation processing

The hMSCs of passage 3 or 4 were cultured in three T75 flasks. When cells became confluent after 3-4 days, PCL-DPP-PCL NPs (0.2 mg/mL) were added to the culture medium. Regular cells without treatment were regarded as control. After further incubation for 3 and 7 days, the excess NPs were removed away by washing with PBS for three times before the cells were trypsinized by 1 $\times$ TrypLE™ Express Enzyme. The cell suspension in DMEM medium from each group was placed on 4-well plates for osteogenic and adipogenic differentiations respectively, while other cells were seeded in sterile conical tubes for chondrogenic differentiation. The DMEM medium was replaced by differentiation induction medium after one day.

3.2.6 MSC differentiations

6.0×10^4 and 6.0×10^3 hMSCs were seeded on 1.9 cm^2 Nunc™ 4-well plates (Thermo Fisher Scientific, Singapore) in expansion medium for adipogenic and osteogenic differentiations respectively. Cells were first incubated overnight for effective attachment. Then the expansion medium was replaced by differentiation medium on the following day for induction of adipogenic or osteogenic differentiations. The adipogenic medium comprised high glucose DMEM (without L-glutamine) supplemented with 10% FBS, 1 mM sodium pyruvate (Life Technologies, Singapore), 100 units/ml penicillin-streptomycin, 0.1 μM dexamethasone (Sigma-Aldrich, Singapore), 10 $\mu\text{g/ml}$ insulin (Life Technologies, Singapore), 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, Singapore) and 200 μM indomethacin (Sigma-Aldrich, Singapore). The osteogenic medium included low glucose DMEM, 10% FBS, $1 \times$ Glutamax™ (Life Technologies, Singapore), 100 units/ml penicillin-streptomycin, 1 mM sodium pyruvate, 50 $\mu\text{g/ml}$ ascorbic acid (Sigma-Aldrich, Singapore), 10 mM β -glycerophosphate (Sigma-Aldrich, Singapore) and 0.1 μM dexamethasone. The cells were cultured under 37°C under 5% CO_2 humidified conditions with the differentiation medium refreshed every 2-3 days. After adipogenic and osteogenic differentiation, the cells were washed twice by PBS and then trypsinized by $1 \times$ TrypLE™ Express Enzyme. Then the cells were mixed with DMEM medium and spun down. The cell pellets were resuspended in 500 μl PBS for flow cytometry.

Induction of hMSC chondrogenic differentiation was performed in a 3-dimensional culture condition. 2.0×10^5 hMSCs were transferred to a 15 ml conical tube and then centrifuged to form a cell pellet, which was cultured in expansion medium at 37°C and 5% CO_2 humidified conditions to allow pellet stabilization. The expansion medium was replaced by chondrogenic medium on the second day. The chondrogenic medium consisted of high glucose DMEM, $1 \times$ Glutamax™, 100 units/ml penicillin-streptomycin, 1 mM sodium pyruvate, 50 $\mu\text{g/ml}$ ascorbic acid, 0.1 μM dexamethasone, 4 mM proline (Life

Technologies, Singapore) and ITS (BioLab, Singapore) was supplemented with 10 ng/ml transforming growth factor β 3 (TGF- β 3) (R&D Systems, Singapore). The medium was changed every 2-3 days. The chondrogenic pellets after differentiation were firstly homogenized in collagenase type I for 2 h at 37°C with 5% CO₂ and then spun down. Then 1×TrypLE™ Express Enzyme was added to the pellet for several minutes and cells were centrifuged after adding DMEM medium. Finally, the cells were resuspended in PBS and measured by flow cytometry.

3.2.7 Histological analysis

The adipogenic and osteogenic differentiation samples were collected at day 21, while the chondrogenic pellets were harvested at day 28. The samples were washed with 1× PBS before being fixed overnight with 10% formalin (Sigma-Aldrich, Singapore). The fixed samples were rinsed with 1× PBS prior to histological staining.

The adipogenic lineage was stained with Oil Red to identify intracellular oil droplet accumulation that can be found in mature adipocytes [137]. The staining solution was prepared by adding 4 ml distilled water to 6 ml of 0.5% Oil Red O stock solution. After 15 min incubation, the samples were washed with 70% ethanol and distilled water to remove the excess staining solution before being counterstained with hematoxylin (Sigma-Aldrich, Singapore) for 30 secs and rinsed with distilled water. For osteogenic lineage, Alizarin Red S solution (Sigma-Aldrich, Singapore) was used to stain the cells for 5 min, followed by gentle agitation with distilled water to remove non-specific staining. The cells subjected to osteogenic differentiation presented brick-red color due to calcium deposition. The chondrogenic pellets were embedded in FSC 22 Mounting Media (Leica, Singapore) and frozen at -20°C for about 2 h. The tissue sections of 5 μ m thick were sliced using a Leica CM1900 cryostat (Leica Microsystems, Singapore) and were collected using Mezel-Glaser Superfrost Plus slides (Thermo Fisher Scientific, Singapore).

The collagen type II expression was analyzed by immunohistochemistry staining using UltraVision™ Quanto Detection System HRP DAB kit (Thermo Fisher Scientific, Singapore). The samples on the slides were first incubated with digestive enzyme containing 0.5% pepsin (Sigma-Aldrich, Singapore) for 20 min at 37°C in humid condition, followed by treatment of hydrogen peroxide for 10 min and Ultra V Block reagent (Thermo Fisher Scientific, Singapore) for 5 min to reduce non-specific background staining. The samples were then incubated overnight with Mouse monoclonal anti-Col2 antibody (Merck Millipore, Singapore) with a dilution factor of 1:500 at 4°C. Following the staining protocol of UltraVision™ Quanto Detection System HRP DAB kit, samples were incubated in Primary Antibody Amplifier Quanto for 10 min, HRP Polymer Quanto for 10 min with minimum or no light exposure, and finally a mixture of 30 µl DAB Quanto Chromogen and 1 ml of DAB Quanto Substrate until color development was evident (approximately 5 min). The chondrogenic sections were counterstained with hematoxylin (Sigma Aldrich, Singapore). The coverslips were dehydrated after staining.

3.2.8 Gene expression

Adipogenic and osteogenic samples were harvested at day 14, while chondrogenic pellets were only collected at day 21. Prior to RNA isolation, chondrogenic pellets were incubated in 0.25% collagenase type I for 2 h at 37°C. The RNA was extracted using RNeasy mini kit (Qiagen, Singapore) and quantified using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Singapore). RNA was reverse transcribed into cDNA with 5× iScript Reverse Transcription Supermix (Biorad Laboratories, Singapore). Real-time Polymerase Chain Reactions (RT-PCRs) were performed with SYBR Green PCR Master Mix kit (Life Technologies, Singapore) using StepOnePlus Real-Time PCR System (Life Technologies, Singapore) to quantify the specific gene expression of mRNA in the samples. Samples were first denatured at 95°C for 10 min, followed with a 40-cycle amplification consisting of 95°C denaturation step for 15 sec and 60°C extension step for 1 min. Primers used to amplify

cDNA of adipogenic cDNA includes GAPDH, PPAR- γ and Leptin. Meanwhile, GAPDH, Col1, OCN and ALP primers were used for osteogenic cDNA, and GAPDH, Col1 and Col2 primers were used for chondrogenic cDNA. The primer sequences are listed in Table 4. The expression levels of the mRNA of target genes were normalized according to the reference gene GAPDH, and then expressed as fold change with reference to their respective levels before induction of differentiation (day 0), which is calculated by the widely used comparative C_T method using the $2^{-\Delta\Delta C_t}$ formula[138].

Table 4. Primer sequences for specific genes used in RT-PCR. (This table is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
GAPDH	ACAACCTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
PPAR- γ	TACTGTTCGGTTTCAGAAATGCC	GTCAGCGGACTCTGGATTTCAG
Leptin	TGCCTTCCAGAAACGTGATCC	CTCTGTGGAGTAGCCTGAAGC
OCN	CGCCTGGGTCTCTTCACTAC	CTCACACTCCTCGCCCTATT
ALP	ACCACCACGAGAGTGAACCA	CGTTGTCTGAGTACCAGTCCC
Col1	CAGAACGGCCTCAGGTACCA	CAGATCACGTCATCGCACAAAC
Col2	CCAGATGACCTTCCTACGCC	TTCAGGGCAGTGACGTGAAC

3.2.9 Confocal laser scanning microscopy

The adipogenic and osteogenic lineages (day 21) were washed twice by PBS, fixed by formalin and then permeabilized by 0.1% Triton-X. Then the samples were blocked by 1% BSA and stained with Alexa Fluor[®] 633 phalloidin for F-actin and DAPI for nucleus. We used three laser channels (405 nm, 561 nm and 633 nm) in a confocal microscope (Carl Zeiss, LSM 710) to excite the fluorophores of DAPI, PCL-DPP-PCL NPs and Alexa Fluor[®] 633 phalloidin. Their fluorescence emission signals were detected in three individual channels (410-549 nm, 566-622 nm and 638-747 nm).

For chondrogenic lineage, the cell pellets were washed with PBS twice and fixed with formalin. The fluorescence of the labelled samples were observed by confocal microscope (excitation: 561 nm; emission: 566-622 nm). We applied z-stack function to image different layers of the pellet. Additionally, the cryosection slides of pellets were also imaged with confocal microscopy.

3.2.10 Cytotoxicity assay

The cytotoxicity of PCL-DPP-PCL NPs on hMSCs during cell labeling was tested by PrestoBlue assay. Firstly, unlabeled hMSCs were plated on a 96-well plate with density of 6000 cells/well. Then the original medium was replaced with new medium containing 0.2 mg/mL PCL-DPP-PCL NPs. The cells were incubated with the NPs for 3 and 7 days respectively before the absorbance (measurement wavelength: 570 nm; reference wavelength: 600 nm) were measured by a micro-plate reader (Infinite M200 Pro). Cell viability was calculated based on the manufacturer's instructions. The unlabeled hMSCs were regarded as control with relative cell viability as 100%.

3.2.11 Fabrication of porous scaffold

Porous scaffold was fabricated using salt leaching and solvent casting techniques. Poly(L-Lactide-co-Epsilon-Caprolactone) (70:30) (Purasorb®, Singapore), also known as PLCL, was dissolved in 10% w/v chloroform (Fischer Scientific, analytical grade, Singapore). NaCl (Sigma-Aldrich) with particle size of 150-200 μm was mixed in the PLCL solvent, which was then casted at a constant rate to form approximately 200 μm thick scaffold and dried. The salt component was then leached by washing with water forming a porous scaffold.

The PLCL scaffolds were pretreated with 3 mg/ml chitosan (Sigma-Aldrich, Singapore) in 4 mM HCl using aminolysis method [22] before they were soaked in 50% ethanol and dried. Subsequently, these scaffolds were immersed in 10% (w/v) 1,6-hexanediamine (Alder BioPharmaceutical, Singapore) in isopropanol (Fischer Scientific, Singapore) and then 1% glutaraldehyde (Sigma Aldrich,

Singapore) after washing with 1× phosphate buffered saline (PBS) (Life Technologies, Singapore). Finally, the scaffolds were incubated in 3 mg/ml chitosan (Sigma Aldrich, Singapore) in 4 mM HCl at 4°C overnight. Chitosan-modified PLCL scaffolds (2 mm diameter) were sterilized using 70% ethanol for 2 h and washed. Approximately 5×10^4 hMSCs were seeded on each scaffold and cultured.

3.2.12 Statistical analysis

All experiments were performed at least thrice. The data were analyzed following Student's t-test to determine the statistical significance between two experimental groups using One-Way ANOVA and Tukey HSD test. The difference between data was considered statistically significant if the p-value was less than 0.05.

3.3. Results and Discussion

For abbreviation, the PCL-DPP-PCL NPs are simply referred to as NPs henceforth. The brief workflow of this study is presented in Figure 18. Firstly, the original hMSCs were incubated with NPs for 3 days (short term, “ST”) and 7 days (long term, “LT”) with blank cells as control. Then, these labelled cells were induced into adipogenic, chondrogenic and osteogenic differentiation. To study the influence of NPs on hMSC proliferation and multipotency, we divided the MSCs into 3 major experimental groups, i.e., unlabeled control, short-term staining for 3 days (NP-ST) and long-term staining for 7 days (NP-LT).

3.3.1 Cell labeling with NPs

We firstly investigated the cellular uptake of NPs. The confocal fluorescence microscopy in both individual channels and ortho-views (Figure 21A and Figure 19) indicated that NPs were internalized into the cytoplasm of hMSCs, which was consistent to the finding in the previous work [106]. Additionally, prolonged incubation time from 3 days to 7 days for cell staining enhanced the cellular intake of NPs with increased fluorescence intensity (Figure 21A), which

was further verified by flow cytometry showing the peak intensity shift (Figure 21B). The staining process did not show any considerable effect on the cell viability compared with the unlabeled control (Figure 20), indicating that the NPs had minimum cytotoxicity to the hMSCs. For long-term tracking of cell differentiation, the NP labeling is desired to have minimum effect on cell proliferation to maintain the multipotency of hMSCs. We used PrestoBlue assay to measure the reduction ability of live cells for up to 2 weeks (Figure 21C), which indicated the cell proliferation rate. The results revealed that the short-term labeled group (NP-ST) and long-term labeled group (NP-LT) did not show any inhibition effect on hMSC proliferation as compared to the unlabeled control. Interestingly, the nanoparticle labelling rather enhanced cell proliferation at day 7 and day 14, while the underlying mechanism is still not clear and needs further investigation.

Figure 18. Schematic illustration of long-term tracking human mesenchymal stem cells during adipogenic, chondrogenic and osteogenic differentiations using PCL-DPP-PCL nanoparticles. (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

Figure 19. Corresponding ortho-views by taking different z stacks for the confocal laser scanning microscope images of NP-ST (A) and NP-LT (B) in Figure 21A. Cell nuclei were counterstained with DAPI (blue), F-actin were stained with Alexa Fluor® 633 phalloidin (red), and nanoparticles were represented as yellow. (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

Figure 20. Relative cell viability of hMSCs during NP-labelling (concentration: 0.2 mg/mL) detected with PrestoBlue assay. The viability of blank cells without labelling was set as 100%. The data were expressed as means $\pm$ SD ($n = 3$). (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

Figure 21. (A) Cellular uptake of NPs in hMSCs imaged by confocal microscopy. Cell nuclei were counterstained with DAPI (blue), F-actin were stained by Alexa Fluor® 633 phalloidin (red) and the nanoparticles were represented as yellow. Scale bar: 100 μ m. (B) Quantitative analysis of cellular uptake of NPs by flow cytometry. Normalized MFI: 1143.5 for NP-LT, 691.1 for NP-ST. (C) Cell proliferation measured by PrestoBlue assays. Data presented as mean $\pm$ SD. *p-value <0.05, **p-value < 0.01. n = 3. (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

3.3.2 Multilineage differentiations of labeled hMSCs

Figure 23 showed the long-term effect of NP labeling on hMSC-derived adipogenic differentiation after 3 weeks. The confocal microscopy indicated that differentiated adipocytes retained strong fluorescence due to NP staining (yellow, Figure 23A) for both NP-ST and NP-LT groups.

The observation was further quantified by flow cytometry analysis, which also showed relatively stronger fluorescence of NP-LT than NP-ST (Figure 23B). Although the normalized MFI decreased considerably after 3 weeks of adipogenic differentiation, the NP-labeled adipocytes still exhibited high fluorescence intensity ratio (394.7 for NP-LT, 208.8 for NP-ST) compared to unlabelled controls indicating the strong photostability of the labeling NPs.

The variation of cell morphology and fluorescence of NP labeling at intermediate stages (day 6, 13 and 20) in the adipogenic differentiation were shown in Figure 22. Figure 23D displayed the accumulation of intracellular lipids in the differentiated adipocytes by histological staining, providing the qualitative evidence of successful adipogenesis in the NP-labeled cells. Image analysis of the positive area of Oil Red staining indicated similar levels of lipid formation in the NP-labeled groups as compared with the unlabeled control (Figure 24).

To evaluate the adipogenic differentiation quantitatively, we measured the gene expression of two important adipose biomarkers including leptin and peroxisome proliferator-activated receptor γ (PPAR- γ), which regulate fatty acid storage and glucose metabolism. The RT-PCR assay (Figure 23C) revealed that the time of NP staining before induction of adipogenic differentiation influenced the adipose gene expression. Specifically, the NP-ST group exhibited a similar level of leptin and an upregulation of PPAR- γ as compared to the unlabeled control. In contrast, the gene expressions of leptin and PPAR- γ in the NP-LT group were both downregulated significantly. These results implied that prolonged NP staining and higher concentrations of internalized NPs could interfere with the hMSC-derived adipogenic differentiation, for which the underlying mechanism of cellular transduction was still unclear and required further investigation. Nevertheless, we have demonstrated that short-term NP staining for 3 days (NP-ST) could provide strong fluorescence labeling without compromise of adipogenesis for long-term tracking of hMSC differentiation. Therefore, we applied short-term NP staining in the following chondrogenic and osteogenic differentiations.

Figure 22. Microscopic images of the NP-labeled hMSCs during adipogenic differentiation. The images of NP-ST and NP-LT groups were obtained by merging the bright field contrast images and the corresponding fluorescent images. Scale bar: 250 μm . (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

A

Figure 23. MSC differentiation in adipogenic lineage. (A) Confocal fluorescence images of the differentiated adipocytes (day 21). Scale bar: 100 μ m. (B) The retained fluorescence due to NP staining in the differentiated adipocytes (day 21) analyzed by flow cytometry. Normalized MFI: 394.7 for NP-LT, 208.8 for NP-ST. (C) The mRNA expression of leptin and PPAR- γ (day 14) quantified by RT-PCR assay. The results were normalized to the data on day 0 (before differentiation). *p-value < 0.05, **p-value < 0.01. Data presented as mean $\pm$ SD. n = 4 for leptin and n = 3 for PPAR- γ . (D) Oil Red staining for oil droplets in the differentiated adipocytes (day 21). Scale bar: 250 μ m. (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

Figure 24. Analysis of the total positive area of Oil Red O (ORO) staining against the reference area of the image frame according to a threshold range set with ImageJ. There is no significant difference among three groups (p -value > 0.05 , $n = 7$). The area is measured based on an arbitrary unit (a.u.). (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

The chondrogenic lineage was analyzed after 3-4 weeks of induced differentiation as shown in Figure 25. We observed strong fluorescence within the formed cartilage tissue pellet labeled with NPs (Figure 25B) in addition to its cryosection (Figure 25A). We further homogenized the tissue pellet, digested the matrix proteins and obtained the suspension of chondrocytes for flow cytometry, which showed that the individual hMSC-derived chondrocytes retained strong fluorescence of the NP labels even after 4 weeks of chondrogenesis (Figure 25C).

Three important chondrogenic markers, including aggrecan (AGG), collagen type I (Col1) and collagen type II (Col2), were analyzed using RT-PCR assay for their gene expressions in the chondrogenic pellets at day 21. The gene expressions of Col1, Col2 and AGG in the labeled chondrogenic pellets were similar to those in the unlabeled control (Figure 25D-E, no significant difference statistically, p -value > 0.05). As the most abundant collagen in native cartilage tissues [140], Col2 exhibited very high level of expression (Figure 25E) that led

to Col2/Col1 ratio above 1,500 (Figure 25F) in both labeled and unlabeled groups, which was a feature of hyaline-like cartilage tissue formation[140, 141].

Immunohistochemistry staining further demonstrated the similar density of Col2 in labelled and control tissue pellets after 4 weeks of chondrogenesis (Figure 25G). Therefore, the NP-labelling was also proved to be amicable for long-term tracking of chondrogenic differentiation of hMSCs.

Figure 25. MSC differentiation in chondrogenic lineage. (A) The cryosections of the hMSC-derived tissue pellets (day 28). Scale bar: 100 μ m. (B) Confocal fluorescence images of the original pellets (day 28). Scale bar: 200 μ m. (C) Flow cytometry analysis of the trypsinized chondrocytes (day 28). Normalized MFI: 80.4 for NP-ST. (D–F) The mRNA expression of Col1, Col2 and aggrecan (day 21) quantified by RT-PCR assay. Data were normalized according to the results at day 0 (before differentiation). Data presented as mean $\pm$ SD. $n = 5$ for Col1, Col2 and Col2/Col1, $n = 3$ for AGG. (G) Immunohistochemistry staining of Col2 in the chondrogenic pellets (day 28). Scale bar: 250 μ m. (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

The last differentiated group was the osteogenic lineage. Confocal fluorescence microscopy after 3 weeks of osteogenic differentiation (day 21) indicated strong fluorescence in the hMSC-derived osteocytes (Figure 26A,D). However, the NP-labelled osteocytes exhibited much lower intensity of Alizarin Red staining as compared to the unlabelled control (Figure 26B), suggesting that the presence of NPs suppressed the calcium deposition considerably during osteogenesis.

We also quantified the gene expressions of three important osteogenic markers, including Coll1, osteocalcin (OCN) and alkaline phosphatase (ALP), using RT-PCR assay at day 14 after induction of differentiation. The RT-PCR data revealed significant downregulation of all three osteogenic gene markers in the NP-labelled group (Figure 26C). These results implied that the NP labelling, even by short-term staining of 3 days, had inhibited the osteogenic differentiation of hMSCs. It was previously reported that TiO₂ nanoparticles exhibited adverse effects on the viability, proliferation and osteogenic differentiation of MSCs by influencing the gene expressions of ALP, OCN and osteopontin (OPN) in a size-dependent and dose-dependent manner [142]. In the present study, on the other hand, we showed that the synthetic polymer-based NPs with staining concentration of 0.2 mg/ μ L did not affect the cell viability and proliferation, while they compromised the osteogenic differentiation of MSCs.

Figure 26. MSC differentiation in osteogenic lineage. (A) Confocal fluorescence images of the differentiated osteocytes (day 21). Scale bar: 100 μ m. (B) Alizarin Red histological staining for calcium deposition after osteogenic differentiation (day 21). Scale bar: 250 μ m. (C) The mRNA expression of Col1, OCN and ALP (day 14) quantified by RT-PCR assay. Data were normalized according to the results at day 0 (before differentiation). *p-value <0.05, **p-value <0.01. Data presented as mean $\pm$ SD. n = 4. (D) Flow cytometry analysis of the hMSC-derived osteocytes (day 21). Normalized MFI: 94.6 for NP-ST. (This figure is reprinted with permission from[139]. Copyright (2016) American Chemical Society)

3.3.3 MSC labeling in 3D scaffold

All above results were based on MSC labelling for conventional 2-dimensional (2D) cell cultures or pellet culture. In the recent advances of tissue engineering, 3-dimensional (3D) scaffolds have become more popular due to many advantages in better mimicking the native cellular environment, which consists of a 3D network with a complex extracellular environment and a highly porous nanotopography [143].

Cells cultured in 3D scaffolds tend to behave more like their in-vivo counterparts, which may work as a better model for tissue engineering and other applications such as drug discovery and cellular signalling [144]. The special mechanical properties of these 3D scaffolds also provide supports to the cells and newly formed tissues, which is important for weight-bearing tissues such as cartilage and bone [136, 145].

As a simple proof-of-concept study, we seeded the hMSCs on a Poly(L-Lactide-co-Epsilon-Caprolactone) (PLCL) porous scaffold (Figure 27) and cultured the cells for 14 days. The 2D and 3D images in Figure 28 obtained by confocal fluorescence microscopy demonstrated the distribution of NP-labelled MSCs scattered in the scaffold.

The cell nuclei stained with DAPI was indicated as blue dots in the magnified inset of Figure 28. It was however difficult to distinguish the nuclei from the strong background fluorescence under excitation of 405 nm due to the autofluorescence of PLCL scaffold [146]. Meanwhile, the bright fluorescent NPs clearly indicated the distribution of labelled hMSCs in the scaffold under excitation of 561 nm. This simple test showed that these NPs could also be used as an excellent contrast agent for cell tracking in the 3D scaffold environment, providing important information on the location, distribution and migration of the labeled cells.

Figure 27. Scanning electron microscope images for PLCL porous scaffold under different magnifications: (A) 100 $\times$, (B) 250 $\times$ and (C) 500 $\times$. (This figure is reprinted with permission from [139]. Copyright (2016) American Chemical Society)

Figure 28. Confocal fluorescence images of hMSCs seeded in a 3D scaffold (day 14). 3D images were reconstructed using ZEN 2012 software by taking different z-stack images. Scale bar: 100 μm . (This figure is reprinted with permission from [139]. Copyright (2016) American Chemical Society)

3.4. Conclusions

In summary, PCL-DPP-PCL nanoparticles are able to label hMSCs by internalization without affecting the cell viability and proliferation behaviors. It was found that PCL-DPP-PCL NPs labeling for 3 days at concentration of 0.2 mg/ μL did not show any negative effect on MSC adipogenic and chondrogenic differentiations, while the osteogenic differentiation was compromised compared to the unlabeled control. Furthermore, the labeling NPs can maintain

strong fluorescence even after 4 weeks, indicating these NPs can work as efficient fluorescent probes for long-term tracing during adipogenic and chondrogenic differentiations. The PCL-DPP-PCL nanoparticles do not require any additional bioconjugation or surface modification, while showed minimum cytotoxicity and negative effects on adipogenic and chondrogenic differentiations under appropriate concentrations. Moreover, the PCL polymer skeleton of this probe has proved to be biocompatible and biodegradable, and has been approved by US Food and Drug Administration (FDA) for specific use in human body. Whereas the potential toxic fluorophore DPP composes only about 1.3% in weight fraction of this probe, thereby further reducing its potential cytotoxicity. By replacing DPP with other near infrared dye in this system, this strategy could be extended for long-term stem cell tracking applications *in vivo*.

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Chapter 4: Peptide Delivery with Poly (ethylene glycol) Diacrylate Microneedles for Keloid Scar Treatment

4.1 Introduction

In addition to long-term stem cell tracking problem, there is another tough issue in the regenerative medicine field, which is keloid scar treatment for skin regeneration. Keloid scars, as a complex abnormal scar, cause not only aesthetic disfigure to the patients, but also have led to pruritus, infection, and much distress. Traditional measures to help management of keloids, such as surgery removal, intralesional injection of drugs, or silicon sheet dressing, may cause pain, infection, and less compliance to patients. High rate of recurrence and frequent follow-up result in many inconveniences. Therefore, it's very meaningful to develop a non-invasive method to deliver the drugs which patients can apply by themselves.

Microneedles are emerging as a proficient transdermal delivery system. It allows a variety of molecules to be transported into skin in a non-invasive way, which overcomes the limitations of conventional needle injection[147-149]. Polymer-based microneedles are specifically attractive due to their excellent biocompatibility, biodegradability and nontoxicity[150-152]. Drugs can be loaded into the microneedle tips and/or base, or coated on the microneedle tips[147, 150, 151]. One example is poly(ethylene glycol) diacrylate (PEGDA) based microneedles[153-155]. PEGDA is biocompatible and can be cross-linked in a short time under UV exposure (a few seconds), which facilitates the control of morphology and dimension of microneedles[153, 156]. Small molecule drugs like 5-fluororacil (5-FU) and curcumin have been integrated within microneedles during the fabrication process[157], in which drugs are mixed with the monomer before the mixture is placed in a mold and exposed to UV light. For example, our group has utilized PEGDA-made microneedles to deliver the 5-FU for treating keloid scar cells[158]. PEGDA microneedles have

sufficient mechanical strength to penetrate skin and release drugs to inhibit keloid fibroblast growth. In addition, other small molecule drugs, such as hydrophobic camptothecin (CPT) – that targets DNA topoisomerase I (Topo I) and inhibit collagen synthesis[43] can also be encapsulated and delivered in a similar manner.

In recent years, peptide-based therapeutics have played a major role in new drug development[159-162]. However, this process has not been without difficulties. Peptides face hurdles including instability under high temperature, light, and high/low pHs, and digestion by gastrointestinal enzymes during oral delivery. Microneedles have been suggested as a means to overcome this delivery hurdle since it can bypass oral uptake routes. The easiest way to load the peptide is to coat them on the surface of needles, however, it often requires optimization and adjusting the coating formulations by adding excipients to increase viscosity and decrease surface tension in order to achieve uniform and sufficient coating[93]. Preloading drugs into the needles during the fabrication is an alternative way. However, preloading processes should not disrupt or significantly compromise peptide activity. PEGDA microneedles have been used to deliver several small molecular drugs that are pre-loaded into the polymer matrix during the fabrication. However, this procedure is unsuitable for peptides since high energy UV rays can denature peptides thus compromise the biological activity of peptide-based therapeutics[163, 164].

This chapter introduces a gentle strategy to load peptides into the PEGDA microneedles without the above complications based on the swelling ability of PEGDA in aqueous solution. We discovered that substances with molecular mass less than 4kDa readily enter the PEGDA matrix during swelling in aqueous solutions. Furthermore, this process is dependent on the period of UV exposure to crosslink PEGDA monomers (part of the microneedle fabrication process). As a proof of concept, the model peptide (Gap 26, a connexin mimetic peptide that inhibits cellular gap-junction)[80, 81, 84, 86, 87, 165-167], is

loaded into the PEGDA microneedles using this PEGDA swelling strategy (Figure 29). After examining the loading capacity and release profile of the peptide-loaded microneedles, the potential therapeutic effect is explored on a keloid scar model comprising of cells and *ex vivo* skin.

Figure 29. Schematic showing the loading of hydrophilic peptides into poly(ethylene glycol) diacrylate microneedles by the swelling effect. (This figure is reprinted with copyright from[168])

4.2 Materials and Methods

4.2.1 Materials

Fluorescein Diacetate (FDA), Calcein-AM, Vybrant® DiI Cell-Labeling Solution, and Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix were purchased from Thermo Fisher Scientific Inc. Goat Anti-Mouse IgG (H+L) Antibody and Rhodamine conjugate were from Sigma Aldrich. Keloid fibroblasts (KF110) were ordered from Cell Research Corporation (Singapore). Dulbecco's modified eagle medium (DMEM) (w/4.5 g/L D-glucose, w/phenol red), fetal bovine serum (FBS), penicillin-

streptomycin (10,000 U/ml), Phosphate buffered saline (1x, PBS), and trypsin-EDTA (0.25 %, 10x) were obtained from Gibco Life Technologies (USA). All reagents were of analytical reagent grade and used without further purification.

4.2.2 Fabrication of blank and CPT loaded PEGDA microneedles

Firstly, a negative polydimethylsiloxane (PDMS) mold of microneedles was prepared by curing PDMS (base: curing agent = 10:1) on the stainless microneedle template for 3 h at 70 °C. In order to make blank PEGDA microneedles, the mixed solution of PEGDA (MW=250) and photoinitiator 2-hydroxy-2-methylpropiophenone (HMP) (0.5% v/v) was added to the negative PDMS mold pre-treated with plasma. The gas bubbles in the solution were removed by vacuum before the whole sample was exposed to UV (Intensity: 10 mW/cm²) for crosslinking. Finally, PEGDA microneedles patch was peeled off from the PDMS mold.

The CPT loaded PEGDA microneedles were fabricated in a similar way as the blank microneedles except that the CPT was present in PEGDA solution. CPT loaded microneedles were imaged by confocal laser scanning microscope (Carl Zeiss, LSM 710) with excitation at 405 nm laser and emission at 410 nm ~ 585 nm. Mechanical properties of microneedles were measured by Instron 5543 Tensile Meter with a speed of 0.5 mm/min.

4.2.3 Swelling ratio and mesh size determination

The microneedle patches were placed in distilled water for 22 h. Then the samples were taken out and gently dried on the paper, and the weight in the swollen state (W_{swell}) was obtained. Later, the patches were transferred into an oven preheated to 50 °C and dehydrated until a stable dry weight (W_{dry}). The swelling ratio can be calculated by equation (1).

$$Q = \frac{W_{\text{swell}} - W_{\text{dry}}}{W_{\text{dry}}} \times 100\% \quad (1)$$

To calculate the mesh size [169], the polymer volume fraction of the swollen state v_{2s} was firstly obtained by equation (2).

$$v_{2s} = \frac{1}{Q \frac{\rho}{\rho_{H_2O}} + 1} \quad (2),$$

where Q is the swelling ratio, ρ is polymer density, and ρ_{H_2O} is water density.

The average molecular weight between two consecutive crosslinks (M_c) can be calculated by equation (3), where M_n is the average molecular weight of the starting polymer (250 g/mol) here, V_1 is the molar volume of water (18 cm³/mol), χ is the Flory-Huggins polymer solvent interaction parameter and assumed equal to 0.426, v_{2r} is the polymer volume fraction in the relaxed state (i.e. right after crosslinking and before full swelling), and the polymer volume fraction in the precursor solution was assumed as indicative of v_{2r} .

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\frac{1}{V_1} [\ln(1-v_{2s}) + v_{2s} + \chi v_{2s}^2]}{\rho v_{2r} \left[\left(\frac{v_{2s}}{v_{2r}} \right)^{\frac{1}{3}} - \frac{1}{2} \left(\frac{v_{2s}}{v_{2r}} \right) \right]} \quad (3)$$

Finally, mesh size can be calculated by the equation (4).

$$\xi = l \left(2 \frac{M_c}{M_r} \right)^{\frac{1}{2}} C_n^{\frac{1}{2}} v_{2s}^{-\frac{1}{3}} \quad (4),$$

where l is the bond length (1.5 Å), M_r is the molecular weight of the PEG repeating unit (44 g/mol), and C_n is the characteristic ratio for PEG, equal to 4.

4.2.4 Fabrication of hydrophilic molecules (FITC, FITC-Dextran, Gap 26) loaded PEGDA microneedles

Aqueous solutions containing FITC and FITC-Dextran with different molecular weight (i.e. 4kDa, 10kDa, 20kDa, and 70kDa) were prepared with DI water and diluted to have the same FITC fluorescence intensity. A specific reservoir with flat bottom was made similarly by PDMS using the stainless microneedles to

allow one patch of microneedles to be immersed in the hydrophilic solution (100 μ L) for loading. After 22 h incubation, the microneedle patch was washed with water twice to remove the molecules on the surface before being dried naturally. The FITC and FITC-Dextran loaded microneedle patches were imaged by confocal laser scanning microscope (Carl Zeiss, LSM 710) with excitation at 488 nm laser and emission at 493 nm~634 nm.

FITC-labeled Gap 26 (FITC-C6-VCYDKSFPISHVR, MW=2,053.3) and unlabeled Gap 26 (VCYDKSFPISHVR, MW=1550.8) were acquired from Sigma. Similar to the loading of FITC and FITC-Dextran, Gap 26 was loaded into blank PEGDA microneedles through the incubation in the reservoir for 22 h. FITC conjugated Gap 26 loaded microneedles were imaged by confocal laser scanning microscope (Carl Zeiss, LSM 710) with excitation at 488 nm laser and emission at 493 nm~634 nm. The loading efficiency was calculated by comparing the total amount of peptides and the remaining in solution.

$$\text{Loading Efficiency} = \frac{\text{Loaded amount}(\mu\text{g})}{\text{Initial amount}(\mu\text{g})} \times 100\%$$

4.2.5 Release profile of CPT and Gap26 from microneedles

Microneedle patches loaded with drugs were immersed in DI water at 37 °C on a shaking device with a speed of 500 rpm. At desired time points, 50 μ L of solution was transferred to Greiner 96 well plate and the fluorescence intensity was examined with Synergy H4 (CPT: excitation 365 nm/ emission 428 nm; FITC-Gap 26: excitation 495 nm/emission 525 nm). The concentrations were calculated based on standard curves and cumulative released amount of drugs were calculated for different time points.

4.2.6 Cell viability test by AlamarBlue® assay

Keloid fibroblasts were seeded in 96 well plate at a density of 1,500 cells per well. After overnight culturing, culture medium was replaced with new culture medium containing different concentrations of free CPT (0.1 μ M, 10 μ M) for 24 h. After 24 h, the medium was removed and cells were gently washed by

PBS for 3 times. Finally, cell medium with Alamar Blue reagent was added. 4 hours later, fluorescence of the solution (emission/excitation: 555/585 nm) was examined and analyzed according to manufacturer's protocol. All the data was normalized by taking the viability of untreated cells as 100%.

4.2.7 FRAP assay

Keloid fibroblasts were seeded at 24 well plate at a density of 40,000 cells per well. Once they reached confluence, cells were incubated with serum-free medium for starvation for 1 h. Then medium was replaced with serum-free medium containing different concentrations of Gap 26 (0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL) for 2 h. Later, cells were stained with calcein-AM for 20 min. After washing twice by PBS, cells were imaged through confocal laser scanning microscope (Carl Zeiss, LSM 710) with excitation at 488 nm and emission at 493 nm ~ 616 nm. Thereafter, FRAP assay was performed at a selected specific region with 50% of 488 nm laser intensity to bleach the fluorescence for 3 cycles and immediately continuous imaged for 20 min to record the fluorescence recovery (imaged once per minute).

4.2.8 Real-time Polymerase Chain Reactions for collagen I expression

Keloid fibroblasts were seeded at the 24 well plate at a density of 40,000 cells per well. Once they reached confluence, culture medium was replaced with culture medium with different concentrations of free CPT (0.1 μ M, 10 μ M) for 24 h. Later cells were washed by PBS for 3 times before being lysed in Lysis buffer with β -mercaptoethanol. Subsequently, RNA was extracted by RNAeasy mini kit and the concentration was detected by NanoDrop UV-vis Spectrophotometer. The complementary DNA was synthesized according to the templates of RNA by Bioline SensiFAST cDNA Synthesis Kit. Finally, in order to quantify the gene expression of samples, Real-time Polymerase Chain Reactions (RT-PCRs) were conducted with SYBR Green PCR Master Mix kit by Step One Plus Real-Time PCR System. The process includes denaturation at 95 °C for 10 min, and amplification for 40 cycles with 95 °C denaturation for

15 s and 60 °C extension for 1 min. The forward and backward primer sequences of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and collagen I are listed in Table 5. The mRNA expression level of collagen I was normalized to the housekeeping gene GAPDH, and then calculated as fold change according to comparative CT method using the $2^{-\Delta\Delta C_t}$ formula.

Table 5. Primer sequences for real-time polymerase chain reactions (This table is reprinted with copyright from[168])

Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
Collagen I	CAGAACGGCCTCAGGTACCA	CAGATCACGTCATCGCACAAAC

4.2.9 3D agarose hydrogel model

400,000 keloid fibroblasts were suspended in 75 µL complete medium. Then 75 µL of 3% agarose pre-heated for melting and cooled to 37 °C was mixed with the cell suspension, in which the bubble formation was avoided. Subsequently, 10 µL of the suspension was added to each well of the 48 well plate. After being placed at 4 °C for 4 min, the agarose gel formed and was placed in the incubator overnight. Later, CPT loaded PEGDA microneedles were inserted into agarose beads for 24 h. After the removal of microneedles, cells were stained with FDA for 20 min and imaged with confocal microscope (LSM 710).

4.2.10 *Ex-vivo* keloid model

Full-thickness human skin derived from operations to remove excess tissue was purchased from Cell Research Corporation (Singapore). The skin samples were cut to 1.3 cm×1.3 cm and maintained in complete medium (DMEM containing 10% FBS). During the maintenance in the incubator, the epidermis layer was exposed to the air while the dermis was submerged in the medium. In order to identify the location and distribution of injected cells, keloid fibroblasts were stained with DiI (1 mL complete medium with 3 µL DiI labeling solution) for 2 h, followed by washing with PBS. Following overnight incubation, DiI-labelled

cells were trypsinized before resuspension in 100 μ L cooled medium (15,000 cells per μ L). The cell suspension was mixed with 100 μ L GelTrex matrigel gently, to avoid the generation of bubbles. Thereafter, the cells – matrigel mixture was injected from the dermis-side of the skin sample using a 30G needle. After resting at room temperature for 15 min, 200 μ L of medium was added and the samples were incubated at 37 °C overnight. After 24 h, the skin samples were fixed in formalin for 72 h, dehydrated by 30% sucrose for 8 h, and 4% sucrose for 18 h. Finally, the samples were frozen in Tissue Freezing Medium and cryosectioned at a thickness of 10 μ m by Leica CryoStat CM1950. The frozen slides were stored at -20 °C before further characterization.

The therapeutic efficacy of drug-loaded microneedles was examined with the skin samples injected with unlabeled keloid fibroblasts. The CPT and FITC loaded PEGDA microneedles were pressed into the skin sample by an applicator (Micropoint Pte Ltd). There were four groups: “CPT MN twice” (CPT MNs (low loading concentration: 18 nmol) were applied at day 14 and 21), “Gap 26 MN twice” (Gap 26 MNs (loading amount: 125 μ g) were applied at day 14 and 21), “CPT MN once” (CPT MNs (low loading concentration: 18 nmol) were applied once at day 21), and “Gap 26 MN once” (Gap 26 MNs (loading amount: 125 μ g) were applied once at day 21). At day 28, the patches were removed from all samples. The skin was fixed in formalin and processed as above for cryosection and immunostaining for collagen I examination.

4.2.11 Immunostaining for collagen I

The sliced skin samples were treated by 0.2% Triton-X 100 in PBS for 10 min, followed by 0.1% Triton-X 100 in PBS for 5 min. After being washed with PBS 3 times, they were placed in the 1% BSA in PBS for 30 min and labeled with primary antibody solution (mouse anti human collagen I, 1 to 100) at 4 °C overnight. Later, the slides were washed by 0.1% Triton-X 100 in PBS for 3 times and stained with secondary antibody (Goat anti mouse IgG, Rhodamine conjugate, 1 to 100) at room temperature for 4 h. Finally, Hoechst (10 μ g/mL)

was added to stain the nuclei. The stained slides were examined by confocal microscope (LSM 710) (Rhodamine: excitation at 561 nm and emission at 570 nm to 660 nm; Hoechst: excitation at 405 nm and emission at 410 nm to 543 nm.)

4.2.12 Statistical analysis

All values were expressed as means±standard deviation. One-way ANOVA and Tukey HSD test was used for statistical analysis with $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***) as significant.

4.3. Results

4.3.1 PEGDA microneedles loaded with hydrophobic camptothecin

10 μM CPT was shown to inhibit the proliferation of keloid fibroblasts (Figure 30A) and suppress the collagen I expression (Figure 30B). Even only after 24 hours, the proliferative activity of CPT of fibroblasts was reduced by 18.21 %. Simultaneously, the cellular collagen I expression per cell decreased ~55 % under the treatment.

Figure 30. The inhibition of (A) the proliferation and (B) collagen I expression of keloid fibroblasts by camptothecin (CPT). ** $p<0.01$, *** $p<0.001$. (This figure is reprinted with copyright from[168])

As a hydrophobic molecule, CPT can be loaded into PEGDA microneedles during microneedle fabrication by dissolving it with PEGDA monomer. The PEGDA microneedles exhibited homogeneous pyramid-like structures with a

base diameter of 300 μm and a depth of ~ 850 μm following photopolymerization (Figure 31A, B). The fluorescence from microneedles confirmed the presence of CPT (Figure 32A). The release profile of CPT was determined at desired time points by measuring fluorescence intensity and calculating the released amount based on a standard curve of pre-determined drug concentrations (Figure 32B). Within 24 hours, 0.37 nmol CPT was demonstrated to be released. In 3D agarose gels containing keloid fibroblasts, CPT-loaded microneedles similarly inhibited fibroblast proliferation (Figure 32C, D). Specifically, CPT loaded microneedles treated groups reduced cell proliferation by 30.1% compared to untreated cells in 24 hours.

Figure 31. (A) Stereomicroscope images of blank PEGDA microneedles, scale bar: 500 μm . (B) Scanning electron microscopy (SEM) images of blank PEGDA microneedles, scale bar: 100 μm . (This figure is reprinted with copyright from[168])

4.3.2 Swelling effect of PEGDA microneedles in aqueous solution

PEGDA exhibits swelling behavior in aqueous solutions[169]. As shown in Figure 33, PEGDA microneedles fabricated using different UV exposure exhibited different swelling properties. Generally, longer UV exposures resulted in smaller swelling ratios (Q). Microneedles were observed to increase their volume by up to 18.88%. Mesh size can be calculated using the equations 1-4. A higher swelling ratio suggests a larger mesh size (Figure 34). Specifically, the mesh size was 2.0 nm when the patch was prepared with a short UV exposure time (18s). PEGDA microneedle patch crosslinked with UV exposure for a period of 18s was selected for the subsequent experiments.

Figure 32. (A) Confocal images of CPT loaded microneedles. Scale bar: 100 μm . (B) Release profile of CPT from the microneedle patch in PBS. (C) Confocal images of agarose hydrogel containing keloid fibroblasts after the treatment with PEGDA microneedle patches for 24 h, scale bar: 100 μm . (D) Anti-proliferative effect of CPT loaded microneedles against keloid fibroblasts encapsulated in agarose gel; * $p < 0.05$. (This figure is reprinted with copyright from [168])

Figure 33. Swell performance of PEGDA microneedles and loading molecules with different molecular weight. (A) Swelling ratio of PEGDA microneedles prepared with different UV exposure time. ** $p < 0.01$. (B) Confocal images of PEGDA microneedles loaded with FITC and FITC-Dextran with different molecular weight. Scale bar: 100 μm . (C) Quantified microneedle fluorescence intensity in B relative to that with free FITC. (This figure is reprinted with copyright from [168])

In the swelling process, molecules like FITC and FITC-Dextran can enter the polymer matrix through diffusion (Figure 33). Obviously, this process is size-dependent, with smaller molecules enter the polymer matrix more readily. In addition, the distribution of loaded FITC and FITC-Dextran molecules within microneedles was inhomogeneous, with more observed at the edge. Increased

cargo size (4kDa to 70kDa), led to a reduction in loading efficiency as reflected in a decrease in microneedle fluorescence signal (Figure 33B, C). Above 4kDa, the molecules exhibit poor diffusion into the polymeric matrix. Based on the total fluorescence signal from microneedle samples, their relative loading efficiency (compared to free FITC) was found to be 21.7%, 8.1%, 3.7%, and 2.4% for FITC-Dextran 4kDa, 10kDa, 20kDa, and 70kDa, respectively.

Figure 34. Mesh size of PEGDA microneedles prepared with different UV exposure time. ** $p < 0.01$. (This figure is reprinted with copyright from [168])

4.3.3 Peptide loaded PEGDA microneedles

Since the characterization of PEGDA swelling showed that hydrophilic molecules smaller than 4kDa can be loaded into microneedles, we hypothesized that the peptide Gap 26 (MW=1550.8) could similarly be loaded. Gap26, as a representative connexin mimetic peptide, can mimic specific amino acid sequences of two well-conserved extracellular loops of connexins and interfere with their functions. Gap junctions are formed by intercellular channels composed of connexin proteins, which are known to impact inflammatory response, wound closure, and scar formation[80-84]. Suppressing connexins activity by antisense oligodeoxynucleotide was found to reduce the spread of tissue damage, accelerate wound closure, and reduce scarring[85]. So, we hypothesized Gap 26 is able to disturb connexins functions, interfere with gap

junction based intercellular communications, thus exhibit potential for keloid scar treatment.

Fluorescence recovery after bleach (FRAP) assay was performed to characterize the inhibitory effect of Gap 26 in gap junction based intercellular communication[170]. Fibroblasts were initially incubated with Gap26 before calcein staining. Subsequently, selected area of the cells was photobleached using high power laser (the red box of Figure 35A) and the recovery of calcein fluorescence at that region was recorded (Figure 35B). The fluorescent intensity during the recovery period was compared with the intensity immediately after bleaching. In 20 min, the fluorescence of untreated cells increased to 5.89 folds. However, the presence of Gap 26 peptide significantly delayed this recovery. For example, only a 1.56-fold increase in the fluorescence intensity was observed in keloid fibroblasts treated with 0.5 mg/mL Gap 26. This suggests considerable attenuation of intercellular communication by gap junctions, indicating the efficacy of Gap26 against connexins, intercellular channels, showing promise for the improvement of scar treatment.

Figure 35. Effect of Gap 26 on FRAP of keloid fibroblasts and loading of FITC-Gap 26 into PEGDA microneedles. (A) Confocal images of keloid fibroblasts in FRAP experiment with the treatment of Gap 26. Scale bar: 20 μm . (B) Recovered fluorescence intensity change during fluorescence recovery. * $p<0.05$, ** $p<0.01$. (C) Confocal images of Gap26 loaded PEGDA microneedles. Scale bar: 100 μm . (D) Quantitative analysis of C. (This figure is reprinted with copyright from[168])

Subsequently, we loaded PEGDA microneedles with Gap26 through the swelling effect of PEGDA. Gap26 was labeled with FITC to facilitate quantification. As shown in Figure 35C, D, a higher peptide concentration in the incubation process provided a higher peptide loading in the microneedles. By quantifying the peptides in the loading solution before and after incubation, the loading efficiency of peptide was calculated (Table 6). For example, $252.13\pm5.94\ \mu\text{g}$ of Gap26 can be loaded to one patch of microneedles (10 by 10 tips) with $50.43\%\pm1.19\%$ loading efficiency for the initial loading amount of 500 μg . As the loading process was performed with only the microneedle tips immersed inside the hydrophilic solution, the drugs were only localized in the tips without excess drug trapped in the base. This maximizes the utilization of the drugs. In this example, each tip was loaded with $2.52\pm0.06\ \mu\text{g}$ of Gap26.

Table 6. FITC-Gap 26 loading amount and efficiency into PEGDA microneedles by swelling effect. (This table is reprinted with copyright from[168])

Initial amount in solution (μg)	500	250	125	62.5
Initial concentration in solution ($\mu\text{g}/\mu\text{L}$)	5	2.5	1.25	0.625
Loaded amount (μg)	252.13 ± 5.94	130.99 ± 15.13	51.75 ± 19.85	27.81 ± 1.40
Loading efficiency (%)	50.43 ± 1.19	52.40 ± 6.05	41.40 ± 15.88	44.50 ± 2.25

The loaded peptides were promptly released once the microneedles were placed in the aqueous solution. As shown in Figure 36, $13.47\pm4.52\ \mu\text{g}$ of FITC-Gap 26 can be released from the tips of microneedles that contained $252.13\pm5.94\ \mu\text{g}$ peptides in total.

Figure 36. Cumulative release of Gap26 from the microneedle patch. (This figure is reprinted with copyright from[168])

4.3.4 Therapeutic effect of Gap26 loaded microneedles in *ex-vivo* keloid model

A keloid disease model was next used to explore the potential efficacy of Gap26-loaded microneedles. Briefly, the model was built by injecting keloid fibroblasts into the dermis region of an *ex vivo* human skin sample (Figure 37A). Keloid fibroblasts were labeled with the lipophilic dye DiI (Figure 38) to enable identification (Figure 37B).

Before the study commenced, keloid fibroblasts were injected into the dermis region of the skin. At day 14, the microneedle patch was applied on the skin for one week. The patch was then replaced with a new one at day 21 for another week to sustain drug release and maintain drug amount within the skin. We studied two types of microneedle patches, CPT- and Gap26- loaded microneedles. At day 28, skin samples were fixed and sectioned to examine the collagen I expression.

Figure 37. *Ex-vivo* keloid scar model and injection of keloid fibroblasts. (A) Illustration of the *ex-vivo* keloid model. (B) Presence of DiI labelled keloid fibroblasts in the dermis region of skin samples. Scale bar: 100 μ m. (This figure is reprinted with copyright from[168])

Figure 38. Keloid fibroblasts labelled with DiI. scale bar: 100 μ m. (This figure is reprinted with copyright from[168])

The presence of keloid fibroblasts increased the expression of collagen I in the dermis layer of the skin (Figure 39A), which is reminiscent of native keloid scars[165, 171].

Keloid scar is characterized by abundant deposition of collagen, especially collagen type I[172]. The treatment with Gap 26 loaded microneedles twice (“Gap 26 MN twice”) reduced the collagen I expression to 36.9% (Figure 39B). Treatment with CPT loaded microneedles twice (“CPT MN twice”) also suppressed collagen I expression to 47.3% (Figure 40). The data reflected a promising trend although there is no significant difference ($p>0.05$), probably due to the individualized difference and limited number of skin samples and more samples can be tested in the further studies. The PEGDA microneedles still maintained their homogeneous pyramid tips structure after application in the skin samples and didn’t dissolve or deform in the skin to leave any remnants as shown in Figure 41. These results indicated the CPT and Gap26-loaded microneedles were effective to inhibit the collagen I expression from keloid fibroblasts, demonstrating their potential efficacy for scar treatment.

Figure 39. Effect of Gap 26 loaded microneedles on collagen I expression of skin samples in the *ex vivo* model. (A) Immunostaining of Collagen I expression after the treatment with Gap26-loaded microneedles. Scale bar: 100 μ m. (B) Quantitative analysis of collagen I expressions in A by normalization to the modified skin without treatment. (This figure is reprinted with copyright from[168])

Figure 40. (A) Immunostaining of Collagen I expression in the *ex vivo* keloid model after the treatment with CPT loaded microneedles. Scale bar: 100 μ m. (B) Quantitative analysis of collagen I expressions in A by normalization to the keloid model without treatment. (This figure is reprinted with copyright from[168])

Figure 41. Stereomicroscope images of CPT and Gap26 loaded PEGDA microneedles after application in the *ex vivo* skin samples, scale bar: 500 μ m. (This figure is reprinted with copyright from[168])

4.4 Discussion

In recent years, there has been increasing interest to apply biomolecules including peptide/protein as targeted therapeutics. Delivery these molecules by microneedles can avoid their digestions by gastrointestinal enzymes during oral delivery. Previously reported methods to load these biomolecules in the microneedles have been limited. In this paper, we developed a facile, effective, and gentle strategy to load peptide into biocompatible PEGDA microneedles. A model peptide, Gap26 (connexin mimetic peptide) was chosen as a candidate therapeutic and applied on an *ex vivo* keloid scar model to prove this concept.

To date, PEGDA microneedles have mainly been used to deliver small molecular drugs (e.g. 5-fluororacil (5-FU) and curcumin) that are chemically stable to high temperature, light, and extreme pH conditions. These stable molecules can be mixed with the monomer before UV exposure or heating to generate microneedles. For example, CPT can reduce collagen biosynthesis, a key step in scar formation[35, 43]. When this drug was encapsulated in PEGDA microneedles through photolithography, no noticeable change was observed in its efficacy to inhibit cell proliferation and collagen I expression.

Subsequently, we loaded hydrophilic sugar and peptide molecules by swelling PEGDA microneedles in an aqueous solution. Upon hydration, the PEGDA matrix allows solutes to diffuse into the hydrogel matrix. As discussed above, the mesh size of PEG hydrogel[173, 174], related to its crosslinking density determines whether molecules can readily diffuse into the matrix. Thus, molecules larger than the mesh size cannot be efficiently loaded into PEGDA. Here, we controlled the mesh size of PEGDA microneedles by tuning the UV exposure time during the fabrication and ended up with a PEGDA matrix with a mesh size of 2.0 nm. Thereafter, we tested the loading of various molecules with distinct molecular weights (FITC, FITC-dextran 4kDa, 10kDa, 20kDa). Due to the difference in molecular weight, their hydrodynamic sizes vary from angstrom scale to 1.3 nm to 2.0 nm to 2.8 nm[157]. In this case, FITC-dextran

molecules bigger than 10kDa are larger than the mesh size of PEGDA. Thus, it is expected that minimal quantities of larger molecules were observed in microneedles. Furthermore, Stokes-Einstein equation indicates diffusion is related with the radius of the solute on a fixed temperature and solvent[175, 176]. So, it is reasonable to observe higher signals for the needles loaded with FITC than FITC-dextran 4kDa.

Next, we chose the connexin mimetic peptide, Gap 26 (MW=1550.8) as a model drug to be loaded into PEGDA microneedles and applied for keloid scar therapy. Inhibition of cell-cell communications through gap junctional couplings is an emerging concept to treat scar[84]. Connexin mimetic peptides like Gap 26 are valuable tools to inhibit connexin functions, making it promising for scar reduction[87, 177]. We confirmed Gap26 can suppress the cell-cell communication between keloid fibroblasts and loaded Gap 26 into PEGDA microneedles. The loading of Gap26 is similar to that of dextran. As indicated in Fick's law, $J = -D\nabla\varphi$, where J is the diffusion flux of the amount per unit area per unit time, D is the diffusion coefficient, $\nabla\varphi$ is the concentration gradient. The peptide solution with higher concentration generates bigger concentration gradient, which allows more peptide molecules to diffuse into the polymeric network given a similar time period and surface area. An interesting phenomenon is that FITC and FITC-dextran 4kDa were mainly located on the edges of microneedles, while Gap26 exhibited more homogenous distribution after loading. This may be related to their different molecular structures, which requires the detailed further studies. The amount loaded to each tip of microneedles through this strategy can reach 2.52 ± 0.06 μg of Gap26, which is similar to the previously reported coating method[93]. However, our loading strategy is comparatively more straightforward since formulation viscosity or surface tension does not need adjustment.

After 7h, 13.47 ± 4.52 μg of FITC-Gap 26 was released from microneedles with 252.13 ± 5.94 μg loaded. As the release was detected by immersing the whole

patch inside water at 37 °C, the swelling occurs throughout the whole patch. As such, released Gap26 from the tips may enter the matrix at the base of microneedles as well, possibly resulting in the relatively low proportion of Gap 26 release. Nonetheless, this situation is not likely to occur in the future therapeutic applications, as skin penetration is limited to microneedle tips.

The efficacy of Gap26-loaded microneedles for keloid scar treatment was investigated on an *ex vivo* keloid scar model. Application of Gap 26-loaded microneedles (especially when applied twice in the treatment period; 4 weeks) could inhibit collagen I expression efficiently, demonstrating the possibility of this peptide loading system in the treatment of keloid scar. This signifies an interesting transdermal drug delivery system, which is potentially for loading other small molecular weight hydrophilic drug molecules in the applications of various diseases.

4.5. Conclusions

In summary, a facile, effective, and gentle strategy is reported to load peptides into PEGDA microneedles by exploiting the swelling phenomena of PEGDA in aqueous solutions. By regulating UV crosslinking time, the swelling ratio and mesh size of PEGDA microneedles can be controlled. This allows loading of molecules less than 4kDa. As a proof of concept, the model peptide, Gap 26 was loaded into the PEGDA microneedles to suppress the gap junction based intercellular communication between keloid fibroblasts which leads to reduced collagen I expression in an *ex vivo* model. This exhibits its therapeutic potentials for keloid scar.

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Chapter 5: Conclusions and Future Work

5.1 Conclusions

In this thesis, two problems existed in regenerative medicine are solved including long-term tracking mesenchymal stem cell differentiation by biocompatible and highly photostable fluorescent nanoparticles and keloid scar treatment by transdermal delivering connexin mimetic peptide through PEGDA microneedles.

First of all, a new type of highly fluorescent and bioresorbable polymeric NPs were prepared through nanoprecipitation from a well-defined polymer, PCL–DPP–PCL, in the presence of Pluronic® 127 as the stabilizer. Covalent insertion of DPP as a highly fluorescent moiety into the middle of each PCL chain and generated steric effect effectively suppress the π – π aggregation of DPP in the PCL matrix to reduce the bleaching effect. The NPs prepared were highly fluorescent and could be internalized by MSCs with little cytotoxicity. Most importantly, the PCL–DPP–PCL NPs exhibited significantly enhanced photostability compared to several commercial organic fluorophores, the physically blended DPPHT/PCL NPs and Qtracker® for imaging stem cells.

Secondly, these PCL-DPP-PCL nanoparticles were applied for long-term tracking of mesenchymal stem cell differentiation. *In vitro* cell experiments exhibited the nanoparticles labelling did not compromise the cell viability and demonstrated different performances on the multilineage potency of the MSCs during differentiation into three lineages. Specifically, compared to the unlabeled control cells, this probe labeling compromised MSC osteogenic differentiation, but didn't show any negative effects for adipogenic and chondrogenic as verified by gene expressions and histological staining. Furthermore, these fluorescent probes were able to maintain their strong fluorescence intensity even after 4 weeks of differentiation. This study

demonstrated that PCL-DPPPCL nanoparticles could be used for long-term cell tracking in MSC differentiation into adipogenic and chondrogenic lineages.

Finally, regarding to the keloid scar treatment issue in the regenerative medicine field, we introduced a facile, effective, and gentle strategy to load PEGDA microneedles with hydrophilic peptide, such as connexin mimetic peptides (Gap26) without compromising peptide potency through the swelling effect of PEGDA in the aqueous solution. By regulating UV crosslinking time, the swelling ratio, and mesh size of PEGDA microneedles can be controlled. Gap 26 was loaded into the PEGDA microneedles to suppress the gap junction-based intercellular communication between keloid fibroblasts which leads to reduced collagen I expression in an *ex vivo* model. This exhibits its therapeutic potentials for keloid scar.

5.2 Limitations and future work

Up to now, this thesis has managed to develop a biocompatible and photostable fluorescent probe, which demonstrated superb photostability compared to organic dyes, could maintain fluorescent signals at a higher ratio compared to commercial quantum dots, and exhibited much potentials for long-term tracking MSCs' adipogenic and chondrogenic differentiation (up to 4 weeks) without disturbing their viability, proliferation, and differentiation performance. However, they showed negative effects on the osteogenic differentiation of MSCs with reducing the representative mRNA expressions. The detailed reason is still unknown based on the current results and requires further studies, but the different influences of NPs on each specific lineage differentiation indicated it's necessary to investigate their effects individually before the final application.

On the other hand, the biosafety and degradation studies should be performed to investigate *in vivo* before their real application *in vivo*. Besides, the current fluorophore-DPP has limitations for the long-term tracking applications *in vivo* since its emission is still in the visible range. Recent decades witnessed the

advanced development of near infrared (NIR) dyes, which are more attractive for *in vivo* imaging with two main advantages. Firstly, NIR light is able to penetrate deeper into tissue compared to the visible light, which allows to interpret deeper tissue structure information. Secondly, NIR dye can provide better signal to noise ratio because there is less autofluorescence in the NIR range compared to visible light. So the future work can apply this strategy to enhance photostability of NIR dye for *in vivo* studies.

Chapter 4 reported a facile and non-disruptive strategy to load hydrophilic peptide into polymeric microneedles and demonstrated effects for keloid scar treatment. This system can be also used for loading other hydrophilic molecules, such as sugar, siRNA by the swelling of microneedles. However, this delivery system is currently only for the hydrophilic molecules with molecular weight less than 4 kDa. Loading bigger molecules above this threshold will be achieved by further optimizations and controls to increase the swelling ratio of the microneedles but maintain the mechanical strength to penetrate the skin.

In addition, an *ex vivo* keloid scar model was developed in this thesis to test the efficacy of drug loaded microneedles. However, the effect is still not satisfying enough for the clinical applications. In the future, to obtain better effects, combination of two or more drugs can be explored. This thesis has demonstrated both hydrophobic and hydrophilic drugs can be loaded inside the PEGDA microneedles. The synergistic effect of two or more drugs have much possibility to demonstrate better results for the treatment of keloids. In addition, a well-recognized animal model of keloid scar should be established although with difficulties for testing all kinds of transdermal delivery systems.

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