

Covalent immobilisation of proteins for biomaterial and biosensing applications

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Supervisors:

Associate Professor Nicolas H. Voelcker (Flinders University)

Associate Professor Sunil Kumar (University of South Australia)

Professor Roger St. C. Smart (University of South Australia)

Doctor Mark DeNichilo (TGR Biosciences)

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Declarations

'I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.'

Endre J. Szili

Date

'I believe that this thesis is properly presented, conforms to the specifications for the thesis and is of sufficient standard to be, *prima facie*, worthy of examination.'

Associate Professor Nicolas H. Voelcker

Date

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Abbreviations List

A	ampere
ABTES	triethoxysilyl butyraldehyde
APTES	3-aminopropyl triethoxysilane
AFM	atomic force microscopy
anti-IGF-1	biotinylated anti-human IGF-1 antibody
ASD	anodic spark deposition
ASTM	American Society for Testing and Materials
at.%	atomic percentage
Avg	average
$A\lambda$	absorbance at a given wavelength
Bal	balance of the elemental composition (wt.%) of the metal alloy
BSA	bovine serum albumin
BSP	bone sialoprotein or BioSpark™
C	coulomb
CCD	charge-coupled device
CDS	cell dissociation solution
cm	centimetre
CPS	counts per second
C3b	complement-activated fragment
DCM	dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dr	Doctor
ECM	extracellular matrix
EGFs	epidermal growth factors
ELISA	enzyme-linked immunosorbent assay
EOT	effective optical thickness

ERK	extracellular-signal-regulated kinase
etc	et cetera
EtOH	ethanol
eV	electronvolt
F-actin	filamentous actin
FBS	foetal bovine serum
FEAM	2,2,2-trifluoroethylamine
FFT	Fast Fourier Transform
FGFs	fibroblast growth factors
FPTMS	3,3,3-trifluoropropyl trimethoxysilane
FT-IR	Fourier transform-IR
FWHM	full-width-half-maximum
GDP	guanosine diphosphate
GPTMS	3-glycidoxypropyl trimethoxysilane
GTP	guanosine triphosphate
h	hour
HA	hydroxyapatite
HBSS	Hank's balanced salt solution
HF	hydrofluoric acid
HOB	human osteoblast-like
HQ	hydroquinone
HRP	horseradish peroxidase
HRP-Strep	peroxidase conjugated streptavidin
h ν	Planck's constant (h) times by the frequency of the exciting radiation (ν)
ICP-AES	inductively coupled plasma atomic emission spectrometry
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IGF-1	insulin-like growth factor-1
IGFs	insulin-like growth factors

IR	infrared
IPTES	3-isocyanatopropyl triethoxysilane
kV	kilovolt
LBNL	Lawrence Berkeley National Laboratory
M	molar
mA	milliamps
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MEM	minimal essential medium
mer	nucleotide
MFC	Mass flow controller
MG63	immortalised cell line of fibroblast morphology with adherent growth properties derived from an osteosarcoma of human bone
MHz	megahertz
min	minute
ml	millilitre
mM	millimolar
MPa	megapascal
$m\lambda$	spectral order of the Fabry-Pérot fringe (m) times wavelength of the incident light striking the surface at an incident angle of 0° (λ)
ng	nanogram
NHS	<i>N</i> -hydroxysuccinimide
nL	average refractive index of a porous silicon layer (n) times porous silicon layer thickness (L)
nm	nanometre
n-type	phosphorous or arsenic doped
OIRS	optical interferometric reflectance spectroscopy
OPD	<i>o</i> -phenylenediamine dihydrochloride
OPG	osteoprotegerin

OX	oxidised
PBS	phosphate buffered saline
PBS-T	PBS-Tween [®] 20
PDEPMA	poly(3,3'-diethoxypropyl methacrylate)
PDGFs	platelet-derived growth factors
PECVD	plasma enhanced chemical vapour deposition
PECVD-Si	silica film deposited on a material by the technique of PECVD
PECVD-Si-Ti	titanium coated with a film of PECVD-Si
p-ERK	phosphorylated ERK
PGE ₂	prostaglandin E ₂
pH	potential of hydrogen
Pty Ltd	proprietary limited
p-type	boron doped
RANK	nuclear factor κ -B
RCF	relative centrifugal force
Red	reduced
RGD	Arginine-Glycine-Aspartic acid
RMS	root mean square
SAGA	smart apertured grazing angle
sccm	standard cubic cm per min
scfh	standard cubic feet per h
SEM	scanning electron microscopy
SMP	<i>N</i> -succinimidyl-3-maleimidopropionate
Strep.-HRP	streptavidin conjugated HRP
TCPS	tissue culture polystyrene
TEOS	tetraethoxysilane
TFAA	trifluoroacetic anhydride
TGF- β_1	transforming growth factor β_1
TGFs	transforming growth factors
TM	trademark
TMB	3,3',5,5'-tetramethylbenzidine

TNBS	2,4,6-trinitrobenzenesulfonic acid
TNP	trinitrophenol
μg	microgram
μl	microlitre
μm	micrometre
μM	micromolar
UTS	ultimate tensile strength
UV-Vis	ultra-visible
v/v	volume per volume
WGFE	whey growth factor extract
wt. %	weight percentage
w/v	weight per volume
XPS	X-ray photoelectron spectroscopy
~	approximately
°	degrees
°C	degrees celsius
\emptyset	diameter
>	greater than
\geq	greater than or equal to
3T3 balb/c	immortalised cell line of fibroblast morphology with adherent growth properties derived from mouse embryo
<	less than
\leq	less than or equal to
3-MPTS	3-mercaptopropyl trimethoxysilane
ε	molar extinction coefficient
Ω	ohms
%	percentage
®	registered
λ	wavelength

Abstract

This thesis focuses on surface science and bioengineering investigations, first for the development of an improved biomaterial for orthopaedic implant applications, and second, for the development of a biosensor device for biomedical diagnostics. A key component considered in this thesis was the covalent linkage of proteins to the material's surface for retaining the protein's immunological and biological activities and for generating a functional interface.

Part 1 of this thesis investigated surface modification procedures for improving the bioactivity of titanium substrates. Titanium is first coated with a bioactive silica film grown by plasma enhanced chemical vapour deposition (PECVD), referred to as PECVD-Si-Ti. In previous studies, the bone-implant integration process was enhanced 1.6-fold for titanium implants coated with PECVD-Si films compared to uncoated titanium implants *in vivo*. However, *in vitro* studies carried out in this thesis showed that the growth of MG63 osteoblast-like cells was 7-fold higher on uncoated titanium compared to PECVD-Si coated titanium. Therefore, to improve cell growth on the surface and, by inference, the integration of PECVD-Si-Ti implants into bone tissue, the implant's surface was functionalised with a mitogenic factor, insulin-like growth factor-1 (IGF-1). This was accomplished by modifying the PECVD-Si-Ti surface with an alkoxysilane, 3-isocyanatopropyl triethoxysilane (IPTES), and then by covalent bioconjugation of IGF-1 through isocyanate-amino chemistry. After 72 h of *in vitro* cell culture in serum-free medium, the growth of MG63 cells was enhanced 1.9-fold on IPTES functionalised PECVD-Si-Ti, which was loaded with covalently immobilised IGF-1 compared to IPTES functionalised PECVD-Si-Ti without IGF-1 (isocyanate reactive groups were quenched with ethanolamine hydrochloride). The attachment and adhesion of MG63 cells were also enhanced on PECVD-Si-Ti by the covalently immobilised IGF-1 in serum-free cell culture conditions. Therefore, the bioactivity of PECVD-Si-Ti was improved by covalently linking IGF-1 to the substrate surface through isocyanate-amino chemistry.

Part 2 of this thesis involved the development of a new optical interferometric biosensor. The biosensor platform was constructed from electrochemically-prepared thin films of porous silicon that acted as a sensing matrix and transducer element. By reflective interferometry using white light, an enzyme-catalysed reaction was discovered (horseradish peroxidase (HRP) mediated oxidation of 3,3',5,5'-tetramethylbenzidine (TMB)), which led to an acceleration in the rate of porous silicon corrosion and represented the biosensor's readout signal. We discovered that another substrate, which is also oxidised by HRP, OPD, produces an even more pronounced readout signal. The HRP-OPD system was used in an immunoassay for detecting human IgG from an Intragam solution. An important part in the design of the biosensor was the surface functionalisation approach where anti-human IgG, referred to as the capture antibody, is immobilised on the porous silicon surface. The readout signal (produced from the capture of human IgG) was enhanced 4-fold on the porous silicon biosensing platform functionalised with covalently linked anti-human IgG through isocyanate-amino chemistry compared to the porous silicon biosensing platform functionalised with adsorbed anti-human IgG. The optimised biosensor was used to detect IgG from a total human protein concentration of Intragam to a sensitivity of 100 ng/ml.

In summary, isocyanate-amino bioconjugate chemistry was used to covalently link either IGF-1 to PECVD-Si-Ti for improving the biological activity of the orthopaedic implant and to covalently link IgG to porous silicon for developing a sensitive biosensor for the detection of proteins. This surface chemistry approach is very useful for biomaterial and biosensing applications.