PHYSICO-CHEMICAL PROPERTIES OF STAINLESS STEEL 316L COATED WITH GINSENG-POLY(LACTIC-CO-GLYCOLIC ACID) FOR STENT APPLICATION

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Dedicated to my mother and father, two little sisters,
mentors and friends who are supporting me.
Thank you for all of your care and motivations.

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ABSTRACT

Drug eluting stent is a stent coated with an anti-proliferative drug to prevent in-stent restenosis in blood vessels. Although this stent is superior to the bare stent, another post-stenting complication called late thrombosis, occurs as a result of late re-endotheliazation. Therefore, this project was aimed to develop a drug eluting coating with ginseng extract that contained active ingredients of ginsenosides Rg1 and Re, proven to not only inhibit the proliferation of vascular smooth muscle cells but also to promote the growth of vascular endothelial cells. In this project, poly (lactic-*co*-glycolic) acid (PLGA) matrix was incorporated with ginseng extract at different ratios: 10%, 30% and 50% (w/w). The gelation solvents were then coated on a stainless steel 316L, a substrate, by a dip coating technique. The coatings were characterized by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), scanning electron microscopy (SEM) and contact angle analyses while the drug release profile was studied through one month immersion tests and analyzed by a mass spectrometry instrument (Q-TOF LC-MS). The FTIR analyses confirmed that the coatings were composed of ginseng and PLGA. The SEM images showed that a full coverage and even coating was found on the 30% sample. Higher ratio of PLGA caused higher hydrophobicity but not as high as the bare substrate. The immersion study showed that all PLGA concentrations undergoes initial burst release dependent on the concentrations. The release mechanism for the 30% and 50% samples was a combination of diffusion and swelling-controlled of PLGA whereas the release mechanism for the 10% sample was a Fickian diffusion. The optimum coating was found on the 30% sample as it demonstrated acceptable wettability, even coating coverage and controlled release through the PLGA swelling. The ginseng-PLGA coating by a dip coating technique is practicable and it can be used as a drug eluting coating for stent application.

ABSTRAK

Sten pembebasan ubat ialah sten yang dilapisi dengan ubat anti-pertumbuhan untuk menghalang penyempitan kembali sten di dalam pembuluh darah. Walaupun sten ini lebih berkesan berbanding sten logam tanpa salutan, berlakunya komplikasi lain selepas implantasi yang dikenali sebagai kelewatan thrombosis, kesan kepada kelewatan pertumbuhan semula endotelial. Oleh itu, projek ini bertujuan untuk menghasilkan salutan pembebasan ubat dengan ekstrak ginseng yang mengandungi ramuan aktif ginsenoside Rg1 dan Re, yang terbukti bukan sahaja menghalang pertumbuhan sel otot licin vaskular tetapi juga menggalakkan pertumbuhan sel endotelial vaskular. Dalam kajian ini, matrik asid poli(laktik-*ko*-glikolik) (PLGA) dilarutkan dengan ekstrak ginseng pada pelbagai nisbah: 10%, 30% dan 50% (w/w). Pelarut gel tersebut kemudiannya disalut ke atas substrat keluli tahan karat 316L menggunakan teknik penyalutan celup. Salutan dianalisis menggunakan jumlah pantulan pengecilan-spektroskopi pengubah inframerah Fourier (ATR-FTIR), mikroskop pengimbas elektron (SEM) dan analisis sudut sentuhan manakala profil pembebasan ubat dikaji melalui ujian rendaman selama sebulan dan dianalisis oleh instrumen spektrometri berat (Q-TOF LC-MS). Analisis ATR-FTIR mengesahkan bahawa salutan tersebut mengandungi ginseng dan PLGA. Imej SEM menunjukkan bahawa salutan adalah menyeluruh dan sama rata pada sampel 30%. Nisbah PLGA yang tinggi menyebabkan peningkatan sifat hidrofobik namun tidak setinggi substrat tanpa salutan. Ujian pembebasan ubat menunjukkan bahawa semua nisbah PLGA melalui pembebasan pecah pada permulaan ujian, bergantung kepada kepekatan. Mekanisma pembebasan untuk sampel 30% dan 50% ialah gabungan serapan dan pengembangan berkadar PLGA manakala mekanisma pembebasan untuk sampel 10% ialah serapan Fick. Lapisan optimum dikenalpasti pada sampel 30% kerana ia menunjukkan kebolehbasahan yang baik, salutan menyeluruh dan pembebasan terkawal melalui pengembangan PLGA. Penyalutan ginseng-PLGA menggunakan kaedah penyalutan celup adalah praktikal dan boleh digunakan sebagai salutan pembebasan ubat untuk aplikasi sten.

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LIST OF ABBREVIATIONS

AMPK	-	5'AMP-activated protein kinase
AP	-	Activator protein
ASK1	-	Apoptosis signal-regulating kinase
ATR-FTIR	-	Attenuated total reflectance-Fourier transform infrared spectroscopy
bFGF	-	Basic fibroblast growth factor
BMS	-	Bare metal stent
CDKs	-	Cyclin-dependent kinases
CE	-	Conformité Européenne
cGMP	-	Cyclic guanosine monophosphate
Co-Cr	-	Cobalt-chromium
CVD	-	Cardiovascular diseases
DES	-	Drug eluting stent
DMEM	-	Dulbecco's Modified Eagle's Medium
DNA	-	Deoxyribonucleic acid
EC	-	Endothelial cells
EDRF	-	Endothelium-derived relaxing factor
EDX	-	Energy disperse X-ray
EGF	-	Epithelial growth factor
eNOS	-	Endothelial nitric oxide synthase
ERK	-	Extracellular signal-regulated kinase
WHO	-	World Health Organization
FDA	-	United States Food and Drug Administration
GR	-	Glucocorticoid receptor
HAEC	-	Human aortic endothelial cells

HCl	-	Hydrochloric acid
HUVEC	-	Human umbilical vein endothelial cells
IAP	-	Inhibitory apoptotic protein
IL-1	-	Interleukin 1
iNOS	-	Inducible nitric oxide synthase
JAK	-	Janus kinase
JNK	-	c-Jun N terminal kinase
KCl	-	Potassium chloride
LST	-	Late stent thrombosis
MAPK	-	Mitogen activated protein kinase
MDM2	-	Murine double minute-2
MMP	-	Matric metalloproteinase
mTOR	-	Mammalian target rapamycin
NaCl	-	Sodium chloride
NO	-	Nitric oxide
NRF2	-	Nuclear factor-erythroid 2-related factor-2
OH	-	Hydroxyl
OG	-	Ongoing
oxLDL	-	Oxidize lipoprotein
PBMA	-	Poly(n-butyl methacrylate)
P. ginseng	-	*Panax ginseng*
P.notoginseng	-	*Panax notoginseng*
P. quinquefolius	-	*Panax quinquefolius*
PAI-1	-	Plasminogen activator inhibitor-1
PC	-	Phosphorylcholine
PCI	-	Percutaneous coronary intervention
PCL	-	Polycaprolactone acid
PDLLA	-	Poly (D, L-lactide) acid
PDGF	-	Platelet-derived growth factor
PDGF-BB	-	Platelet-derived growth factor-BB
PGA	-	Polyglycolic acid
PKA	-	Protein kinase
PKC	-	Protein kinase C

PLA	-	Polylactic acid
PLGA	-	Poly (lactic-*co*-glycolic) acid
PPD	-	Protopanaxadiol
PPT	-	Protopanaxatriol
Pt-Cr	-	Platinum-chromium
PVP	-	Polyvinylpyrrolidone
P13K/Akt	-	Phosphatidylinositol 3-kinase/ protein kinase B
Q-TOF LC-MS	-	Quadrupole time-of-flight liquid chromatography-mass spectrometry
SEM	-	Scanning electron microscopy
SIBS	-	Poly(styrene-b-isobutylene-b-styrene);
SiRNA	-	Small interfering ribonucleic acid
SNAP	-	S-nitroso-N-acetylpenicillamine
SNP	-	Sodium nitroprusside
SOD-1	-	Superoxide dismutase-1
SS	-	Stainless steel
STAT	-	Signal tranducers and activators of transcription family
t-PA	-	Tissue-type plasminogen activator
TRAIL-R1/DR4	-	Tumor necrosis factor-related apoptosis-inducing ligand/death receptor 4.
VEGF	-	Vascular endothelial growth factor
VSMC	-	Vascular smooth muscle cells
NF-κB	-	Nuclear factor κB
TNF-α	-	Tumor Necrosis factor alpha
Ca^{2+}	-	Calcium ion
$CaCl_2$	-	Calcium chloride
C_2H_3N	-	Acetonitrile
CH_2O_2	-	Formic acid
$C_3H_8O_3$	-	Glycerol
$C_2H_6O_4S$ *(DMSO)*	-	Dimethyl sulfoxide
$(HOCH_2)3\ CNH_2$	-	Tris-hydroxymethyl aminomethane
H_3PO_4	-	Phosphoric acid

$K_2 HPO_4.3H_2O$	-	di-potassium hydrogen phosphate trihydrate
$MgCl_2.6H_2O$	-	Magnesium chloride hexahydrate
$NaHCO_3$	-	Sodium hydrogen carbonate
Na_2SO_4	-	Sodium sulfate

LIST OF SYMBOLS

^{o}	-	Degree
$=$	-	Equal
$<$	-	Less than
%	-	Percentage
A	-	Ampere
dL/g	-	Deciliter per gram
g	-	Gram
kV	-	Kilovolt
L/min	-	Liter per minute
mg	-	Milligram
mg/L	-	Milligram per liter
mm	-	Millimeter
mL	-	Milliliter
mm/min	-	Millimeter per minute
mL/min	-	Milliliter per minute
mPa.s	-	Millipascal seconds
m/z	-	Mass-to-charge ratio
V	-	Volt
w/w	-	Mass per mass
w/vol	-	Mass per volume
α	-	Alpha
β	-	Beta
κ	-	Kappa
μm	-	Micrometer
$\mu mol/L$	-	Micromole per liter

μL	-	Microliter
^{o}C	-	Degree celcius
cm^{2}	-	Square centimeter
cm^{-1}	-	Per centimeter

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

In the South Asia region including Malaysia, both morbidity and mortality caused by the cardiovascular disease (CVD) is high and it is expected to increase even more due to high prevalence of diabetes, hypertension and smoking [1]. Statistics from the Ministry of Health Malaysia 2013 showed that diseases of the circulatory system are the number one cause of death in the public and private hospitals with 24.38% and 27.73% of total deaths, respectively [1]. The *Global Atlas on cardiovascular disease prevention and stroke* report stated that over 80% of CVD deaths take place in low and middle income countries [2]. Besides, the World Health Organization (WHO) predicted more than 23.3 million of people would die annually from CVDs by 2030 [3]. With increasing number of cases, especially in the low and middle income countries, it is vital to take active prevention and offer more affordable treatment of CVDs.

Atherosclerosis is a type of arteriosclerosis that leads to several CVDs such as peripheral arterial disease, coronary heart disease, stroke and heart attack [3]. This condition caused by an accumulation of fatty plaque and cholesterol blockage

that later thicken and harden blood vessels [3]. One of the current most used treatments for CVD is the Percutaneous Coronary Intervention (PCI) [2]. This non-surgical procedure involves insertion of a balloon with or without stent through catheter wires at the side of a narrowed blood vessel. Stent is a small metal mesh tube that acts as a scaffold inside narrow weak blood vessels [4]. There are three types of coronary stents: bare metal (BMS), drug eluting (DES), and bioresorbable scaffold or biodegradable stent [5]. All stents and their accessories are expensive as they are imported from United States of America, Europe, Japan and China due to no local manufacturer. According to the chief executive officer of National Heart Institute, Tan Sri Dr. Robaayah Zambahari, the cost for BMS in Malaysia is RM 2000 whereas the DES cost is between RM 6000 and RM 8000 [6].

High-pressure balloon inflation during PCI caused mechanical stress and denudation of the endothelium layer in the blood vessel wall [7]. An inflammatory response at the lesion wall started and progressed earlier at first six weeks than the recovery of the endothelial layer that took approximately three months to complete after stenting [7]. This inflammation will cause rapid proliferation of vascular smooth muscle cells (VSMC) and lead to in-stent restenosis [7]. Drug eluting stent is the second generation of stent, coated with drugs targeted to prevent restenosis by stopping the proliferation of VSMC [8]. However, it has several limitations such as delayed endotheliazation caused by rapid release of anti-proliferative drug and polymer hypersensitivity which leads to late thrombosis [8]. Because of these problems, there are tremendous works on how to develop a multifunctional drug and less sensitive stent design.

Most permanent implants are made of stainless steel 316L because of its biocompatibility, less toxicity and strong structure [9]. Besides stent, the stainless steel 316L is also used to make cranial plates, orthopedic fracture plates, dental implants, spinal cords, joint replacement prostheses catheters and other surgical tools [9]. This low carbon steel and cobalt chromium are traditional materials used to fabricate BMS, the first generation of invented stent [8]. In the human body, the corrosion of stainless steel 316L will release iron, chromium and nickel ions where

incontrollable amount of these ions can act as allergens and carcinogens [9]. The major problem of BMS is the inflammation reaction of human body to this foreign matter that cause rapid growth of VSMC [8]. Thus, the coating of permanent metal implants is necessary to lower the response and the effect of body inflammation at early stage of implantation until the metal is no longer presumed as a foreign body.

Metallic implants' drug coating used biodegradable polymers to control the drug release. Drugs were incorporated into polymer matrix by several techniques such as nano-precipitation, emulsion-diffusion, double emulsification, emulsion-coacervation, polymer-coating and layer-by-layer methods [10]. The polymers hydrolyzed to release drugs and later excreted from the body. Several polymers used in DES as a coating material are polylactic acid (PLA), polyglycolic acid (PGA), poly(D,L-lactide) acid (PDLLA), poly(lactic-*co*-glycolic) acid (PLGA) and polycaprolactone acid (PCL) [11]. Poly(lactic-*co*-glycolic) acid is a copolymer of lactic and glycolic acid that degrades by hydrolysis of its ester linkages in the presence of water and it will be removed by renal excretion. Since its application has been approved by the United States Food and Drug Administration (FDA), PLGA is used in many biomedical device applications such as graft, sutures, implants and nanoparticle [12]

Plants are utilized to alleviate disease since centuries ago. The WHO estimated that 80% of people worldwide rely on herbal medicine for some parts of their primary health care [13]. Nowadays, researchers are emphasizing on evaluating the effects of phytochemicals from the active ingredients of herbs that play major roles in their therapeutic effects on various diseases including CVD. Ginseng root has been used for over 5000 year ago as a medicinal herb in traditional Chinese medicine and still being used as a health supplement worldwide [14].

Ginsenoside is the active ingredient of Asian ginseng, scientifically known as *Panax Ginseng*, and has been studied intensively for its effect on cardiovascular system such as improving circulation and antioxidant activity, modulating vascular

function, improving cardiac function, inhibiting platelet aggregation, and adjusting lipid profile [14]. Certain types of ginsenosides such as Rb1, Rg1 and Rg3 are able to overcome the problem of DES by inhibiting the proliferation of VSMC and promoting the growth of endothelial cells (EC) *in vitro* and *in vivo* [14,18], making them suitable to be used as a herbal drug to treat post-stenting problem. Despite some significant scientific findings of ginsenosides and their effects on CVD, ginseng coating for cardiovascular implant, specifically on stent, has not yet been reported. Therefore, this project was aimed to develop a drug eluting coating on stainless steel 316L by incorporating ginseng extract, a herbal medication that can addresses the drawbacks of current DES. Poly(lactic-*co*-glycolic) acid was utilized as the main matrix to optimize and control the coating formation and ginseng release.

1.2 Problem statement

Heart disease is still the main cause of death in Malaysia. Drug eluting stent is currently a preferred stent for PCI because it reduced the proliferation of VSMC [6]. However, the technology has not been developed, locally, and the price of DES is three times higher than the BMS [6]. Therefore, there is a need to develop our own drug eluting coating as an alternative to the current over-price DES.

The current DES undertakes the BMS problem but it also has several drawbacks such as late endotheliazation and polymer hypersensitivity that later cause abnormal endothelial cells, local hypersensitivity and late thrombosis [8]. Several factors that caused late endotheliazation is the rapid release anti-proliferative drug that can arrests endothelial cells growth as shown in several clinical trials [8] and the usage of permanent, hydrophobic and biostable polymer. New DES design together with several improvements on metal design and drug function that specific for VSMC and EC were developed but no multifunctional herbal-based drug was used as all drugs were derived synthetically.

It can be concluded that, to develop our own drug eluting coating for stent, the coating must be able to release multifunctional drug, proven by many literatures to be anti-proliferative to VSMC yet harmless to EC, in a controlled manner by using a biocompatible polymer.

1.3 Objectives of the study

This study aimed to develop a controlled release ginseng-PLGA drug eluting coating by a dip coating technique for stent application. Fundamental experiments listed below were carried out to develop the coating:

1. To determine optimize drug to polymer ratio that can produces adequate PLGA-ginseng coating on stainless steel 316L.

2. To identify the physico-chemical properties of the PLGA-ginseng coating by identifying the coating composition using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), coating morphology using scanning electron microscopy-energy dispersive X-ray (SEM-EDX) and wettability property using contact angle analysis

3. To investigate the drug release mechanism of the PLGA-ginseng coating using mass spectrometry instrument (Q-TOF LC-MS) and five mathematical models.

1.4 Scopes of the research

The scope of this study was divided into three parts: sample preparation, coating characterization and coating drug release profile. The sample preparation involved the pre-treatment of stainless steel 316L metal started with a cleaning and ended with an electropolishing process. A coating solution with standard total concentration of PLGA and ginseng but with different polymer to drug ratio were made by dissolving them in dimethyl sulfoxide (DMSO), a solvent that can dissolve both, ginseng and PLGA. The metals were coated with different ginseng to PLGA ratio by using a desktop dip coater. The coatings were characterized to visualize the coating surface, coating coverage and coating thickness by SEM. The coatings compositions were then characterized by ATR-FTIR and the wettability of the coatings was measured via contact angle analysis. The coating were immersed in a complete cell medium Dulbecco's Modified Eagle's Medium (DMEM), mimicking the *in vitro* environment to observe the sustainability of the coating to release drug in a month. The liquid chromatography – mass spectroscopy (Q-TOF LC-MS) was used to quantify the drug released. Finally, the quantitative data were analyzed to best fit five mathematical models in determining the drugs release mechanisms..

1.5 Significance of the study

This study utilized the knowledge of ginsenosides' properties that is anti-proliferative to the smooth muscle cells and harmless to the endothelial cells based on reports in many literatures. The novelty of this study is that, despite these known properties of ginsenosides, there was no attempt to coat ginseng on implant to use it for local delivery. Acknowledging the healing properties of ginseng in preventing restenosis and late thrombosis, the study of ginseng coating on implant, generally, and in stent, especially, will provide an alternative to the current drug eluting coating.

This fundamental research will show the capability to coat a herbal-based drug by using an easy and cost-effective coating technique called dip coating. The characterization of this coating can be used to further optimize the coating quality and supports the idea of the possibility to coat herbal-based drug on a metal. The drug release study of this coating will also help to illustrate the coating condition and its quality when immersed in a cell medium.

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