# Covalent immobilisation of proteins for biomaterial and biosensing applications

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## Part 2: Covalent immobilisation of anti-human IgG on porous silicon for the detection of human IgG by reflective interferometry

# 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 |
| hv | Planck's constant (h) times by the frequency of the exciting radiation (v) |
| 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 |
| mλ | 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 | *N*-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 | *o*-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 | *N*-succinimidyl-3-maleimidopropionate |
| Strep.-HRP | streptavidin conjugated HRP |
| TCPS | tissue culture polystyrene |
| TEOS | tetraethoxysilane |
| TFAA | trifluoroacetic anhydride |
| TGF-$\beta_1$ | transforming growth factor $\beta_1$ |
| 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 |
| ø | diameter |
| > | greater than |
| ≥ | greater than or equal to |
| 3T3 balb/c | immortalised cell line of fibroblast morphology with adherent growth properties derived from mouse embryo |
| < | less than |
| ≤ | 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.