A Nanometre Scale Surface Coating to Promote Endothelization of Intracranial Stents

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Declaration

I, Joseph Junjie Zhao, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, they have been properly indicated and referred to in the thesis.

.............................................
Abstract

Introduction: Stroke is the leading cause of disability worldwide and a major cause of death, its mortality is second only to cardiovascular disease. The role of intracranial stents is essential in the management of stroke, but current intracranial stents display unwanted side effects resulting in allergic reactions, chronic inflammation and thrombosis followed by re-narrowing of the blood vessels. Surface modification strategies are now in demand to improve tissue interactions at the neurovascular interface. This study aims to develop a novel nano-surface coating technique to create reproducible nano-metric scale surface topographies and to apply it as a post-modification of intracranial stents, to promote in-situ endothelization.

Methodology: We develop a low-cost procedure to create polymeric nano-island coating by enduring phase separation. SEM and AFM were applied to study the topography profile of the coating. Hemocompatibility tests were performed to assess the thrombogenicity of the coating. HUVECs were also cultured on the coating to assess the toxicity and biocompatibility of the coating. SEM was carried out on the cells after culturing on the coatings to reveal the material-cellular interaction on the coating surface. Coating procedure was then optimised to be applied onto bare metal stents (BMSs). The coated BMSs were mounted onto balloon catheters, sterilised with Ethylene Oxide and implanted into a pilot rabbit model for 28-days. Patency data was collected, histological analyses were then performed following termination.

Results: SEM and AFM confirmed the presence of a uniform coating layer with nano-islands of 20 nm in height and 100 nm in radius. Hemocompatibility tests revealed the coating was inert towards human blood component and showed lower thrombogenicity than control. HUVECs also demonstrated enhanced growth and proliferation on the coating. SEM morphology study hinted that the nano-islands on the coating could be promoting the HUVECs migration and growth by providing contact guidance to the cells. We successfully apply the coating onto commercially available BMSs. Delamination study revealed only minimal surface cracks on the optimised coated BMSs, suggesting the coating remained stable. Finally, two coated BMSs were implanted into two healthy New Zealand White rabbits, and their patency was maintained over 28 days.

Conclusion: We have developed a simple and reproducible polymeric nano-island coating that showed low thrombogenicity and promote in-situ endothelization for vascular stents. Preliminary in vivo data suggest non-inferior early patency compared to uncoated BMSs with no apparent adverse effect.
Impact Statement

Thromboemboli and in-stent stenosis have long been the major problems for intracranial stents and have resulted in the high post-procedural complication rate, morbidity and mortality. Such problems are also commonly presented in coronary artery stents. Currently, all patients who go through intracranial stenting procedures are required to undergo a therapeutic regime of dual antiplatelet therapy for a minimum period of 6 months, and some for the remainder of their life accompanied by side effects and adverse reactions. We have developed a nano-surface coating for intracranial stents that promote in-situ endothelialisation at the tissue-implant or neurovascular interface as a strategy to prevent thromboembolic and in-stent stenosis. This technology can be easily applied to intracranial stents as a nanometre scale coating. Following testing to international guidelines and approval by regulatory bodies, it can be translated into the clinic and be released into the market. We anticipate that such new technologies can result in a much lower complication rate and long-term in-stent stenosis rate, thereby decreasing morbidity and mortality in patients in need of intracranial stenting.

The impact on the general public and healthcare sector will include benefiting healthcare professionals, who will be able to implement new treatments and therapies in patients with a neuro-vascular disease and also benefit patients in general, improving clinical outcomes. NHS systems will also benefit (through cost and efficiency) from simpler/shorter surgery, recovery and rehabilitation times and will ease the strain on vital healthcare resources. In addition to its clinical influence, this technology would have a significant impact on the global economy, as individuals affected will be active in the workforce for longer, enjoy a greater quality of life and reduce the strain on vital healthcare
resources. We have already achieved some tangible impact on public awareness as we have already disseminated our research at conferences (and related published articles) to scientists, clinicians and industrial partners.

The impact in the commercial sector will be extended to fields beyond cardiovascular disease and stroke management as our research had demonstrated the potential of the nanometer scale or nanosurface coating platform to be applied on various metallic, as well as, polymeric biomaterials. The fact that these coating platforms can be applied onto various biomaterials suggests that the technology can be applied to other applications in the human body, focusing on two main categories: 1) other neurosurgical implantable devices, such as electrical stimulatory devices such as deep brain stimulation, cerebral cortex electrodes and ventriculoperitoneal shunts, and 2) — other cardiovascular implantable devices, e.g. transcatheter heart valves, stent grafts, haemodialysis equipment and cardiac patches. Currently, we are collaborating with scientific, industrial and clinical project partners who have a keen interest in developing this technology further to commercialise coatings for stent devices. We have approached UCL Business to explore any potential intellectual property and establish contacts within the specialist healthcare industry, and we have also approached UCLB for potential investment in any new knowledge that is generated in this thesis.
# Table of Contents

Acknowledgements ............................................................................................................. 3
Declaration ............................................................................................................................ 5
Abstract ............................................................................................................................... 6
Impact Statement .................................................................................................................. 7
Table of Contents .................................................................................................................. 9
List of Figures ....................................................................................................................... 14
List of Tables ......................................................................................................................... 22
Abbreviations ....................................................................................................................... 23

Chapter 1  Introduction ........................................................................................................ 26
  1.1  Background – Stroke statistics ................................................................................. 26
  1.2  Stroke management ................................................................................................. 28
      1.2.1  Ischemic stroke ............................................................................................... 29
      1.2.2  Haemorrhage stroke ......................................................................................... 32
      1.2.3  Current clinical guidelines ............................................................................... 34
  1.3  Neurovascular devices and ICS .............................................................................. 34
      1.3.1  Current commercially available ICS .............................................................. 35
      1.3.2  Biomaterials for current ICS ........................................................................... 39
      1.3.3  Problems with current ICS .............................................................................. 39
  1.4  In search of a solution ............................................................................................. 42
      1.4.1  Neurovascular biomaterials for ICS ............................................................... 42
      1.4.2  The neurovascular interface ........................................................................... 44
  1.5  Surface modification strategies ................................................................................. 45
      1.5.1  Physical and mechanical approaches ............................................................. 46
      1.5.2  Electrical approaches ...................................................................................... 48
      1.5.3  Surface chemical approaches .......................................................................... 50
      1.5.4  Surface coating strategies for ICS ................................................................. 51
      1.5.5  Bio-active coatings for stents .......................................................................... 54
5.2.3 Immunofluorescent staining.................................................................158
5.3 Results .................................................................................................159
  5.3.1 Cell migration studies .................................................................159
  5.3.2 Gross morphology of HUVECs on different substrates.............160
  5.3.3 Immunofluorescent staining.........................................................163
5.4 Discussion .........................................................................................171

Chapter 6 Translation of the nano-surface coating platform onto other biomaterials 175

6.1 Introduction ..........................................................................................175
  6.1.1 What are SMA and Nitinol®?.......................................................175
  6.1.2 PtCr BMS .....................................................................................178
  6.1.3 ePTFE for small diameter vascular bypassing grafts ..................179
6.2 Material and methods .........................................................................180
  6.2.1 Surface characterisation of coated samples .............................181
  6.2.2 Haemocompatibility tests ..............................................................181
  6.2.3 HUVECs culture, maintenance and cell viability tests .............182
  6.2.4 Statistical analysis .........................................................................182
6.3 Results ..................................................................................................182
  6.3.1 Contact Angle (θ°) and Surface Energy (γ) Measurements ........182
  6.3.2 AFM .............................................................................................185
  6.3.3 Hemocompatibility tests .................................................................191
  6.3.4 HUVECs viability tests .................................................................198
6.4 Discussion .............................................................................................200

Chapter 7 Optimisation of poly-SIBS nano-island coatings using ultrasonic spraying techniques on BMS: Steps towards Translation .................................................202

7.1 Introduction ..........................................................................................202
  7.1.1 Ultrasonic atomization .................................................................202
  7.1.2 Ultrasonic atomisation spray coating techniques (UASC) .........205
  7.1.3 Animal models for testing endovascular stents .........................206
7.1.4 Small animal models for testing the efficiency of small diameter stents 208

7.2 Material and methods........................................................................................................210

7.2.1 Manufacturing of poly-SIBS nano-surface coatings.................................210
7.2.2 Characterisation of the coated stents .................................................................212
7.2.3 Assembly of the coated BMS system and dry run .................................212
7.2.4 Poly-SIBS coating delamination study .................................................................215
7.2.5 Sterilisation of poly-SIBS nano-island coatings on BMS ......................215
7.2.6 Development of rabbit model for testing small diameter stents..............215
7.2.7 Evaluation of the nano-island coating ICS in the pilot rabbit model...226

7.3 Results ..........................................................................................................................226

7.3.1 Manufacturing of poly-SIBS nano-surface coated stents ......................226
7.3.2 FE-SEM .................................................................................................................226
7.3.3 AFM .........................................................................................................................228
7.3.4 Delamination study.................................................................................................234
7.3.5 In vivo NZW rabbit model for testing small diameter stents.................236
7.3.6 In vivo testing of poly-SIBS nano-island surface coatings .................238

Chapter 8 Conclusion & Future Work................................................................................240

Future work and limitations ...............................................................................................244

Chapter 9 Reference............................................................................................................249

Appendix ..............................................................................................................................264

Studies arise from the result this research - 1.................................................................265

Studies arising from the result this research - 2...............................................................267

Awards, qualifications, conference presentation and publications arising from this research .................................................................270
List of Figures

Figure 1-1. Schematic drawings of two subtypes of stoke. A. Haemorrhage stroke; B. Ischemic stroke. ................................................................. 29
Figure 1-2. Schematic diagram of endovascular stenting angioplasty. .................. 31
Figure 1-3. Schematic drawings of endovascular thrombectomy......................... 32
Figure 1-4. Schematic drawings of stent assisting coil embolisation for ICA........... 33
Figure 1-5. Nitinol® surfaces after 400 and 600 °C heat treatment. A) Atomic force microscope (AFM) images and line profiles; B) HOS cell viability results Confocal microscopy fluorescence images of the test samples after 1 and 7 d in culture; C) and D) Cytoskeleton and focal adhesion staining of HOS cells after cultured on samples for 3 d (actin and tubulin). ......................................................................................................................... 47
Figure 1-6. HUVECs cultured on MP600 and EP Nitinol® samples A. B. Immunofluorescent staining of 4’,6-diamidino-2-phenylindole (DAPI, nuclei stain, blue), connexin 43 (red) and von Willebrand factor (vWF, green) in HUVECs cultured on different samples; C, D. DAPI (blue) and endothelial nitric oxide synthase (eNOS, red). Bar: 40μm. A, C. HUVECs cultured on MP600 Nitinol® samples; B, D. HUVECs cultured on EP Nitinol® samples................................................................................................................... 49
Figure 1-7. PLGA/PVA coated drug-eluting Nitinol® Stent. .............................. 54

Figure 1-8. Images at 4 and 26 weeks after the placement of different stents. Neointimal thickness at 4 weeks was similar among BMS, BMS Plus S-SIBS, DES, and DES Plus S-SIBS groups. By 26 weeks, DES plus S-SIBS showed similar ability to DES to inhibit neointimal hyperplasia in contrast with BMS (P < 0.05), which showed a steady increase in neointimal thickness. Sections shown were stained with hematoxylin and eosin (H&E). ................................................................................................................................. 57

Figure 1-9. Scanning electron microscopy (SEM) images of fibroblasts cultured on different size nano-islands. A) On the 13nm islands, many cells were seen to be in various stages of division; this image is of a cell rounding up to divide (R) among cells
that are generally spread flat (F); B) On the 35nm islands, cells were clearly starting to interact with the islands (arrowheads show filopodia–island interactions); C) On the 95nm islands, the cells appear to be moving along the tops of the islands, using “pseudopodial-like” processes (arrows show lamellipodia –island interactions).

Figure 2-1. The reaction of organosilanes with hydroxylated surfaces with a variety of mineral, inorganic and metallic substrates.

Figure 2-2. Chemical structure of poly-SIBS containing tri-block copolymer structure composed of polystyrene (hard segments) and polyisobutylene (soft segment) chemistry.

Figure 2-3. Selected organosilane precursors common in biological systems to yield 1) \(-\text{CH}_3\); 2) \(-\text{NH}_2\); 3) \(-\text{SH}\); 4) \(-\text{COOH}\); 5) \(-\text{F}\) terminal functional groups.

Figure 2-4. Electrostatic coatings are generated by fluorinated groups present in CFMTS, which have been net negatively (-) charged, which are attracted to positively (+) charged phenyl rings (arrows) in the poly-SIBS backbone to yield covalently bound thin films.

Figure 2-5. Schematic representation of passive and phase separated coatings on negatively charged substrates with cationic poly-SIBS coatings.

Figure 2-6. The SCK-200 spin coater from Instras Scientific Inc, USA. Highlighted boxes showed spin speed controller, spin coat chamber and sample loading platform.

Figure 2-7. SIGMA FE-SEM and schematic image of the electron gun and beam pattern (Carl Zeiss UK, Ltd). The highlighted box showed the schematic structure of electron gun inside the SEM sample loading chamber.

Figure 2-8. Schematic image of the operating modes of the AFM as shown in A) contact mode – tip making continuous contact with the sample surface, and B) tapping mode – tip making intermittent contact with the sample surface.

Figure 2-9. Contact angle (\(\theta^\circ\)) and surface energy (\(\gamma\)) results on silanized samples and selected droplet images. Upper left: \(\theta\) results (n=9 per condition in 3 repeats); Upper
right: $\gamma$ results (n=9 per condition in 3 repeats); Lower: A. KRÜSS DSA 100 machine and EasyDrop DSA20E Drop Shape Analysis software; B ~ D. Selected droplet images showed the different modified surface varying from hydrophilic to hydrophobic. .....82

Figure 2-10. Contact angle ($\theta^\circ$) and surface energy ($\gamma$) results on poly-SIBS coated samples. Left: $\theta^\circ$ (n=9 per condition in 3 repeats); Right: $\gamma_s$ results (n=9 per condition in 3 repeats)..................................................................................................................................................................................................................................................................................................................84

Figure 2-11. FE-SEM images of organosilanized glass substrates. Images A-E were captured at random locations of samples at x10,000 magnification; while the highlighted image was captured at x35,000 magnification. ..................................................85

Figure 2-12. Representative FE-SEM images of poly-SIBS coated glass samples. Images A-D were captured at random locations of samples at x10,000 magnification; while the images underneath were matching images captured at x35,000 magnification. One of the nano-islands were highlighted in the added red circle in D. .................87

Figure 2-13. AFM images of representative poly-SIBS coated glass samples. A. UC; B. Dip coated; C~E. Spin coating at 1000, 3000, and 6000 rpm. All scanning sizes were set at 25$\mu$m X 25$\mu$m, 3D images underneath matching 2D ones. The bright areas represented elevated features and protrusions. ..................................................................................................................................................................................................................................................................................................................89

Figure 2-14. AFM surface roughness results of all samples (n=9 per condition in 3 repeats). Left: Organosilanised glass samples; Right: Poly-SIBS coated samples. .....90

Figure 3-1. The intrinsic and extrinsic blood coagulation cascade: Left – the intrinsic pathway; Right – the extrinsic pathway..................................................................................................................................................................................................................................................................................................................................................................................97

Figure 3-2. The effects of the intrinsic pathway platelet adhesion, activation and aggregation..................................................................................................................................................................................................................................................................................................................................................................................98

Figure 3-3. Structure of a platelet. ..................................................................................................................................................................................................................................................................................................................................................................................99

Figure 3-4. Before and after centrifugation of citrated whole blood to obtain platelet-rich plasma (PRP). A. Donated citrated whole blood layered on top of Histopaque™ before centrifugation; B. plasma components after centrifugation; and C. digital image
showing the isolation of PRP and platelet poor plasma (PPP) with Lymphoprep™ to remove residual WBCs ................................................................. 103
Figure 3-5. The Speedy 100R laser cutter, A and B the platform in which the samples are manufactured................................................................. 104
Figure 3-6. Standardised TEG® trace displaying the key parameters used to measure haemostasis such as R time, K time, α-angle, and MA leading to clot lysis. ............... 107
Figure 3-7. BCI of all organosilaned and poly-SIBS nano-coated substrates (n=12 per condition in 4 repeats)......................................................................................... 110
Figure 3-8. PAI of all organosilanised and poly-SIBS nano-coated substrates (n=9 per condition in 3 repeats)......................................................................................... 111
Figure 3-9. FE-SEM images of organosilanised samples and on untreated glass control after platelet adhesion tests. ................................................................. 112
Figure 3-10. FE-SEM images of plates on poly-SIBS nanosurface coated samples and using collagen type 1 and ePTFE as positive controls............................................... 113
Figure 3-11. TEG results of all samples (n=9 per condition in 3 repeats)...................... 116
Figure 3-12. Cooper’s platelet index highlighting five key stages of platelet morphology upon contact with biomaterials. ................................................................. 123
Figure 4-1. Schematic diagram of cell viability tests on to different modified substrates and on poly-SIBS nanosurface coatings. ............................................................. 129
Figure 4-2. The total DNA standard curve generated using calf thymus. ................. 134
Figure 4-3. MTT and total DNA assays with HUVECs culturing on different modified and nano-surface coating glass samples on day 1, day 7 and day 10 (n=12 per condition in 4 repeats)......................................................................................... 135
Figure 4-4. HUVECs on organosilanised glass substrates after ten days of culture and stained with methylene blue................................................................. 137
Figure 4-5. HUVECs on poly-SIBS nanosurface coatings after ten days of culture stained with methylene blue................................................................. 138
Figure 4-6. hASMCs on organosilanised glass samples after ten days of culture and stained with methylene blue.................................141

Figure 4-7. hASMCs on nano-surface coating samples after ten days of culturing and stained with methylene blue.................................142

Figure 4-8. HUVECs on nano-surface coating samples for prolonged culture and stained with methylene blue.................................145

Figure 4-9. hASMCs on the poly-SIBS nanosurface coated samples after prolonged studies and stained with methylene blue.................................147

Figure 5-1. Migration studies of HUVECs: Left. Half coated CDTFS samples; Right. Half dip coated poly-SIBS samples.................................160

Figure 5-2. FE-SEM images of HUVECs on A. UC; B. CDTFS (-F) modified; C. poly-SIBS nanoislands; and D. dip coated nanopits and nanopores.................................161

Figure 5-3. HUVEC growth on model glass substrates: A. UC; B. (-F) organosilane; C. poly-SIBS nano-islands (6000 rpm); D. poly-SIBS dip coated sample. Color indicator: Blue: DAPI (DNA); Green: Phalloidin (F-actin); Red: vWF.................................165

Figure 5-4. HUVEC growth on model glass substrates: A. UC; B. (-F) organosilane; C. poly-SIBS nano-islands (6000 rpm); D. poly-SIBS dip coated sample. Color indicator: Blue: DAPI (DNA); Green: Phalloidin (F-actin); Red: VEGFR-2.................................167

Figure 5-5. HUVEC growth on model glass substrates: A. UC; B. (-F) organosilane; C. poly-SIBS nano-islands (6000 rpm); D. poly-SIBS dip coated sample. Color indicator: Blue: DAPI (DNA); Green: Phalloidin (F-actin); Red: Vinculin.................................170

Figure 6-1. Contact angle (θ°) and surface energy (γ) results on to biomaterials before and after coating with Poly-SIBS nano-islands. Left: WCA (n=12 per condition in 4 repeats); right: Surface energy (n=12 per condition in 4 repeats)..................................184

Figure 6-2. AFM images of poly-SIBS nano-island coatings on different samples (Part one). A. Untreated glass; B. 6000 rpm spin coated glass; C. Nitinol®; D. 6000rpm spin coated Nitinol®.................................187
Figure 6-3. AFM images of poly-SIBS nano-island coatings on different samples (Part Two). E. Plain Pt-Cr; F. 6000 rpm spin coated Pt-Cr; G. ePTFE; H. 6000rpm spin coated ePTFE.

Figure 6-4. AFM surface roughness results of nano-island coatings on different samples (n=12 per condition in 4 repeats).

Figure 6-5. BCI of poly-SIBS nano-island coatings on different samples (n=12 per condition in 4 repeats).

Figure 6-6. PAI of poly-SIBS nano-island coatings on different samples (n=12 per condition in 4 repeats).

Figure 6-7. TEG results of poly-SIBS nano-island coatings on different samples (n=12 per condition in 4 repeats).

Figure 6-8. MTT and total DNA assays with HUVECs cultured on different poly-SIBS nano-island coatings on day 1, 7 and 10 (n=12 per condition in 4 repeats).

Figure 7-1. CFD stimuli induced ultrasonic atomization. A. Atomization phenomenon creates different surface tensions generated on the liquid surface resulting in capillary waves; B. The capillary waves cause changes in amplitude.

Figure 7-2. Ultrasonic atomization of liquids. A: Ultrasonic waves conduct energy onto the surface of the liquid droplets and generate different surface tensions resulting in the stretching of the droplets; B: by applying ultrasonic frequency and atomization power results in different surface tensions on the liquid droplet cause stretching and deformation.

Figure 7-3. Spray coating procedure of BMS. A: MediCoat DES 1000 ultrasonic atomising spray coating system; B. Stents being cleaned and mounted on to a mandrill and loaded into a programmed spray cycle; C. Stents were transferred to an oven after spray coating.

Figure 7-4. Digital images of the MSI SC. A. Front view, shows the crimping chamber on the left and push quill on the right; B. and C. Side views; and D. Shows the dial on
the pressure handle of the crimping chamber to adjust stent crimping to a designated size from 0-5 mm.................................................................213

Figure 7-5. NZW Rabbit model for testing small diameter stents (step one): skin preparation and position for general anaesthesia.................................................................219

Figure 7-6. NZW Rabbit model for testing small diameter stent (step two): incision made and exposing the femoral artery.................................................................220

Figure 7-7. NZW Rabbit model for testing small diameter stent (step three): arterial sheath insertion.................................................................221

Figure 7-8. Innova 3100 DSA (General Electric, USA): Left – rotatable radiation pole; Right – automatic bed and real-time image display system........................................223

Figure 7-9. Tissue harvesting after animal termination: A. Postmortem incision; B Locating the stent inside the abdominal aorta.................................................................224

Figure 7-10. Schematic drawing of the morphometric measurements of the vessel lumen......................................................................................................................225

Figure 7-11. Representative images of control and sprayed coated BMS. A. BMS (control); B. BMS coating after a single spray cycle; C. two spray cycles; and D. four spray cycles..........................................................................................................................227

Figure 7-12. Control and sprayed coated BMS. A. BMS (control); B. BMS after two cycles; C, and four cycles........................................................................................................229

Figure 7-13. Stent crimping procedures. A. Structure of the stent delivery catheter and positioning of the stent; B. Spray coated stent loaded on to a balloon catheter, before crimping; C. Stent crimped under maximum pressure; D. Spray coated BMS crimped down on to the balloon catheter; E. The spray coated stent delivery system installed in the protective sheath prior to sterilization with EtO.............................................................231

Figure 7-14. A diagram describing a dry run of stent deployment. Left: Schematic diagram of balloon expansion and deployment in vivo; Right. The ex vivo dry run of spray coated BMS matching the in vivo stenting protocol. A. The stent catheter is passed through a 5F guiding catheter; B. Balloon inflated to deploy the spray coated BMS; C.
Balloon deflation; D. Balloon catheter is withdrawn leaving behind the spray coated BMS.............................................................233

Figure 7-15. FE-SEM images of the spray coated stents after three cycles of crimping and expansion. A. Stent 01; B. Stent 02; and C. Stent 03..........................................................235

Figure 7-16. Representative images of BMS histological samples. Upper: H&E staining of the stent deployed in the aorta forming an enveloped segment; Lower: MT staining. ........................................................................................................................................237

Figure 7-17. Representative images of DSA analysis of poly-SIBS nano-island coated BMS: Left – before stent deployment; Right – after stent deployment.................238

Figure 8-1. A flow chat of future work........................................................................................................................................248
List of Tables

Table 1-1. The summary of currently available ICSs .................................................. 38
Table 1-2. The summary of adverse effect rates for currently available ICS .......... 41
Table 2-1. AFM measurements of surface features obtained with dip and spin coating procedures (n=9 per condition in 3 repeats). ................................................................. 90
Table 3-1. TEG® parameters and definitions............................................................. 108
Table 6-1. AFM surface measurement of different spinning speed poly-SIBS coated substrates (n=12 per condition in 4 repeats) ................................................................. 189
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>Abdominal aorta aneurysm</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
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<tr>
<td>AMR</td>
<td>Action medical research</td>
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<tr>
<td>APRE-19</td>
<td>Human retinal pigment epithelial cells</td>
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<td>APTS</td>
<td>3-aminopropyltrimethoxysilane</td>
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<tr>
<td>ASA</td>
<td>American stroke association</td>
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<tr>
<td>ASC</td>
<td>Adipose stem cell</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<td>BCI</td>
<td>Blood compatibility index</td>
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<td>BMS</td>
<td>Bare metal stent</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CDF</td>
<td>Computational fluid dynamics</td>
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<tr>
<td>CDTFS</td>
<td>Chloro-dimethyl(3,3,3 trifluoropropyl)silane</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CSF</td>
<td>Cerebral spinal fluid</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DAPT</td>
<td>Dual anti-platelet therapy</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep brain stimulator</td>
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<td>DCDMS</td>
<td>Dichlorodimethylsilane</td>
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<td>DES</td>
<td>Drug eluting stent</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>EP</td>
<td>Electro-polishing</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>EPSRC</td>
<td>Engineering and physical sciences research council</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expandable polytetrafluoroethylene</td>
</tr>
<tr>
<td>ETO</td>
<td>Ethylene oxide</td>
</tr>
<tr>
<td>FE-SEM</td>
<td>Field emission scanning electron microscopy</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>FDS</td>
<td>Flow diverter stent</td>
</tr>
<tr>
<td>hASMC</td>
<td>Human aortic smooth muscle cell</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HOS</td>
<td>Human osteosarcoma</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SSA</td>
<td>Stent strut area</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture polystyrene plate</td>
</tr>
<tr>
<td>TEG</td>
<td>Thromboelastography</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLA</td>
<td>Theoretical lumen area</td>
</tr>
<tr>
<td>TMPM</td>
<td>Trimethoxysilylpropyl methacrylate</td>
</tr>
<tr>
<td>TPI</td>
<td>Thrombodynamic potential index</td>
</tr>
<tr>
<td>TRIAS</td>
<td>TRI-stent adjudication study – high risk of restenosis</td>
</tr>
<tr>
<td>UASC</td>
<td>Ultrasonic spray coating technique</td>
</tr>
<tr>
<td>UC</td>
<td>Untreated control</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVV</td>
<td>Ultraviolet vision spectrometry</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>V-P Shunt</td>
<td>Ventriculoperitoneal shunt</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Chapter 1   Introduction

In 1970, the World Health Organization defined a stroke as ‘rapidly developed clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin’.

Although the term ‘stroke’ has been widely used over the past few decades, the World Health Organization (WHO) definition relies heavily on clinical symptoms and had been considered outdated by the American Stroke Association (ASA) due to significant advances in the “nature, timing, and clinical recognition of stroke and its mimics, and anatomical imaging findings that require an updated definition.” Besides its definition, the treatment strategies, as well as, management guidelines are also evolving continuously. In this chapter, we explore up-to-date stroke statistics, currently guideline and management strategies, and current research and future trend.

1.1   Background – Stroke statistics

Cardiovascular disease (CVD) is the number one cause of death globally and it is estimated that by 2030, the annual incidence rate of cardiovascular-related diseases will increase to 23 million (WHO, 2017, Hansson and Libby,
Atherosclerosis is a subset of CVD and is characterised by a buildup of fatty deposits in the arteries. Left untreated, occluded and blocked arteries can lead to myocardial infarction (heart attack), stroke and even death. The current definition of a stroke from 2014 is “caused by the interruption of the blood supply to the brain due to a blocked blood vessel by a blood clot or blood vessel rupture.” This cuts off the supply of oxygen and nutrients, causing damage to the brain tissue.” Stroke is the leading cause of disability worldwide and a major cause of death. According to the WHO and stroke association UK joint report in 2018, there are more than 100,000 strokes in the UK each year, and around one stroke every five minutes. Around one in six men would have a stroke in their lifetime, while in women, this is approximately one in five. Age is a major risk factor for stroke patients, and reports claim that people are most likely to have a stroke after the aged of 55. Stroke rate in children are also higher than in normal adults; these are usually associated with various congenital defects and other diseases, e.g. moyamoya disease. In the UK, there are roughly 400 cases of childhood stroke occurring each year. The mortality of stroke in the UK alone is 38.22 in every 100,000 people, claiming almost 38,000 lives annually. On average, there is a life lost every 13 minutes. Stroke causes almost twice as many deaths in women as breast cancer; 5,000 more deaths a year than prostate cancer in men. One in eight strokes is fatal within the first 30 days. In the UK, approximately 10-15% of patients die before they reach a hospital, and
of those who survive 42% will be dependent, 46% will have some form of
disability, and 12% will be left severely impaired.

There are over 1.2 million stroke survivors in the UK. Most stroke survivors
have to endure neurological dysfunction and may be dependent on care to
various degrees. A survey in 2015 suggested that four in ten stroke survivors
consider the physical impact of stroke the hardest to deal with, and the mental
impact of stroke on its survivors is even higher. Moreover, approximately one
in four stroke survivors will experience another stroke within the next five years
following the first episode. It is clear that at current time, the life quality for stroke
patients are quite poor and it calls for improved management strategies
(StrokeAssociationUK, February 2018).

1.2 Stroke management

Stroke can be broadly classified into two major subtypes: 1) ischemic stroke
and 2) hemorrhagic stroke (figure 1-1). Ischemic strokes are caused by
blockages which cut off the blood supply to parts of the brain. These blockages
are usually caused by atherosclerosis and the formation of a blood clot due to
plaque rupture. They can occur in a major brain artery or a small blood vessel
deep within the brain. Without a fresh supply of blood, the neurons and glial
cells within the brain begin to die. This damage can have different effects,
depending on where it happens in the brain. Hemorrhagic strokes are caused
when a blood vessel bursts or ruptures within or on the surface of the brain. Hemorrhagic strokes are generally described as subarachnoid haemorrhage (SAH) - bleeding on the surface of the brain, and intracerebral haemorrhage (ICH) - bleeding within the brain. They are usually considered to be more severe, and are associated with a considerably higher risk of dying within the first three months and beyond when compared to ischemic strokes. In fact, one in ten patients with hemorrhagic stroke dies before reaching a hospital. About 85% of strokes are ischemic while the remaining are hemorrhagic.

![Figure 1-1. Schematic drawings of two subtypes of stroke. A. Hemorrhage stroke; B. Ischemic stroke.](image)

Due to their different pathological nature, the management of both forms of stroke is different, which is summarised in the following sections.

1.2.1 Ischemic stroke

The majority of ischemic stroke cases are caused by clot embolisation and
vascular stenosis (narrowing of the blood vessels) following plaque rupture and intimal hyperplasia (IH) caused by over-proliferation of smooth muscle cells (SMCs). Conventionally, there are three different management strategies for this type of stroke, they are: 1) conservative treatment; 2) endovascular stenting angioplasty; 3) endovascular thrombectomy. Conservative treatment refers to general medical treatment, including secondary prevention with antiplatelet therapy to stop in-situ blood clot formation, as well as aggressive medical treatment such as thrombolysis therapy to dissolve blood clots (Sugiyama et al., 2015, Li et al., 2012, Al Hasan and Murugan, 2012). For ischemic strokes caused by IH, endovascular stenting angioplasty is the most commonly used management strategy (Bang, 2014, Arkuszewski et al., 2012). This approach involves the use of an intracranial stent (ICS) positioned via digital subtraction angiography (DSA) and deployed at the narrowing site of the blood vessel, to reopen narrowed blood vessels and restore blood flow (figure 1-2).
Figure 1-2. Schematic diagram of endovascular stenting angioplasty.

Endovascular thrombectomy is another endovascular approach performed under DSA (Hopf-Jensen et al., 2013, Bae et al., 2012, Castro-Afonso et al., 2012). It is mostly for ischemic strokes caused by blood clot formation. It describes the procedure of using an ICS to catch and subsequently remove the blood clot formed inside the cerebral artery (figure 1-3).
1.2.2 Haemorrhage stroke

For ICH, the most common cause is hypertension, therefore the treatment strategy for this type of stroke is mostly controlling the risk factors, long-term monitoring and management of hypertension to control the blood pressure to a safe level (Araki et al., 2015, Lanzino et al., 2013, Rose et al., 2012). SAH strokes are caused by intracranial aneurysms (ICAs). Therefore the management strategies for SAH is mostly based on the management for ICAs (Zhao et al., 2017). There are two major approaches for managing ICAs, 1) surgical clipping and 2) endovascular embolisation. Surgical clipping was once the classical approach for treating ICAs. This approach requires a craniectomy to be performed to expose the entire ICA and its aneurysmal neck before an
aneurysmal clip was inserted to cut off the blood flow into the aneurysmal sack, isolating it from active circulation, to prevent the risk of bleeding. Nowadays, this approach is being used less and less due to its invasiveness, it is only recommended in certain cases of ICAs where the other approaches are difficult to manage (Germans and Macdonald, 2013, Lad et al., 2013, Li et al., 2013, Liao et al., 2013, Rahal and Malek, 2012).

Endovascular embolisation is an approach performed under DSA and requires the navigation of a micro-catheter to the ICA site and the embolisation of the aneurysmal sack using a special coil. This technique usually requires the assistance of the ICS (figure 1-4). Due to its minimally-invasive nature, it is currently being recommended as one of the first line treatment strategies for ICAs (Gupta et al., 2012, King et al., 2012).

Figure 1-4. Schematic drawings of stent assisting coil embolisation for ICA.
1.2.3 Current clinical guidelines

Although different countries have their own medical regulatory system and their own medical guidelines towards various diseases, for stroke, the most recognized guidelines in the international community are the ASA guidelines and the UK national institute of clinical excellence (NICE) guidelines (Zhao et al., 2017, Thompson et al., 2015, Connolly et al., 2012, Hussain et al., 2012, Macdonald, 2012). Both guidelines are constantly updated as clinical evidence emerges from major clinical studies and trials from across the globe.

Currently, for most ischemic stroke cases, both guidelines suggest a conservative medical intervention, and if the risk persists after medical intervention, endovascular stenting is recommended. While for most hemorrhagic strokes – SAH caused by ICAs, endovascular coiling with/without ICS is the first line treatment strategy (Excellence, July 2004, Excellence, May 2003).

1.3 Neurovascular devices and ICS

Neurovascular devices are implantable surgical devices targeting the vasculature of the central nervous system (CNS) that is both the brain and the spinal cord. ICS is one of the major types of neurovascular devices for managing stroke to correct the vascular abnormalities in the brain and to restore the disruptive blood flow. A marketing report by New Meditech described the
average growth rate in the global market for ICS as being 45% over the past five years. It is also forecasted that the global market of intracranial stents will be close to $1.5 billion by 2020 (New Meditech).

1.3.1 Current commercially available ICS

There are generally four major types of ICSs based on their different designs, 1) open-cell balloon-expandable ICS, 2) close-cell self-expandable ICS, 3) flow diverter stents (FDSs), and 4) covered stents (Zhao et al., 2014b). Open-cell balloon-expandable ICSs are ICSs with a continuous branch-like metallic configuration forming the main stent structure; they are compressible in their spring-loop arrangements. The Neuroform stents (Boston Scientific Neurovascular Fermont, California, USA), and the Wingspan stents (Striler/Boston Scientific SMART Fermont, California, USA.) are examples of this category. Their loose configuration allows them maximum flexibility, good vessel-wall positioning after deployment, which makes them extremely ideal for tortuous blood vessels. However, this design also comes with disadvantages like weak radial supporting force requiring the assistance of ballooning to expand it in the vessels of atherosclerotic plaques. Also, their branch-like metallic structures also contribute to free pointy terminals, which constitutes risks for vessel perforation during stent deployment. This type of ICSs is currently widely used both following angioplasty, as well as, assisting embolisation for ICAs (Gu et al., 2012, Samaniego et al., 2012, Shin et al., 2012,
Close-cell self-expandable ICS have a web-like metallic scaffold or tubular structure, and they are compressible via the folding of their cell windows. The Enterprise stent (Cordis, Florida, USA), the LEO stent (Balt, Montmorency, France) and Solitaire stents (Ev3, Irvine, California, USA) are ICS in this category. Their web-like structure greatly improves their stability, allowing maximum radial supporting forces. They can also be retrieved after semi-deployment or even after fully deployed, making them retrievable ICSs. This feature not only allows them to re-adjust their position after deployment, but also enables them to be used in many advance stenting techniques, such as the stent-jack technique for assisting ICA embolization (de Paula Lucas et al., 2008) and for thrombectomy by catching and retrieving the clot as shown in figure 1-3 (Hopf-Jensen et al., 2013).

On the contrary, this type of ICSs is not as flexible as the open-cell types and would face risks of structural compromises when facing tortuous vessels. Currently this type of ICSs is used in both angioplasty as well as assisting embolisation for ICAs, and it is the only type of ICSs that is recommended by the US Food and Drug Administration (FDA) to perform thrombectomies (Padalino et al., 2013, Huang et al., 2012, Lv et al., 2011). FDSs are ICS with a fine mesh metallic tube structure. The Silk flow diverter (Balt Extrusion, Montmorency, France) and the Pipeline Embolisation device (Ev3, Irvine, California, USA).
California, USA) are ICS in this category. Their fine mesh metallic wall structure has limited flow dynamics passing through the structure, which redirects the blood flow in one direction while limiting little flow to past the fine mesh wall. Their main purpose is to treat fusiform ICAs, however, there are also reports of FDSs being applied on general ICAs, as well as, for acute vessel perforations (Rouchaud et al., 2016, Szmuda et al., 2016, Aydin et al., 2015, Arrese et al., 2013).

Similar to the FDS, covered ICS are stents with a thin layer of polymer membrane covering itself entirely, and allowing no blood flow through its wall structures. The Willis covered stent (MicroPort Medical Company, Shanghai, China) is an example of this type. It is a bare metal stent (BMS) wrapped around the stent with expandable polytetrafluoroethylene (ePTFE) with an organic agglomerate as an adhesive layer. Currently, studies and trials are still underway to access the efficiency and safety of this type of ICSs (Fang et al., 2017, Liu et al., 2017b, Tang and Qi, 2017, Lai et al., 2013, Zhu et al., 2013). Details of current commercially available ICS are summarised in table 1-1.
<table>
<thead>
<tr>
<th>Neuroform</th>
<th>Wingspan</th>
<th>Enterprise</th>
<th>LEO</th>
<th>Solitaire</th>
<th>SILK</th>
<th>PED</th>
<th>Willis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composition</strong></td>
<td>Nitinol®</td>
<td>Nitinol®</td>
<td>Nitinol®</td>
<td>Nitinol®</td>
<td>Platinum</td>
<td>Cobalt platinum alloy (w/w: 3/1)</td>
<td>Cobalt chromium alloy, ePTFE</td>
</tr>
<tr>
<td><strong>Design</strong></td>
<td>6-8 linked radiolucent pointy branch</td>
<td>Linked radiolucent pointy branch</td>
<td>Parallelogram cells, flared ends</td>
<td>Braided wire formed cells</td>
<td>Honeycomb pattern cells</td>
<td>48 braided strands with 35μm microfilaments</td>
<td>Cylinder with 48 woven microfilaments</td>
</tr>
<tr>
<td><strong>Cell Design</strong></td>
<td>Open-Cell</td>
<td>Open-Cell</td>
<td>Close-Cell</td>
<td>Close-Cell</td>
<td>Close-Cell</td>
<td>Close-Cell</td>
<td>Open-Cell with covering membrane</td>
</tr>
<tr>
<td><strong>Surface coverage (%)</strong></td>
<td>6.5-9.5%</td>
<td>-</td>
<td>10%</td>
<td>-</td>
<td>5-7%</td>
<td>35%</td>
<td>30%</td>
</tr>
<tr>
<td><strong>Retrievability (%)</strong></td>
<td>0%</td>
<td>0%</td>
<td>70%</td>
<td>90%</td>
<td>100%</td>
<td>90%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Radial Force</strong></td>
<td>Median</td>
<td>Median</td>
<td>Median</td>
<td>Lowest</td>
<td>Highest</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Stiffness</strong></td>
<td>Lowest</td>
<td>Low</td>
<td>Highest</td>
<td>Median</td>
<td>Median</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cell Size</strong></td>
<td>Median</td>
<td>Median</td>
<td>Median</td>
<td>Smallest</td>
<td>Largest</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
1.3.2 Biomaterials for current ICS

Although ICS designs have been evolving over generations, the biomaterial for ICS has remained almost the same over the past few decades. Most of the existing ICS are of medical grade Nitinol® (Zhao et al., 2014b). Developed at the US naval ordnance laboratory (Silver Spring, Maryland, USA) in 1963 by Buehler and colleagues, Nitinol® was the legendary shape memory alloy (SMA) (BUEHLER et al., 1963). Nitinol® shows superiority as a biomaterial for ICS in many ways (BUEHLER et al., 1963), for example, its super-elasticity allows its use for making small stent structures of less than 1.5 mm and for use in tortuous intracranial vessels, and its shape memory effect (SME) enables the self-expandable or even semi-self-expandable feature which could reduce the need for a balloon expanding procedure, or even allow decreased balloon pressures during that procedure. This is generally considered helpful as it reduces the risks of in-situ endothelial cell (EC) damage that is induced by the effect of balloon expansion (Jani et al., 2014).

1.3.3 Problems with current ICS

Despite its desirable material and mechanical properties, Nitinol® as a biomaterial demonstrates poor biocompatibility towards vascular tissues. It contributes to three of the most outstanding problems for current ICS regardless of their designs. First, they are highly thrombogenic; under the current
guidelines, all stenting procedures are recommended to be regularly followed by a lifetime prescription of dual antiplatelet therapy (DAPT). DAPT is associated with unwanted side effects like gastric reactions and bleeding complications (Akbari et al., 2012). Secondly, Nitinol® ICSs have a high rate of in-stent restenosis. Currently, ICSs demonstrate poor biocompatibility towards endothelial cells (ECs), and the damage of the in-situ EC layer leads to the subsequent over-proliferation of smooth muscle cells (SMC) underneath, causing thickening of the vessel wall, as well as, shrinking of the vessel lumen. This, in most cases, is the cause of stent failure in the long-term (Liu et al., 2017b, Chalouhi et al., 2012, Lee et al., 2012). Third, nickel sensitivity is the most common form of body rejection towards Nitinol® ICS. The immune response is triggered by nickel (Ni) ions released from the stent surface resulting in localised allergic reactions (Darlenski et al., 2012, Ryhanen et al., 1997). Besides these three major problems, there are also other not so common, stent-related complications such as stent deployment failure, stent migration, vessel trauma during stenting leading to hemorrhagic events (Zhao et al., 2014b). These complications are not related to ICS biomaterials. On average, all the current commercially available ICSs come with an adverse event rate of over 8.5%. The summary of adverse effect rates, morbidity and mortality for current commercially available ICSs are listed in detail as following in table 1-2.
Table 1-2. The summary of adverse effect rates for currently available ICS

<table>
<thead>
<tr>
<th>Stent</th>
<th>Deployment Failure Rate (%)</th>
<th>Stent Migration Rate (%)</th>
<th>Restenosis Rate (%)</th>
<th>Thromboembolic Event Rate (%)</th>
<th>Haemorrhagic Event Rate (%)</th>
<th>Morbidity (%)</th>
<th>Morality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroform</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Wingspan</td>
<td>2</td>
<td>0</td>
<td>0.96</td>
<td>4</td>
<td>6.25</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Enterprises</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Leo</td>
<td>5</td>
<td>2</td>
<td>-</td>
<td>14</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Solitaire</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>17-22</td>
</tr>
<tr>
<td>Silk</td>
<td>3</td>
<td>&gt;1</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>PED</td>
<td>2</td>
<td>-</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Willis</td>
<td>1.9</td>
<td>-</td>
<td>11.3</td>
<td>1.9</td>
<td>1.9</td>
<td>13.2</td>
<td>1.9</td>
</tr>
</tbody>
</table>
1.4 In search of a solution

Promoting *in situ* endothelialisation is one strategy that is being explored to overcome some of the problems associated with Nitinol® ICS. By promoting rapid endothelization on the ICS surface will minimise thrombosis and restenosis. Theoretically, rapid endothelialisation of the luminal surfaces is a novel means of increasing implant patency and preventing in-stent stenosis by inhibiting intimal hyperplasia, as well as, the only material that can prevent the reoccurrence of thrombosis (Nakazawa et al., 2010).

1.4.1 Neurovascular biomaterials for ICS

Since it is recognised that the three major problems of ICS are due to poor biocompatibility of biomaterials towards cell-material interactions, the search for more desirable neurovascular biomaterials for ICS has never ceased. An ideal biomaterial for ICS should be inert, non-toxic to the host and therefore minimizing the immune response, as well as, having sufficient material properties such as the mechanical strength under physiological stress associated with any form of micro-movement (Adiga et al., 2009), and most importantly be capable of promoting integration with minimal time-dependent deterioration of the biomaterial (Ivanova et al., 2014a). A biomaterial is a substance that has been engineered to take a form, which is used to direct, by control of interactions with components of living systems, the course of any
therapeutic or diagnostic procedure in human or veterinary medicine (Williams, 2009). According to this definition, nanomaterials, gene/drug delivery systems, and tissue engineered materials (e.g. acellular or cell-seeded scaffolds, tissues, organs) fall into this category. In general, biomaterials comprise of polymers, ceramics, metallic alloys, composite (with or without cells) or natural materials (Ordikhani et al., 2017). Singular or combined, with proper engineering and design, they make up various medical devices. For ICS, past studies have explored a series of metallic options, such as 316L surgical stainless steel (Buhagiar et al., 2012), Cobalt-based (Cr-Co-X) alloys (Bahraminasab et al., 2012), titanium-based materials (Shadanbaz and Dias, 2012), noble metal (Au, Pt) alloys (Mehl et al., 2011), biodegradable metals (Mg, Zn) (Hort et al., 2010); and polymeric options, like polyethylene terephthalate (PETE, also known as Dacron), PTFE and ePTFE.

However, despite their many desirable properties as biomaterials, there are currently still many limitations to all three metallic alloys. They are thrombogenic, unstable in long-term applications, demonstrate poor local tissue integration and endothelialisation and can be toxic due to Ni ion releasing after their surface pacification has corroded. Similarly, polymeric biomaterials are relatively inert, but also demonstrate poor localised tissue integration and are highly thrombogenic when manufactured to the miniature size of ICS (Fang et al., 2017, Zhu et al., 2013). Currently, many more studies are being carried out
to look into novel designs and engineering of combined biomaterials, in the hope of overcoming the restrictions of current ICS.

1.4.2 The neurovascular interface

The neurovascular interface refers to the unique interface between ICS biomaterial surface and the host tissues. On its outer surface, the ICS is in direct contact with the blood and is exposed to blood components, plasma proteins, water and counter-ions to create a conditioned interface in which the cells respond. ICS is in direct contact with the vascular tissue within the CNS and influences cell-material interactions, as well as, other bio-interactive structures, e.g. the blood-brain barrier (BBB), cerebral spinal fluid (CSF) and the nerve tissue of the brain and spinal cord. On its inner surface, it is constantly challenged by circulating blood flow, and in direct contact with active blood cells and blood components such as red blood cells (RBCs), white blood cells (WBCs), platelets and plasma proteins composed of immunoglobins. Controlling cellular events at the neurovascular interface is the key to determining whether the biomaterial is compatible or incompatible towards local tissue, and so determines the success or failure of the implantable device.

For this reason, much research on protein-surface and cell-surface interactions on different biomaterials has been carried out over the past decade. On a physiological level, cells interact with their microenvironment through adhesive serum proteins, e.g. fibronectin that relays biochemical, cellular
signalling events in response to external guidance cues (Nguyen et al., 2016b).

*In vivo*, cells are surrounded by a complex microenvironment of extracellular matrix (ECM) (Dvir et al., 2011). The ECM enriches biochemical and physical guidance cues. Biochemical cues such as growth factors and hormones can influence cell fate by activating or deactivating cellular signaling pathways. While mechanical cues such as the micro- and nanoscale (1-100 nm) topographic patterns and features presented in the ECM, e.g. collagen fibers presented in the hierarchical structures, which interact with cells by providing the basis for mechanical signaling (Dalby et al., 2014, Solomonov et al., 2014). Together, these two major external guidance cues are the most direct stimuli affecting individual cells and contribute to major cell behaviours such adhesion, migration, proliferation, growth, differentiation and apoptosis (Curtis et al., 2006). Similarly, cell-material interactions are governed by the surface properties of the biomaterial such as surface chemistry (charge), energy (wettability) and topography of the underlying substrate (Son et al., 2015, Nguyen et al., 2016a, Kshitiz et al., 2015). Currently, means to improve the neurovascular interface on the ICS surface are still being explored as one of the key research themes to improve the long-term performance and patency of ICS clinically.

### 1.5 Surface modification strategies

A variety of surface modification techniques have been applied to current
ICS biomaterials to improve the neurovascular interface and is described in the following sections in detail.

1.5.1 Physical and mechanical approaches

Heat treatment is one of the most common methods for improving the performance of Nitinol®. Heat treatment results in thermal oxidation, which in return preventing the Ni ions from releasing and entering the circulation system. However, with super high-temperature heat treatment (above 600 °C), the Nitinol® surface generates a thick oxide layer of around 0.5μm, which was prone to cracking and delamination (Siu and Man, 2013). Heat treatment of around 400 °C results in low Ni-ion release. Human osteosarcoma cell lines (HOS-TE85) demonstrated enhanced cell proliferation and metabolic activity on the 400 °C heat-treated Nitinol® surfaces (figure 1-5) (Chrzanowski et al., 2012, Chrzanowski et al., 2010).
Figure 1-5. Nitinol® surfaces after 400 and 600 °C heat treatment. A) Atomic force microscope (AFM) images and line profiles; B) HOS cell viability results Confocal microscopy fluorescence images of the test samples after 1 and 7 d in culture; C) and D) Cytoskeleton and focal adhesion staining of HOS cells after cultured on samples for 3 d (actin and tubulin).
Besides heat treatment, many mechanical surface treatments have also been studies. For example, surface etching was commonly used in creating an etched line, folds and holes on Nitinol® surfaces. However, studies have proven that had little influence over surface chemistry and topography (Kuribayashi et al., 2006). Besides etching, mechanical polishing (MP) is also frequently used to generate different surface roughness. MP for Nitinol® surfaces can be achieved via sandblasting, shot peening or grooving. Each of these methods can induce a specific topography and roughness to facilitate cell adhesion, proliferation and migration (Wirth et al., 2005, Armitage et al., 2003). Studies have shown that grooved Nitinol® surface would promote human umbilical vein endothelial cells (HUVECs) migration (Palmaz et al., 1999).

1.5.2 Electrical approaches

Electro-polishing (EP) is used to generate smooth surface profiles on various metallic biomaterials. A study by Petrovic and colleagues compared EP Nitinol® and conventional MP Nitinol® surfaces with 600 grit finishing. Findings showed that MP600 surfaces noticeably suppressed EC proliferation by up to 30% compared with the control EP surfaces (figure 1-6) (Shabalovskaya et al., 2008).
Electrophoretic deposition is another approach to modify metallic biomaterial surfaces. Strauss and colleagues explored coating titanium, nickel and silver nanoparticles or nickel-titanium colloidal nanoparticles on to Nitinol® surface in a monolayer coating. *In vitro* tests revealed that such coatings are non-toxic, and promote adipose stem cells (ASCs) adhesion and differentiation (Strauss et al., 2013). Sun and colleagues used this modification strategy to coat heparin on to Nitinol® substrates via a nanoparticle comprised of hydroxyapatite, bioglass and chitosan resulting in improved biomaterial thrombogenicity (Sun et al., 2008). Besides those mentioned above, other electrical approaches such
as laser surface treatments, electro-grafting, plasma ion implantation and plasma electrolytic oxidation can also induce alteration on material surfaces.

1.5.3 Surface chemical approaches

Chemical etching via erosive agents is one of the most basic chemical surface treatments. For Nitinol®, acid treatment can influence the surface roughness, while alkali treatment showed great potential in changing both the pits and pores influencing the surface topography and chemistry. However, both types of treatments are known to damage the passive oxide layer resulting in a burst release of Ni ions from the surface, which will cause a decrease in surface wettability as well as unfavourable cellular reactions, e.g. decreased proliferation and growth with various cell types have been confirmed in multiple studies (Chrzanowski et al., 2012, Chrzanowski et al., 2010).

Surface treatment with organosilane precursors is another principal method of surface modification converting mineral or inorganic surfaces of many different types of materials. The modification could enable cross-linking for attaching particles or fibres that provide reinforcement or molecules of interests for biofunctionalisation. Organosilane treated substrates contain hydrolysable substituents on its silicon centre that could react through condensation reactions with water via hydroxyl (-OH) groups present on the Nitinol® surface, while reactive substituents on the silane species can be tailored for specific
applications. Nitinol® silanization could be applied as a way to cross-link or immobilise bioactive molecules on to the surface or create cross-linked materials (Roy et al., 2009, Yeh and Lin, 2009, Cousins et al., 2004).

1.5.4 Surface coating strategies for ICS

The goal of ICS coatings is to find a non-toxic and more biocompatible biomaterial to be manufactured into a thin layer to coating cover the surface of ICS materials. The rationale behind this concept is that by maintaining the metallic framework underneath, the favourable material and mechanical properties can be retained, while the coating covers the metallic framework leaving behind more favourable surface properties to influence cell-material interactions and minimise complications of post-stenting ICS. Currently, there are no commercially available coated ICS, nor have extensive R&D been carried out in this area, however, there are many examples from cardiovascular stents or peripheral stents with drug-eluting stent (DES) polymers, and we can align such approaches to enhance the function and performance for ICS.

1.5.4.1 Non-biodegradable polymeric coatings for stents

Polyethylene terephthalate (PET) or Dacron as mentioned previously is used as both a coating and a cover for abdominal aorta aneurysm (AAA) stents, while PTFE is used as coating or cover material for stents or bypass grafts with diameters > 6 mm in diameter (Willigendael et al., 2005, Solouk et al., 2011a,
Duda et al., 2002). However, both materials demonstrate poor biocompatibility and high thrombogenicity in smaller diameter stents that include ICS (Lai et al., 2013). Polyurethane (PU) and poly(caprolactone-urea) urethane (PCU) has been studied as one of the durable polymer coatings for Nitinol® stents since 1981 (Rechavia et al., 1998). Tepe and colleague suggested in their study that PU is a non-toxic and biocompatible biomaterial, with great potential in enhancing cell spreading and adhesion. Both PU and PCU as biomaterials demonstrate enhanced hemocompatibility in the coated devices (Rechavia et al., 1998, Han et al., 2002, Tepe et al., 2006).

1.5.4.2 Biodegradable polymeric coatings for stents – drug-eluting stents (DES)

Biodegradable polymer coatings for Nitinol® stents are being investigated as new approaches to fabricate stents with drug-eluting capabilities. Currently, there is no commercially available DES for intracranial use. The advantage of having an intracranial DES could be enormous. Polylactide-co-S-caprolactone (PLA) has been explored as a drug-eluting coating for Nitinol®. It is degraded in vivo via hydrolysis of polymer chains into non-toxic lactic acid and is proven to be suitable as a matrix for loading many different drugs, such as dexamethasone or sirolimus. It had a high drug-carrying capacity that only a minimal amount of polymer would be required for assembling a mid-to-long term drug-eluting device. It can maintain a steady drug-eluting profile for a minimum of 6 weeks up to a year (Indolfi et al., 2011). Modified PLA coating
has already been used in an FDA approved sirolimus DES, the DEVAX (Irvine, California, USA) to inhibit in-situ SMC proliferation. In pre-clinical studies, 28 days after implantation following histomorphometry showed a 50% reduction in stenosis and IH when compared with controls (Cilingiroglu et al., 2006).

Poly(Lactic-Co-Glycolic) acid (PLGA) is another common biodegradable polymer, while poly(vinyl alcohol) (PVA) is a non-biodegradable hydrogel that is highly stable under a range of temperature and pH values. Siddhesh and colleagues proposed in 2011 to submerge dexamethasone-loaded PLGA microspheres into PVA hydrogel composites to deliver dexamethasone. The in vitro and in vivo release kinetics showed a perfect linear correlation. The composites were capable of controlling both the acute and chronic phases of the inflammatory response (e.g. IL-4, IL-6 and TNF-α) and minimisation of fibrosis in the vicinity of the device (Patil et al., 2011). This PLGA/PVA eluting layer can be coated on to a Nitinol® stent, which results in a bio-inert stent with controlled drug-eluting features (figure 1-7) (Bhardwaj et al., 2010)
1.5.5 Bio-active coatings for stents

In 2001, research by Yvette and colleagues investigated a novel coating Persantin®, which is an immobilised dipyridamole coating. It is a potent non-toxic inhibitor of platelet activation and aggregation, and also a strong inhibitor of SMC proliferation. In their study, they coated the Persantin® onto a "biodurable" PCU vascular graft called Chronoflex®. The coated vascular graft was studied in vitro for hemocompatibility, platelet studies and endothelial cell culture, as well as, in two established in vivo models. The results demonstrate that Persantin® coating was a non-toxic and biocompatibility material for ECs. Moreover in vivo studies demonstrate success in promoting in-situ endothelization while inhibiting SMC proliferation (Aldenhoff et al., 2001).

Heparin coatings for Nitinol® stents are also popular. One approach uses a simple layer-by-layer deposition of positively charged chitosan and negatively charged heparin on to the native surface of both Nitinol® and titanium without
any previous treatments. Nitinol® stents treated this way also demonstrated antibacterial property through the surface deposition of chitosan, as well as ability to suppress blood clot formation via heparin (Meng et al., 2009, Schweizer et al., 2010). The mechanism of Nitinol® stents may inhibit bacterial growth is most likely due to the free nickel ion exposed on the biomaterial surface after the surface deposition process. Matanovic and colleagues showed that a mixed coating of heparin and chitosan with layer-by-layer technique successfully promoted a fast endothelialisation and intimal healing after stent implantation (Radivojša Matanovic et al., 2015).

1.5.6 Bioengineering coating strategies for stents

The idea of engineering a coating that is theatrically stable and can last forever after implantation has been explored. The rationale of a long-term stable polymeric material in living tissue is when both the polymeric backbone and pendant groups are devoid of unprotected ester, amide, ether, carbamate, urea, or any other groups that are prone to oxidation, hydrolysis, or enzymatic cleavage. Further, secondary carbon-containing polymers such as polyethylene, and secondary-and-tertiary carbon-containing polymers such as polypropylene, are also to be avoided as double bond formation leads to embrittlement and stress cracking. The ideal polymer for implant application should contain only oxidatively, hydrolytically and enzymatically stable alternating secondary-and-quaternary carbons in the backbone, and equally
stable primary carbons as pendant groups. The basic structures of this nature are those comprised of polyisobutylene (PIB). The less biodegradation, the less inflammation would be caused.

Kennedy’s laboratory at The University of Akron had synthesised a PIB-based thermoplastic elastomer in 2005 (Kennedy, 2005). This PIB-based tri-block copolymer, poly(styrene-block-isobutylene-block-styrene) (poly-SIBS), is one of the ideal biomaterials for stent coating. It is currently being evaluating as a drug carrier for eluting paclitaxel to suppress SMCs in a drug-eluting coronary stent, the TAXUS® (Boston Scientific Corporation, Natick, MA, USA), in both pre-clinical and clinical trials (Kamath et al., 2006, Wang et al., 2010, Pinchuk et al., 2008). In 2012, Zhu and colleagues proposed a modification to produce sulfonated SIBS (S-SIBS). Sulfonic acid could increase proton transport on the surface and therefore increase water uptake. Incorporating sulfonic acid (anionic group) on to SIBS would change the material’s wettability significantly from hydrophobic to hydrophilic properties. The result of their study showed that the S-SIBS-coated DES successfully delivered sirolimus and demonstrated the ability to inhibit long-term neointimal hyperplasia (Zhu et al., 2012). Moreover, the S-SIBS-coated DES exhibited reduced inflammation within the vessel wall and better able to support the attachment of HUVECs (figure 1-8) (Kotani et al., 2006, Virmani et al., 2004).
Figure 1-8. Images at 4 and 26 weeks after the placement of different stents. Neointimal thickness at 4 weeks was similar among BMS, BMS Plus S-SIBS, DES, and DES Plus S-SIBS groups. By 26 weeks, DES plus S-SIBS showed similar ability to DES to inhibit neointimal hyperplasia in contrast with BMS (P < 0.05), which showed a steady increase in neointimal thickness. Sections shown were stained with hematoxylin and eosin (H&E).
1.5.7 Stem cells capturing techniques for stents

Since endothelial progenitor cells (EPCs) were first isolated from peripheral blood in 1997, there has been extensive research focusing on immobilization of EPCs via surface embedded specific molecules such as anti-CD133 and VEGF-R2, and promoting them to differentiate and proliferate into an EC monolayer (Andukuri et al., 2013, Matsuda et al., 2013). The Genous® stent (OrbusNeich Medical Technologies, Hong Kong) is the first EPC capturing stent. It is a 316L surgical stainless steel stent functionalised with anti-CD34 antibodies (anti-CD34Ab) via a biocompatible polysaccharide matrix (Andukuri et al., 2013, de Mel et al., 2012, Rossi et al., 2010, Shirota et al., 2003). However, in a pilot study, the TRIAS HR (TRI-stent adjudication study – high risk of restenosis) trial, the Genous® stent did not demonstrate superiority to currently-used DES in patients with high risks of restenosis (Beijk et al., 2011, Klomp et al., 2011, Klomp et al., 2009).

1.5.8 Nanotechnology strategies for ICS

Surface topography on the nanometre (nm) scale defined as 1-100 nm has been shown to influence cell fate such as adhesion, migration, growth, proliferation and differentiation of a variety of different cell types (Lima and Mano, 2015, Le Saux et al., 2011, Jin et al., 2008). For example, much research
has been carried out on the interaction between ECs and nanopodography. ECs have been observed to showed increased cellular proliferation, elongation and enhanced filopodia formation on titanium nano-tubes (Han et al., 2015); ECs on nano-bulges with a height of 13 nm showed increased cell adhesion and spreading (Dalby et al., 2002, Buttiglieri et al., 2003); ECs on nanogrooves with depth around 100–300nm showed enhanced cell elongation, increased cell orientation and migration along grooves (Ranjan and Webster, 2009, Biela et al., 2009, Bettinger et al., 2006); ECs on nanocones with a height of 300 nm showed cellular adhesion (Carpenter et al., 2008). It is suspected that these nanotopography influence cell response via altering the production of ECM materials to increase or reduce cell adhesion and response and to promote or suppress the ability of cells to form focal contacts and develop cytoskeletal arrangements (Bigerelle et al., 2011).

Dalby and colleagues showed that fibroblasts had the most developed actin, tubulin, and vimentin cytoskeletons, as well as, the most proliferative cell population on the surface with 13 nm polymer de-mixed nanoislands. The cells on the 95 nm islands, however, showed poorly developed cytoskeletons, as well as, low levels of cell proliferation. On the 35 nm islands, we can see fibroblasts with protruding filopodia to sense their environment and detect the nanoislands, seemingly using them as “stepping stones” (figure 1-9) (Dalby et al., 2006).
Figure 1-9. Scanning electron microscopy (SEM) images of fibroblasts cultured on different size nano-islands. A) On the 13nm islands, many cells were seen to be in various stages of division; this image is of a cell rounding up to divide (R) among cells that are generally spread flat (F); B) On the 35nm islands, cells were clearly starting to interact with the islands (arrowheads show filopodia–island interactions); C) On the 95nm islands, the cells appear to be moving along the tops of the islands, using “pseudopodial-like” processes (arrows show lamellipodia–island interactions).
1.6 Summary

Stroke is a devastating condition affecting over 100,000 people annually in the UK. It causes more deaths than breast cancer in women and prostate cancer in men and those who survive are likely to face many disabilities and reduced quality of life. ICSs are essential neurovascular devices for stroke management. However, the current ICS are problematic. They are highly thrombogenic, and usually, require a lifetime prescription of DAPT following stent surgery. They have poor compatibility towards local tissue, resulting from poor endothelization and induce over the proliferation of the in-situ SMCs, leading to re-stenosis and eventually stent failure. They can also cause nickel sensitivity due to the release of Ni ions from the device surface. All of these major problems are at some level due to poor biocompatibility of the ICS biomaterial, Nitinol®, towards neurovascular tissue and blood components.

By improving the biocompatibility of ICS, we hope to overcome all the adverse effects mentioned in the above section. Firstly, by improving the adverse effects and complication rate of ICSs, interventional surgery would show superiority over best medical therapy in stroke management and help many more patients who still have a high risk of recurrent strokes under best medical therapy due to their underlying risk factors. Secondly, the efficiency of ICS is deemed to be higher than best medical therapy as it instantly improves blood flow and perfusion, while best medical therapy would take a much longer time to take effective. Third, with better performance of ICS, we can expect patients to be medication free for the rest of their life which can be more economical in the long run as well as avoiding all the adverse effect from long-term medication like bleeding disorders, the resistance of drug etc. Finally, with
all these improvements we expected to achieve by enhancing the biocompatibility of ICS, our overall purpose was still to prolong the life expectancy for stroke patients as well as improving the quality of their life after stroke.

There have long been calls for better stroke management with improved outcomes for stroke patients, along with it comes the need for better neurovascular devices and a new generation of ICSs with better performance and less unfavourable effects. Significant research has been conducted in search of novel methods of improving current ICS. Many new polymeric biomaterials have been developed and tested in order to replace Nitinol®. However, very few have come close to matching their superior mechanical properties. Challenges to finding an alternative to metallic based biomaterials have led to research focusing more on surface modification strategies to improve events at the neurovascular interface of the classic biomaterial while retaining its desirable mechanical properties.

Our original hypothesis was that by developing an electrostatic coating and applying suitable polymeric materials such as poly-SIBS using different coating methods, we could produce a variety of surface topographies onto conventional biomaterials while retaining their desirable properties to explore cellular interactions at the tissue-implant interface. Currently, there are two approaches of incorporating a nanotopography on to different biomaterials. Conventional manufacturing approaches use top-down approaches through mechanical etching, carving away material by using photolithography and electron-beam lithography to create and pattern nanotopography on to different materials. These methods are often expensive, time-consuming, and only create a single
2D geometric pattern on the material and is not always consistency. Another approach is bottom-up construction using novel biomaterials or applying 3D printing techniques of existing biomaterials as nanocomposites. However, each newly fabricated nanocomposite can only process one particular nanotopography, and limitations with the resolution of existing technology can only print to a scale of 100 µm. Current advances in 3D printing have yet to reach the nanometer scale dimensions we desire to interact at the cellular level. There is a significant unmet need in the development of new surface engineering strategies to incorporate nanopatterning of materials using alternative, more economical and efficient methods.

1.7 Aims and objectives

The overall aim of this study is to develop a simple and novel electrostatic coating using poly-SIBS tri-block copolymers that can be optimised towards the development of nanotopographical surfaces to influence the cellular response at the tissue-implant interface, which can then be optimised further and applied bare metal stents (BMS) and ICS. The main objectives and subsidiary aims are as follows:

• To optimise poly-SIBS coatings and characterise their material properties.
• To study the influence of the coatings in vitro, including their interactions with blood components (e.g. platelets), as well as, whole blood and biocompatibility testing to screen their cytotoxicity with vasculature cell types, e.g. ECs, and SMCs in vitro.
• To investigate the effects of varying nanotopographical surface cues.

• To explore the translation of poly-SIBS nanosurface coatings on stent materials such as Nitinol®, platinum-chromium (PtCr), as well as, polymeric biomaterials.

• To develop proof of concept studies and apply the nano-coating on to commercially available stents, and test in vitro and in vivo biocompatibility and safety using a small animal (rabbit) model in a pioneer study over 28 days.

Optimisation of the poly-SIBS nano-coatings will enable a thorough study on the controlled deposition of poly-SIBS nanosurface features on stent biomaterials, which can be tailored towards a variety of cardiovascular and neurovascular cell types, and explore the potential of the coatings on current stent devices.
Chapter 2  An electrostatic coating platform with controllable nano-surface features

This chapter explores the optimisation of novel coatings via surface modification using electrostatic coatings to incorporate chemical and surface features at the nanometer scale on model glass substrates. Here, we describe the use of organosilane precursors, which provides a reliable and convenient method for covalently attaching poly(styrene-block-isobutylene-block-styrene) or poly-SIBS on to surfaces to yield uniform thin film coatings. Water contact angle (θ°) and surface energy (γs) measurements were used to characterise surface properties before and after modification. Field Emission Scanning Electron Microscopy (FE-SEM) and Atomic Force Microscopy (AFM) were used for high-resolution image analysis and characterisation of nanotopography.

2.1 Introduction

2.1.1 Organosilanisation cross-linking reactions

Surface treatments with reactive organosilane precursors (X-Si-R3) is one of the principal methods for converting mineral or inorganic surfaces in to materials bearing covalently bound functional groups, capable of graft formation when used as substrates for attaching particulates or fibres as in reinforced polymer composites, as well as, metallic biomaterials such as titanium, stainless steel and ceramics (Nanci et al., 1998). Organosilanes containing halogen, alkoxide, acrylate, and amino groups attached to silicon and react with surface hydroxylic species to form covalently bound surface
siloxy derivatives as shown in figure 2-1:

\[ \text{OH} + X\text{-Si-R}_3 \rightarrow \text{Si-R}_3 + H\text{-X} \]

**Figure 2-1.** The reaction of organosilanes with hydroxylated surfaces with a variety of mineral, inorganic and metallic substrates.

The reactions of organosilanes with inorganic and metallic surfaces have been extensively studied, and their chemistry and technology have been frequently reviewed especially when attaching nanoparticulate materials on to different substrates (Cousins et al., 2004, Solouk et al., 2011b). For example, silanes can covalently attach to other functional groups such as amines (-NH₂), which can be further used as a cross-linking agent to immobilise bioactive compounds, e.g. peptides or antibodies. They can also be introduced into bulk materials to form reinforced composites to improve material properties such as mechanical strength, stability elasticity and flexibility.

**2.1.2 Poly(styrene-block-isobutylene-block-styrene) - Poly-SIBS**

Poly-SIBS is a non-biodegradable, biostable thermoplastic tri-block copolymer, and is well known for its non-cytotoxicity, immunogenicity and minimal foreign body reactions (Fittipaldi et al., 2014). Multiple studies have addressed the biocompatibility of poly-SIBS and have shown that the biocompatibility of the materials is not affected by changing its tri-block composition, which can be adjusted to elicit any desired property and is shown
For example, the mechanical rigidity can be enhanced by increasing the styrene component while the isobutylene segments can enhance the elastomeric plasticity. It has material properties that are ideal for biomedical applications such as hemocompatibility, biocompatibility and long-term stability in contact with metallic substrates (Pinchuk et al., 2008). Poly-SIBS has been approved for use by the FDA and tested as the key biomaterial for stent-grafts, a carrier for drug-eluting stents (DES), glaucoma shunts and artificial tri-leaflet aortic heart valves (Kamath et al., 2006, Wang et al., 2010).

At present, many researchers have studied medical device-related applications with poly-SIBS and found the following desirable properties: 1) poly-SIBS does not substantially activate platelets leading to thrombosis; 2) polymorphonuclear leukocytes (PMNs) are not observed around poly-SIBS after implantation; 3) the materials do not degrade or crack causing embrittlement; 4) poly-SIBS is resistant to calcification; and 5) creep deformation occurs in certain load-bearing applications. Perhaps, the explanation for such desirable properties and excellent biocompatibility may be due to its inertness and lack of cleavable functional moieties, which are
resistant to degradation of enzymes or chemotactic towards PMNs. Such resistance and inertness properties distinguish it from other SIBS containing polymers such as the acrylates, methacrylates, polyesters, polyethers, polyamides and PUs that contain ester, ether, amide or carbamate groups, which may slowly hydrolyse, oxidise or cleave side groups when in contact with the physiological environment. An abundance of PMNs and macrophages cells are often attracted to degrading polymers and monomers, which can lead to thick fibrous capsules as the body attempts to remove the foreign body leading to implant failure (Pinchuk et al., 2008).

In this study, we explore the use of organosilane precursors to develop electrostatic surface coatings with a variety of surface functional groups on model glass substrates to covalently attach poly-SIBS tri-block copolymers, which are positively charged (+) to form uniform thin film surface coatings. Through modifying different coating procedures using dip and spin coating methods, and phase separation of poly-SIBS under different conditions, we create a range of surface topography at the micro- and nanometer scale.

2.2 Material and methods

All chemicals were purchased from Sigma-Aldrich, UK unless otherwise specified.

2.2.1 Preparation of glass substrates with modified surface chemistries

Organosilane precursors were selected with common functional groups present in many biological molecules to modify surface chemistry using the
following reagents: 1) dichlorodimethylsilane (DCDMS) for methyl (-CH$_3$) groups; 2) 3-aminopropyltrimethoxysilane (APTS) for amine (-NH$_2$) groups; 3) 3-mercaptopropyltrimethoxysilane (MPTS) for sulphur (-SH) groups; 4) trimethoxysilylpropyl methacrylate (TMPM) for methacrylate (-CH$_2$=C-COOR) groups; and 5) chloro-dimethyl(3,3,3 trifluoropropyl)silane (CDTFS) for fluorinated (-F) groups (figure 2-3).

**Figure 2-3.** Selected organosilane precursors common in biological systems to yield 1) -CH$_3$; 2) -NH$_2$; 3) -SH; 4) -COOH; 5) -F terminal functional groups

For DCMDS and CDTFS modification, glass substrates (circular, diameter=13mm, VWR, UK) was rinsed with 100% v/v iso-propanol before reacting 2 ml organosilane for 1 min directly on to the substrate surface. The substrates were then rinsed three times with 100% v/v toluene to remove excessive reagents before being allowed to cure in an air-circulating oven.
(Binder ED23, Cole-Parmer, UK) at 65°C for 5 min. The modified substrates were stored in a Petri dish and placed into a desiccator vessel before use.

For APTS, MPTS and TMPM modified glass substrates; each sample was rinsed with 100% v/v isopropanol and placed into a 1000ml glass beaker placed on a digital ceramic stirring hot plate (IKA 3581401, Cole-Parmer, UK) containing 300ml 100% v/v isopropanol. 2ml organosilane was added to the solvent along with 1g anti-bumping granules, and 1 ml deionised water (dH$_2$O). The reactants were heated to reflux at 70°C for 30 mins. Excess solvents were removed to recover the samples and were placed on to aluminium foil in an air-circulating oven at 65°C for 5 min, and stored in a desiccator before use.

2.2.2 Synthesis of nanostructured coatings on a glass substrate

To apply the poly-SIBS coating on to glass substrates, we developed a primary adhesive or treated base layer following optimisation of the organosilane reactions as mentioned previously to create electrostatic coatings. CDTFS organosilane precursor shown in figure 2-3 (5) was known to generate fluorinated surface groups, which have a net negatively (-) surface charge on model glass substrates (Yeh and Lin, 2009). The aromatic phenyl rings of polystyrene present in the poly-SIBS backbone are positively charged (+), thereby reacting with poly-SIBS coating layer covalently attached to the negatively charged surface through electrostatic attraction as shown in figure 2-4.
Poly-SIBS is a thermoplastic block copolymer, and its chemical structure is made up of the polystyrene and polyisobutylene copolymer segments linked together by the polymer backbone. By manipulating the conditions applied during the coating procedure, e.g. dip or spin coating, we can create various nanosurface features with poly-SIBS coatings. Our original hypothesis was that when casting poly-SIBS under ambient and passive conditions such as dip coating, a densely packed polymer film would be present on the surface. However, if a negatively charged organosilanised substrates were coated with poly-SIBS at high speeds, the difference in adhesion of the cationic layer binding to the negatively charged substrate will cause the polymer segments to stretch and phase separate during the coating procedure. The high speed coating procedure will stretch the polymer backbone causing the polystyrene (hard segment) and polyisobutylene (soft segment) chemistry to phase separate (and pull apart) to create patterned substrates as shown in the schematic figure in Figure 2-4. Electrostatic coatings are generated by fluorinated groups present in CFMTS, which have been net negatively (-) charged, which are attracted to positively (+) charged phenyl rings (arrows) in the poly-SIBS backbone to yield covalently bound thin films.

![Figure 2-4](image-url)
Dip coating procedures can be regarded as passive and ambient coating conditions, whereby the poly-SIBS copolymer simply adheres to the substrate via electrostatics via charge-charge interactions between cationic and anionic functional groups. Glass substrates were handled with microsurgical tweezers, and were completely submerged in 10% (w/v) poly-SIBS solution for 30 secs and gently removed while being held in suspension for 1 min to allow residual copolymers to drip dry before being gently taped onto absorbent paper to remove excess solution. The coated samples were then transferred directly into an air-circulating oven at 65°C for 30 min.
2.2.2.2 Poly-SIBS coatings via spin coating methods

Spin coating techniques often provide a means to create uniform thin film coatings on planar substrates at high speeds. The adhesion of poly-SIBS onto different chemically treated substrates at varying speeds can create a variety of thin films with the level of adhesion depending upon the surface chemistry and overall charge during the coating procedure. Glass substrates were coated and processed using an SCK-200 Spin Coater (Instras Scientific, USA) as shown in figure 2-6. Briefly, each sample was mounted on the circular sample chuck of the spin coater followed by the addition of 100μL 5% (w/v) poly(styrene-block-isobutylene-block-styrene), sulfonated poly-SIBS solution placed on the centre of the sample. The spin speeds of 1000, 3000 and 6000 rpm were selected for a duration of 1 min for each sample. Each of the coated substrates was then transferred to 65°C air-circulating oven to dry for 30 min.
Figure 2-6. The SCK-200 spin coater from Instras Scientific Inc, USA. Highlighted boxes showed spin speed controller, spin coat chamber and sample loading platform.

2.2.3 Surface characterisation of samples

2.2.3.1 Contact Angle (θ°) and Surface Energy (γ) Measurements

Surface wettability of all of the modified samples was determined via a static θ measurement (in degrees °) using the sessile drop method and dH₂O as the solvent. Young’s equation was used to define the balance of forces caused by a water droplet on a dry surface. Surface energy (γₛ) was calculated from θ measurements using Owens–Wendt theory as follows in equation (2-1):

$$\gamma_{lg}(1 + \cos \theta) = 2\sqrt{\gamma_s^d \gamma_{lg}^d} + 2\sqrt{\gamma_s^p \gamma_{lg}^p}$$

Eq. 2-1

Where γ/lg is the surface tension of the liquid, and γ/lg<p and γ/lg>d are polar and disperse components from two selected test liquids. γS is the sum of both polar and disperse components as in equation (2-2):

$$\gamma_s = \gamma_s^p + \gamma_s^d$$

Eq. 2-2

γS<p is correlated to the polar interaction, and γS<d corresponds to the portion of dispersive components in the Lifshitz-van der Waals interactions to both components of γs. Deionised water (dH₂O: γₛ = 72.8, γₛ<p = 51.0, γₛ<d = 21.8mN/m), ethylene glycol (>99.9% purity; γₛ = 47.3, γₛ<p = 29.0, γₛ<d = 18.3mN/m) and diiodomethane (99% purity; γₛ = 50.8, γₛ<p = 48.5, γₛ<d = 2.3mN/m) were used as the solvents to calculate the surface tension of all samples. θ measurements were performed using a KRÜSS DSA 100 machine and the Drop Shape Analysis software (EasyDrop DSA20E, KRUSS GmbH, Germany). The sessile drop method used a 5.0μl droplet volume and dispensing rate of 195.1μl/min at 25°C. Images of the droplets were captured.
after 1 min. Three randomised locations on each sample were recorded from three different experimental samples (n=9 per condition in 3 repeats). Sample surfaces with θ greater than 90° were considered to be hydrophobic (Sirocic et al., 2013, Weng and Shen, 2013, Solouk et al., 2011b).

2.2.3.2 Field-Emission Scanning Electron Microscopy (FE-SEM)

FE-SEM uses a focused beam of high-energy electrons produced by a field emission source to scan solid samples in a zig-zag pattern to generate a variety of signals at the surface of the specimens. In most applications, these signal data are collected over a selected area of the surface of the substrate and an image is generated that displays spatial variations within these properties. With conventional SEM techniques, an FE-SEM can achieve greater magnification ranging from x45 to x1,000,000. It is also capable of performing analyses of selected point locations on the sample, and this approach is especially useful for qualitatively or semi-quantitatively determining the crystalline structure, and crystal orientations (Cazaux, 2012). It is one of the most commonly used techniques in material science to visualise topographic details down to nanometric scale dimensions on the sample surface. The FE-SEM may also be employed for example to study cell organelles and DNA material, synthetic polymers, and coatings on microchips (Cui et al., 2017). Compared with convention scanning electron microscopy (SEM), FESEM produces clearer, less electrostatically distorted images with high spatial resolution.

In FE-SEM, electrons are liberated from a field-emission source (FE gun) and accelerated in a high electrical field gradient. Within the high vacuum column, these so-called primary electrons are focused and deflected by
electronic lenses to produce a narrow scan beam that bombards the object. As a result, the "electron hail" secondary electrons are dislocated from each spot on the object. The smaller the angle of incidence of the electron beam is with respect to the sample surface and the higher a certain point is in the sample, the more secondary electrons can reach the detector and the lighter this dot will appear in the final image following electronic signal amplification and digitalisation. Also, the composition of the sample affects the number of deflected electrons too and thus on the 'grey value' of the corresponding pixel in the final image. Detection of the secondary electrons results in a kind of three-dimensional shadow-cased surface representation of the sample. To prevent that electrons that are not captured by the detector would hang like a cloud masking around the sample, thus masking the image, SEM samples are coated in advance by a very thin layer of conductive material, e.g. gold-palladium or carbon or platinum, to clear away superfluous electrons.

A schematic image of the beam pattern for SIGMA FE-SEM (Carl Zeiss, UK) is shown below in figure 2-7.
Figure 2-7. SIGMA FE-SEM and schematic image of the electron gun and beam pattern (Carl Zeiss UK, Ltd). The highlighted box showed the schematic structure of electron gun inside the SEM sample loading chamber.

FE-SEM was employed to determine the surface morphology of all samples used in this study. Each sample was coated using a platinum target to deposit
a 20 nm thin film layer using a Sputter Coater (Cressington, UK) under argon before image analysis using a SIGMA FE-SEM (Carl Zeiss, UK). High-resolution images were obtained from random locations using an accelerating voltage of 3-5 kV at different magnifications using Atlas 5 software (Carl Zeiss, UK).

2.2.3.3 Atomic force microscopy (AFM)

AFM is another commonly used technique by researchers in biology, chemistry and physics to produce high-resolution images of material surfaces. They are designed to measure local properties, such as height, roughness, friction, magnetism, with an AFM tip (20 nm in diameter) positioned at the end of a cantilever. It operates by measuring forces between a probe and the sample. Normally, the probe is a cantilever with a sharp tip, which is a 3~6µm tall pyramid with 15-40nm end radius. The lateral resolution of AFM is about 2~30nm due to the convolution, and the vertical resolution can be up to 0.1nm (Ando, 2013). Besides imaging and measuring, the AFM can also perform manipulations at the atomic scale. In force measurement, AFM can be used to measure the forces between the probe and the sample as a function of their mutual separation. The manipulations at atomic scale using AFM can be applied to perform force spectroscopy, to measure the mechanical properties of the samples, such as Young's modulus, a measure of stiffness (Neugirg et al., 2016). More advanced manipulation could be applied to samples using the forces between tip and sample to change the properties of the sample in a controlled manner. Examples of this include atomic manipulation, scanning probe lithography and local stimulation of cells.

Traditionally, most AFMs use a laser beam deflection system where a laser
is reflected from the back of the cantilever and on to a position-sensitive detector. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to Hooke’s law. Depending on the situation, forces that are measured in AFM include mechanical contact force, van der Waals forces, chemical bonding, electrostatic forces, and magnetic forces (Leite et al., 2012). AFM can be operated in some modes, depending on the application. In general, the most common modes are static (also called contact) mode and a dynamic (non-contact or “tapping”) mode where the cantilever is vibrated or oscillated at a given frequency. In contact mode, the tip is "dragged" across the surface of the sample, and the contours of the surface are measured either using the deflection of the cantilever directly or, more commonly, using the feedback signal required to keep the cantilever at a constant position. Dynamic contact mode, or more commonly known as tapping mode, the cantilever is driven to oscillate up and down at or near its resonance frequency (typically ~ 300 kHz). This oscillation is commonly achieved with a small piezo element in the cantilever holder. The amplitude of this oscillation usually varies from 20 nm to 100 nm. The frequency and amplitude of the driving signal are kept constant, leading to the constant amplitude of the cantilever oscillation as long as there is no drift or interaction with the surface. A schematic image of the two modes of AFM is shown below in figure 2-8.
Figure 2-8. Schematic image of the operating modes of the AFM as shown in A) contact mode – tip making continuous contact with the sample surface, and B) tapping mode – tip making intermittent contact with the sample surface.

In ambient conditions, most samples develop a liquid meniscus layer. The liquid meniscus layer would cause the tip to stick to the surface when keeping the probe tip close enough to the sample for short-range forces to become detectable. Tapping mode can bypass this problem easily. For this reason, tapping mode is the most frequently used AFM mode nowadays. AFM was performed at room temperature in PeakForce Tapping mode using a Dimension Icon AFM (Bruker Nano Surfaces, UK). Images were taken at 512 × 512 pixels with a PeakForce Tapping frequency of 1 kHz and amplitude of 5nm. Probe–sample contact time was 200μs for each cycle. Automatic gain control was used to improve the feedback for surface tracking. Surface roughness measurement was performed on all of the coated samples. Mean surface roughness (Ra) is defined as the arithmetic mean of the deviations from the centre plane and the root mean square (Rq) or deviation from the centre plane was calculated using...
Nanoscope Analysis v1.70 software (Bruker Nano Surfaces GmbH, Germany). Three random locations on each sample were subject to area, and line scans from three samples per condition (n=9 per condition in 3 repeats).

2.3 Results

2.3.1 Contact angle (θ°) and surface energy (γ) measurements

Surface chemical modification of the glass substrates was achieved by silanization reactions as revealed by alteration of θ° and γS, respectively. The untreated controls (UC), representing in the following result figures as '-OH', exhibited a θ of 65.5 ± 6.22°. In general, θ° increased significantly (P<0.001) after chemical modification. The θ° for TMPM, MPTS, APTS and CMTFS treated samples were 74.6 ± 4.54°, 78.5 ± 5.32°, 81.6 ± 5.47° and 86.0 ± 6.05°, respectively. The DCMDS treated samples produced a hydrophobic surface with the θ° = 101.7 ± 2.19°. The Owens-Wendt equation was applied to estimate the γS of untreated and treated samples. The γS for UC was 37.51 ± 1.25 mN/m. It was also found that the γS altered after each chemical modification step providing confidence that the materials had changed. The γS for TMPM, MPTS, APTS, CMTFS and DCMDS treated samples were 34.82 ± 0.95, 33.61 ± 0.75, 32.06 ± 0.56, 30.77 ± 0.49 and 24.55 ± 0.31 mN/m, respectively. The results indicated the surfaces of glass substrates were successfully modified. θ° and surface energy results on silanized samples and selected droplet images are shown below in Figure 2-9.
Figure 2-9. Contact angle (θ°) and surface energy (γ) results on silanized samples and selected droplet images. Upper left: θ results (n=9 per condition in 3 repeats); Upper right: γ results (n=9 per condition in 3 repeats); Lower: A. KRÜSS DSA 100 machine and EasyDrop DSA20E Drop Shape Analysis software; B ~ D. Selected droplet images showed the different modified surface varying from hydrophilic to hydrophobic.
We also found the $\theta^\circ$ for dip coated poly-SIBS samples was 68.6 ± 1.25°, while the $\theta^\circ$ for spin coated poly-SIBS samples at 6000, 3000 and 1000 rpm are 77.1 ± 2.34°, 99.2 ± 2.32° and 96.8 ± 1.76°, respectively. By altering the hard and soft segment chemistry giving rise to hydrophobic surface properties. The $\gamma_S$ for dip coated poly-SIBS sample, spin coated poly-SIBS samples at 6000, 3000 and 1000 rpm are 35.82 ± 0.72, 28.48 ± 0.39, 27.48 ± 0.36 and 34.18 ± 0.44 mN/m, respectively and lowering of surface energy consistent with hydrophobic properties. Similarly, these results showed surface property of treated glass substrates altered significantly (P<0.001) after we applied the poly-SIBS coating onto them, hinting the successful coating procedure performed. WCA and surface energy results on poly-SIBS coated samples are shown below in figure 2-10.
Figure 2-10. Contact angle (θ°) and surface energy (γ) results on poly-SIBS coated samples. Left: θ° (n=9 per condition in 3 repeats); Right: γ, results (n=9 per condition in 3 repeats).
2.3.2 Characterisation of Poly-SIBS coatings on glass substrates

2.3.2.1 FE-SEM image analysis

FE-SEM image analysis of representative silanized glass samples is showed in figure 2-11. All glass samples, before and after silanization treatment, demonstrate flat surfaces with some imperfections, and few identifiable features under both low and high magnification imaging as expected for clean samples.

![FE-SEM images of organosilanized glass substrates. Images A-E were captured at random locations of samples at x10,000 magnification; while the highlighted image was captured at x35,000 magnification.](image)

**Figure 2-11.** FE-SEM images of organosilanized glass substrates. Images A-E were captured at random locations of samples at x10,000 magnification; while the highlighted image was captured at x35,000 magnification.
The surface morphology and topography after coating with poly-SIBS was investigated to study the surface properties and topographical features as shown in figure 2-12. Compared to the untreated glass controls with a flat surface profile as shown previously with FE-SEM, substrates dip coated with poly-SIBS showed a densely packed configuration of micro and nanofeatures forming irregular nanopores and nano-pits approximately 200nm in diameter with protrusions on the edge of the nano-pits. This is a close-packed configuration followed by nanoporous structures as predicted earlier in our investigations. The interconnectivity, depth profile and permeability of the porous network creating by dip coating with poly-SIBS is relatively unknown at present and warrants future investigation. Moreover, spin coating of poly-SIBS at 6000 rpm samples show a uniform, random array of poly-SIBS nano-islands as a result of adhesion and phase separation with the appearance of polymer hard segment chemistry, presumably enriched with polystyrene. Samples with lower spinning speed (1000 rpm) showed similar poly-SIBS nano-island topographic features on the surfaces of negatively charged substrates. High magnification images show the distance in between of the poly-SIBS nano-islands gradually decreased with lower spin coating speed providing a level of control of the spatial arrangement of nanostructures.

For experimental reasons, we also tried applying the poly-SIBS coating onto other silanized samples. However, none of those samples managed to produce a uniform coating layer. Most of the coatings were observed torn, with large holes and peeling off from the substrate surface. This is expected as the mismatch of surface charges repel the coating layer from the substrate.
Figure 2-12. Representative FE-SEM images of poly-SIBS coated glass samples. Images A-D were captured at random locations of samples at x10,000 magnification; while the images underneath were matching images captured at x35,000 magnification. One of the nano-islands were highlighted in the added red circle in D.
2.3.2.2 AFM

AFM image analysis revealed similar findings to FE-SEM on represented samples as shown in figure 2-13. With AFM software analysis, we determined the dimensions and spacing of poly-SIBS nano-surface features. AFM analysis revealed that spin coating of poly-SIBS at 1000 rpm resulted in the appearance of poly-SIBS nano-islands on the surface, which had a close-packed array and shorter spacing, which were difficult to measure due to their size variation. Spin coating of poly-SIBS at 3000 rpm resulted in nano-islands being pulled further apart due to phase separation increasing the spacing of polymer segments on the surface, and AFM revealed the presence of poly-SIBS nano-islands with a radius of $126.2 \pm 11.0$ nm, a height of $23.7 \pm 4.3$ nm, and spacing of $209.8 \pm 66.4$ nm representing a shorter distance in between the nano-islands. Spin coating at 6000 rpm revealed nano-islands with a radius of $117.2 \pm 12.3$ nm, a height of $16.7 \pm 4.2$ nm and spacing of $658.8 \pm 59.0$ nm demonstrating that spin coating speed influenced poly-SIBS nano-island spacing and packing density. Dip coated substrates also showed irregular nano-pores and nano-pits with densely packed nanostructures.
Figure 2-13. AFM images of representative poly-SIBS coated glass samples. A. UC; B. Dip coated; C–E. Spin coating at 1000, 3000, and 6000 rpm. All scanning sizes were set at 25μm X 25μm, 3D images underneath matching 2D ones. The bright areas represented elevated features and protrusions.
Summary of AFM surface measurements for a different poly-SIBS dip and coated on CFMTS organosilanised glass substrates as shown in table 2-1.

Table 2-1. AFM measurements of surface features obtained with dip and spin coating procedures (n=9 per condition in 3 repeats).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Surface feature</th>
<th>Island Radius (nm)</th>
<th>Island Height (nm)</th>
<th>Spacing (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dip Coated</td>
<td>Nano-pits</td>
<td>Irregular nano-pores and nano-pits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 rpm</td>
<td>Nano-island</td>
<td>Closely packed nano-island</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3000 rpm</td>
<td>Nano-island 126.2 ± 11.0</td>
<td>23.7 ± 4.3</td>
<td>209.8 ± 66.4</td>
<td></td>
</tr>
<tr>
<td>6000 rpm</td>
<td>Nano-island 117.2 ± 12.3</td>
<td>16.7 ± 4.2</td>
<td>658.8 ± 59.0</td>
<td></td>
</tr>
</tbody>
</table>

Surface roughness analysis of untreated and treated samples was also measured and described using Ra and Rq values as shown in figure 2-14. Surface roughness, significantly higher than spin-coated ones (P<0.01).

![Figure 2-14. AFM surface roughness results of all samples (n=9 per condition in 3 repeats).](image)

There was no significant difference in both Ra and Rq with all the organosilane-treated glass substrates (P>0.05). However, with the poly-SIBS coated samples, we
noticed that the Ra increased from UC to dip coated samples with values of 0.2527 ± 0.03 nm to 13.09 ± 2.22 nm, which gradually decreased as the spin speed increased and consistent with spacing and packing density of poly-SIBS nano-islands. The Ra for spin coated samples with 1000, 3000 and 6000 rpm are 11.55 ± 2.312 nm, 8.725 ± 2.101 nm and 3.723 ± 0.4199 nm, respectively. The same trend was also reflected in the mean Rq value, which ranged from 0.4672 ± 0.06790 nm to 17.37 ± 2.557 nm and consistent with the packing and spacing of poly-SIBS nano-islands. UC had a flat surface with significant lower Ra and Rq than the coated surfaces (P<0.01), and the spin-coated samples was moderately rough, while the dip coated samples had the highest surface roughness and were consistent with FE-SEM and AFM image analysis.

2.4 Discussion

We have developed a simple coating procedure using commercially available organosilane precursors and poly-SIBS tri-block copolymers on model glass substrates. We used electrostatic surface coatings as a method to covalently attach the poly-SIBS coating on to the surface of treated model substrates. Although we did not test the surface chemistry in this study using x-ray photoelectron spectroscopy (XPS) to detect organosilane precursors molecules on the substrate following post-modification procedures, we did notice that the θ and the γS increased significantly after each treatment and silanization step (P<0.01). This suggests that the surface properties altered significantly before and after treatment, which implies that the chemical modification process was successful, and the covalently bound siloxy
derivatives were presented on the surface of the model substrate.

Similarly, we found the θ and the γ_s of samples also changed significantly after applying the poly-SIBS coating regardless of the different coating techniques (P<0.01). This also confirmed successful attachment of poly-SIBS coating layer on to the substrate’s surface on top of the CFMTS, ‘F’, treated substrates. This is further verified by the FE-SEM and AFM image analysis by direct visualisation of the coatings on the substrate compared with flat treated glass substrates.

The FE-SEM images revealed that we had produced consistent thin film layers on the treated substrate’s surfaces with each coating technique. We noticed that with 6000 rpm spin coating techniques, we produced a coating with uniform, random arrays of poly-SIBS nano-islands on the substrate surface as predicted in a hypothetical model explained. While dip coating technique produced coatings with irregular nanopores and nano-pits shown under FE-SEM on the surface of the substrate. Based on our original hypotheses, we presumed that the poly-SIBS nano-islands are hard polystyrene segments from the poly-SIBS layer. The adhesion created by the negatively charged surface created by spin coating had stretched its hard and soft segment chains causing it to re-distribute on the substrate surface as a result of phase separation exposing the polymer backbone and underlying chemistry. For the same assumption, with zero tension via dip coating, the hard and soft segment remained elastic and tightly bound together exposing hard polystyrene segments creating a densely packed configuration of poly-SIBS nanofeatures, with the formation of nanopores and nano-pits and was consistent with the changes observed in θ° and γ_s.
Substrates subjected to 1000 and 3000 rpm spin-coated samples showed similar nano-island topographic features on their surfaces under FE-SEM. We noticed that the distances in between of the nano-islands gradually decreased as spin speed decreased. Thus, providing further evidence that phase separation induced by adhesion on electrostatic coatings accurately predicted that higher the spin speed would create greater adhesion and tension followed by phase separation to stretch the polymer hard and soft segments to a greater extent. These observations were verified with FE-SEM data and consistent with AFM findings. For example, increasing the spin coating speed from 3000rpm to 6000rpm increased the average distance between the poly-SIBS nano-islands from around 200nm to proximately 650nm. The fact that our experiment revealed the spacing between the nano-islands increased as the spinning speed increase suggested that the soft segment chains remain elastic within a given range of parameters. We also found that poly-SIBS nano-islands had an average radius of around 100nm and height of around 20nm regardless of the spin coating speed applied. This approach provided confidence that we could achieve an economical method in producing thin film coatings with patterned nanofeatures using electrostatic coatings with poly-SIBS to control the spacing and packing density of nanofeatures.

In comparison with other methods such as top-down approaches using electron-beam lithography to create nanopatterns on materials are expensive, time-consuming, and can only create a single geometric pattern (El Feninat et al., 2002, Shabalovskaya et al., 2008). There is an unmet need for innovation in surface engineering strategies to create nanotopographical features on to biomaterials using more economical,
efficient methods, and our findings suggest that simple coating procedures are capable of producing low-cost alternative methods to influence cell-material interactions at the tissue implant interface.

The dense packing of the nano-feature structure has allowed us to create various nano-size pores on the coating surface and interconnecting porous system underneath. This can be further exploited as a potential vector for control drug delivery. Therapeutic agents, such as antibiotics, growth factors, chemotherapy agents and even steroids could be incorporated into the coating system using complex mixing techniques. By adding steroid into the coating for control release purpose could enable the coating for anti-inflammation effect in the implanting location, to prevent in situ scar formation. We had explored into this anti-inflammatory functionalization of our coating system and result included in appendix 2.

Moreover, the versatility of poly-SIBS chemistry can be used to design and manufacture surfaces with selected chemical and mechanical cues that influence the cellular response and to promote wound healing that occurs during implant and integration within the host tissues. We have successfully demonstrated the manipulation of packing density using this coating technique can be used to create a variety of nanosurface features. Therefore, its potential of being applied on to various neurosurgical biomaterials and implantable devices to improve the cell-to-material interactions would require further studies to explore their overall biocompatibility of poly-SIBS coating platform when in contact with the blood and following in vitro and in vivo assessments in the following chapters.
Chapter 3  Haemocompatibility study of the nano-surface coating

Haemocompatibility tests are a major part of biocompatibility testing and is the evaluation of interactions of foreign materials with blood to explore possible adverse effects, which may represent risks to health arising from the exposure to blood cells and proteins. In arterial blood, hemodynamic flow at high pressure causes shear stress conditions and understanding how platelet interactions at the tissue-implant interface are critical for blood coagulation events, and maintenance of haemostasis (Chong et al., 2010). This chapter investigates the interactions of poly-SIBS nano-surface coatings with blood components.

3.1 Introduction

All surgical implants during implantation come into intimate contact with the blood. Whole blood in the circulation contains a variety of cells and components including red blood cells (RBCs), white blood cells (WBCs), platelets, and plasma, which contains over 300 types of proteins, e.g. albumin, fibrinogen, fibronectin, immunoglobulins, enzymes, clotting factors, hormones, and many others. The initial reaction from of the body toward surgical implants is the rapid absorption of plasma proteins in < 1 sec followed by the interaction of platelets, which adhere, activate and aggregate and interact with components of the blood when exposed to the surface of a medical device.
3.1.1 Events at the tissue-implant interface

Coronary artery stents and ICS are one of the most commonly used and widely known chronic blood contacting devices for cardiovascular and neurovascular applications. The inner surface of stents makes direct contact with active components of circulating blood, while the outer surface is in direct contact with the artery wall and microvascular tissues. The outer surface influences the integrity and function of the vasculature when in contact with a damaged endothelium. Here, we describe the events that take place when a biomaterial or foreign body is placed in direct contact with the blood.

3.1.2 Protein absorption and blood coagulation

The exposure of materials to physiological fluids composed of water, electrolytes and proteins in the blood results in a preferential accumulation of adsorbed proteins at the tissue-implant interface (Xu et al., 2014). Protein adsorption is governed by the physicochemical properties of biomaterial surface such as the surface chemistry (energy, charge), wettability and topography at the micro- and nanometer scale, which is known to influence the structure and composition of electrolytes, as well as, proteins adjacent to the interfacial region to create a conditioned interface in which the cells respond (Zhang et al., 2011). Surface chemistry is the dominant factor and proteins with polar, non-polar, and charged amino acids adhered to surfaces and based on their size and charge undergo dynamic rearrangements described by the Vroman effect.
Immediately following protein adsorption and contact with the implant surface (or after injury), a series of biochemical events are initiated via contact activation (intrinsic system) or through the release of tissue factor (TF) from damaged cells at the site of injury (extrinsic pathway) to activate an enzymatic cascade as shown in figure 3-1 involving many enzymes and co-factors (Nick Pace et al., 2014).

**Figure 3-1.** The intrinsic and extrinsic blood coagulation cascade: Left – the intrinsic pathway; Right – the extrinsic pathway.

The intrinsic pathway is independent of injury. Adsorbed surface proteins form a complex composed of collagen, HMWK, prekallikrein, and factor XII (Hageman factor), which converts prekallikrein in kallikrein. Inactive enzyme precursors (and clotting factors) change conformation and are converted into active enzymes via a biochemical cascade, which, with the addition of certain co-factors. Factor Xa causes the cleavage of prothrombin to thrombin, which in turn converts fibrinogen into fibrin and results in platelet adhesion, aggregation and activation to form a stable blood clot (Gorbet and Sefton, 2004).
3.1.3 Haemostasis and thrombogenicity

Under normal physiological conditions, a confluent layer of endothelial cells (ECs) and healthy endothelium lining the blood vessels actively prevents thrombus formation by presentation of heparin sulphate or the release of anti-coagulants like prostacyclin (PGI2) and nitric oxide (NO), and clotting factors to prevent their overexposure in the blood and maintain haemostasis (Rao and Chandy, 1999). It is important to note that both intrinsic and extrinsic pathways converge during the formation of the prothrombinase complex (Factor X), leading to the activation of platelets by Ca$$^{++}$$ and the generation of the thrombin. Thrombin converts fibrinogen into fibrin and activates platelet surface receptors. Platelets change morphology, activate and aggregate by release growth factors and cytokines to enhance the coagulation cascade, and strengthen the haemostatic plug as shown in figure 3-2 (Gorbet and Sefton, 2004).

![Figure 3-2](image_url)

**Figure 3-2.** The effects of the intrinsic pathway platelet adhesion, activation and aggregation.
Platelets are abundant components of the blood with a disc-like appearance produced from megakaryocytes (e.g. bone marrow cells) as shown in figure 3-3.

![Figure 3-3. Structure of a platelet.](image)

In a non-activated state, platelets have a diameter of 3 µm, and a volume of 1.0 x 10-9 mm3, with an average concentration of 250 x 109 cells per litre of blood (Pocock et al., 1999). Their outer surface contains integrin receptors (e.g. glycoprotein IIb/IIIa), which bind to fibrin and play a pivotal role in platelet adhesion, and aggregation on artificial surfaces, as well as, mediating their activation. Three types of granules are present in the cytoplasm of platelets, which include: 1) α-granules, which contain platelet factor 4 (PF4), β-thromboglobulin, and plasma proteins such as albumin, fibronectin, fibrinogen, and coagulation factors V and VIII, 2) high-density granules containing calcium ions (Ca2+), adenosine diphosphate (ADP), and serotonin; and 3) granules associated with lysosomes containing enzymes such as acid hydrolases. When activated, platelets, combined with the fibrin to develop into a porous mesh to capture circulating RBCs to form a clot as described previously. If the thrombolysis pathway is not activated to dissolve the blood clot, WBCs and fibroblasts are mobilised
and grow into the blood clot forming a white thrombus. This is a dangerous event for patients undergoing stenting as it often results in stent failure and can cause neurological dysfunction (Anderson et al., 2008).

Thrombogenicity refers to the ability of a material to produce a thrombus (clot) and is defined as the ability to induce or promote the formation of thromboemboli (Gorbet and Sefton, 2004). It is known that low rates of thrombosis can be tolerated since the fibrinolytic system (under normal circumstances) exists to break down fibrin during clot lysis. The criterion for anti-thrombogenic materials was originally thought to represent long clotting times followed by minimal platelet adhesion. However, this has been extended to define desirable parameters when considering anti-thrombogenicity: (1) A low thrombin production rate constant (kp < $10^{-4}$ cm s$^{-1}$), (2) low platelet consumption and activation, (3) some platelet adhesion, and (4) low complement and leukocyte activation (Williams 1999). Such criteria highlight essential parameters that identify a strategy for producing anti-thrombogenic biomaterials. However, there is a shortage of direct and simple laboratory-based test methods to measure and evaluate the thrombogenic behaviour of biomaterials used for cardiovascular applications.

3.1.4 Influence of surface topography

The vast majority of surfaces can be considered as highly thrombogenic when there is an abundance of adsorbed plasma proteins, which serve as a platform on which platelets adhere, and the activation of the complement cascade. It is known that surface topography on the nanometre scale can influence protein absorption, cell
adhesion, proliferation and orientation (Dalby et al., 2002, Dalby et al., 2004, Curtis et al., 2006, Dalby et al., 2014). For example, differences in immunoglobulin G (IgG) absorption occurs as a result of varying the surface nanotopography (Tan et al., 2008). Similarly, collagen type I also displays different absorption profiles on surfaces with different nanostructures as a result of colloidal lithography (Gullekson et al., 2011).

Similarly, platelet behaviour on surfaces is dependent on surface characteristics including surface chemistry (energy, wettability, charge) and chemical composition, and surface topography. Recent evidence suggests that hydrophobic surfaces such as fluorinated carbons (-CF$_3$) are less thrombogenic compared to hydrophilic substrates such as hydroxyl functional groups (-OH) and thiol (-SH) groups with anti-thrombogenicity being defined as a situation whereby the materials elicit a minimal thrombogenic response as described previously (Silver et al., 1999). However, other studies have also shown that hydrophilic surface groups show reduced platelet and fibrinogen adhesion (Poussard et al., 2005, Ruckenstein and Gourisankar, 1984, Bhat, 2002, Hamlin et al., 1978). Surface roughness and topography also plays a role in the assessment of thrombogenicity. Rough surfaces are generally considered more thrombogenic than smooth surfaces (DePalma et al., 1972, Goldberg et al., 1981, Hecker and Scandrett, 1985). For example, coronary stents require a highly polished (and hence smooth) hydrophilic metallic surface. Studies have shown that platelets have a net negatively-charged surface (Karagkiozaki et al., 2008). Therefore, it was thought that negatively-charged surfaces inhibit platelet adhesion and aggregation (Jung et al., 1982). However, hemodynamic flow and shear stress conditions in
turbulent blood flow up-regulates pro-thrombogenic factors causing a high turnover of platelet consumption (Yoshizumi et al., 2003).

Based on these considerations when materials make intimate contact with the blood, surface modifications of both chemical and topographical cues can influence the interaction of adsorbed proteins on its surface and platelets to enhance their hemocompatibility and overall thrombogenicity. Therefore, this chapter aims to evaluate the hemocompatibility of poly-SIBS nanosurface coatings and to test their interactions with components of the blood (e.g. platelets) in plasma and whole blood when all of the components are present.

The coagulation activation process is different in vitro and in vivo, as during in vitro process we mainly focus on the reaction of active blood components towards the biomaterial itself; while the in vivo process includes the combination reaction of multiple systems such as local vasculature, in situ endothelium function, etc. In this chapter, our experiment design focus on the in vitro aspect of the activation of coagulation by the biomaterial itself, and we will explore the in vivo aspect of contact activation in the in vivo pilot study, detailed described in chapter 7.

3.2 Material and methods

All chemicals were obtained from Sigma-Aldrich unless otherwise stated and all poly-SIBS nano-surface coatings were manufactured according to the same protocol.
as mentioned in chapter 2, section 2.2.1–2.2.2.

3.2.1 Citrated whole blood collection

Citrated whole blood was collected from healthy volunteers. No anti-platelet or anticoagulants were consumed by the donors within two weeks before each blood collection. Blood was obtained from puncturing the donor’s cephalic vein aseptically with a 21G butterfly needle and collected with BD Vacutainer blood collection tubes containing 0.109M buffered sodium citrate. Platelet-rich plasma (PRP) was obtained from centrifuging citrated blood at 200g for 30 mins (figure 3-4). The top yellowish plasma layer was collected and designated as PRP. The PRP was then diluted in sterilised phosphate buffer solution (PBS) before counting with a hemocytometer for three times to adjust the concentration to of 1x10⁶ platelets/ml. All blood compatibility tests were conducted in an incubator at 37°C, 5% CO₂ and 95% humidified air.

Figure 3-4. Before and after centrifugation of citrated whole blood to obtain platelet-rich plasma (PRP). A. Donated citrated whole blood layered on top of Histopaque™ before centrifugation; B. plasma components after centrifugation; and C. digital image showing the isolation of PRP and platelet poor plasma (PPP) with Lymphoprep™ to remove residual WBCs.
3.2.2 Blood compatibility tests

ePTFE was selected as a positive polymeric control in all blood compatibility tests as this is one of the few commercially available biomaterials used in blood contacting devices such as stent grafts, and small diameter bypass grafts (> 6mm in diameter), as well as, a covered ICS (Fang et al., 2017, Liu et al., 2017b, Tang and Qi, 2017). In this study, ePTFE sheets were purchased from Goodfells (Goodfells Corporation, UK) and cut into circular discs (1cm in diameter) using a Speedy 100R laser cutter with automated computer software as shown in figure 3-5.

![Figure 3-5](image.png)

Figure 3-5. The Speedy 100R laser cutter, A and B the platform in which the samples are manufactured.

3.2.2.1 Blood compatibility index (BCI)

The whole blood reaction was measured by calculating the BCI. Samples were placed into 24-well tissue culture polystyrene plates (TCPs) and equilibrated as described previously (n=12 per condition in 4 repeats). 12.5μl 0.2M CaCl2 was added on the surface of each sample, following by 200 μl of fresh citrated whole blood, and incubated at 37°C, 5% CO₂ and 95% humidified air for 30min. 1ml of deionised water
(dH₂O) was added to each well and allowed to incubate in the same condition for a further 10 mins to allow clot lysis of free RBCs. Retrieved supernatants were diluted with dH₂O 1:50 for UV-visible spectroscopy. The ELx800 Absorbance Microplate Reader machine (BioTek, UK) and Gen5 Reader Control (Version: 2.00.18) software were used for spectral analysis. Absorbance was set at 562nm to measure the extent of lysed haemoglobin. The BCI was calculated as follows in equation (3-1):

\[
\text{BCI (\%) } = \frac{\text{Absorbance of sample}}{\text{Absorbance of baseline}} \times 100\% \quad \text{Eq. 3-1}
\]

Native citrated whole blood was used as a baseline in all experiments. BCI was inversely related to the thrombogenicity of the materials (Lemm et al., 1980).

### 3.2.2.2 Platelet adhesion index (PAI)

All samples were was placed in 24-well TCPs and equilibrated overnight (18 h) in sterilised PBS in an incubator at 37ºC, 5% CO₂ and 95% humidified air (n=9 per condition in 3 repeats). Then, PBS was placed into each well and replaced with 1ml diluted PRP (1x10⁶ platelets/ml) and incubated for 60 mins, and the supernatant in each well was transferred into a sterile eppendorf tube before being counted. The degree of platelet adhesion was measured according to Salzman’s platelet retention index as follows in equation (3-2):

\[
\text{PAI (\%) } = \frac{\text{PLTi} - \text{PLTe}}{\text{PLTi}} \times 100\% \quad \text{Eq. 3-2}
\]

PLTi means the number of platelets before adhesion, and PLTe means the platelets remaining in the supernatant after incubation.

After the supernatant was removed, the samples were retained and fixed for FE-
SEM analysis. Samples were washed with PBS and treated with 4% glutaraldehyde for 30 mins followed by a degrading ethanol series (e.g. 20, 40, 60, 80, 100% v/v), with a duration of 30mins for each step. Samples were air-dried in the laminar flow hood overnight (18h) before imaging (Ko and Cooper, 1993). FE-SEM image analysis was carried out following the protocol mentioned previously in Chapter 2, section 2.2.3.2.

3.2.2.3 Thromboelastography (TEG®)

Whole blood coagulation monitoring was measured via TEG®. The technique can measure all aspects of blood coagulation from protein adsorption to initiate contact, and enzymatic cascade through to clot build up due to platelets and strength of the blood clot through to activation of fibrinolysis and clot breakdown (Peng, 2010, Donahue and Otto, 2005). TEG® is one of the most widely used tools in clinical practice for monitoring haemostasis during surgical operations as a point of care device. A thin wire probe is used to measure, which the clot forms around. The speed and strength of clot formation are measured in various ways, typically by a computer. The speed at which the sample coagulates depends on the activity of the plasma coagulation system, platelet function, fibrinolysis and other factors which can be affected by genetics, illness, environment and medications. The patterns of changes in strength and elasticity in the clot provide information about how well the blood can perform hemostasis, and how well or poorly different factors are contributing to clot formation.

Samples were cut using a standardised mould cutter and gently fixed into the TEG® cups, which were then mounted onto the TEG 5000® Thrombelastograph Haemostasis
Analyzer and calibrated at 37°C (n=9 per condition in 3 repeats). For each set of analysis, 20μL 0.2M calcium chloride was added to each cup to initiate blood coagulation followed by 340μL citrated whole blood.

The following parameters were generated and recorded during the process (figure 3-6): 1) Reaction time (R): the latency period from the first contact until fibrin formation is initiated due to the enzymatic response; Clotting time (K): time that taken for the clot to reach 20 mm in strength; Alpha angle (α): measures the rapidity of fibrin build-up, cross-linking and clot strengthening, and Maximum Amplitude (MA): the maximum dynamic properties of overall clot strength representing platelet GPIIb/IIIa receptors bound to fibrin (~20%) and contribution of platelets (~80%) (Peng, 2010).

**Figure 3-6.** Standardised TEG® trace displaying the key parameters used to measure haemostasis such as R time, K time, α-angle, and MA leading to clot lysis.

Besides the four major parameters mentioned above, some may be calculated to
generate more information on such as the thermogenesis progress (TMA) which is defined as the time to reach the maximum amplitude. Thrombodynamic potential index (TPI) was also calculated as follows in equation (3-3):

$$\text{TPI} = \frac{\text{MA} \times 100 \times (100 - \text{MA})}{2 \times K}$$

Eq. 3-3

The overall clotting Index (CI) was also calculated as follows in equation (3-4):

$$\text{CI} = -0.6516R - 0.3772K + 0.1224\text{MA} + 0.0759\alpha - 7.7922$$

Eq. 3-4

Thrombolysis effects were also measured using LY30 and LY60. They were defined as the percentage decreases in amplitude at 30 and 60 mins post-MA.

TEG® provides information on aspects of blood coagulation and the key parameters, as well as the normal ranges in citrated and native blood, are defined and shown in table 3-1 in the following (Peng, 2010, Shankarraman et al., 2012).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R (min)</strong></td>
<td>R (reaction) time represents the period taken from the start of the test to initial fibrin formation by enzymes and is a representation of standard coagulation studies, and is dependent on intrinsic co-factors and inhibitor activity.</td>
<td>Native: 12.1-26.5</td>
</tr>
<tr>
<td><strong>K (min)</strong></td>
<td>K time is the period to achieve a certain level of clot strength (amplitude = 20 mm) and is dependent on the activity of clotting factors, fibrinogen and platelets.</td>
<td>Native: 3.2-12.8</td>
</tr>
</tbody>
</table>
**α-angle (°)** Measures the rapidity of fibrin build-up and cross-linking to assess the rate of clot formation. Plasma components reside on the platelet surface also contribute to α-angle. Both K and α-angle measure the speed of clot formation.

<table>
<thead>
<tr>
<th></th>
<th>Measures</th>
<th>Range 1</th>
<th>Range 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-angle</td>
<td>Measures the rapidity of fibrin build-up and cross-linking to assess the rate of clot formation.</td>
<td>13.6-46.4</td>
<td>22.0-58.0</td>
</tr>
</tbody>
</table>

**MA (mm)** Maximum amplitude measures the strength of the clot as a measure of the dynamic properties of fibrin and platelet binding via glycoprotein IIb/IIIa receptors. This represents the ultimate strength of the fibrin clot and correlates with platelet function (80% platelets, 20% fibrinogen).

<table>
<thead>
<tr>
<th></th>
<th>Measures</th>
<th>Range 1</th>
<th>Range 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA (mm)</td>
<td>Maximum amplitude measures the strength of the clot as a measure of the dynamic properties of fibrin and platelet binding via glycoprotein IIb/IIIa receptors. This represents the ultimate strength of the fibrin clot and correlates with platelet function (80% platelets, 20% fibrinogen).</td>
<td>41.8-63.6</td>
<td>44.4-63.6</td>
</tr>
</tbody>
</table>

### 3.2.3 Statistical analysis

Parametric data were expressed as mean and standard deviation. SPSS 21.0 (IBM) was used to perform one-way ANOVA with post-hoc Bonferroni’s test for analysis of statistical significance between test groups. *P*-values <0.05 were considered statistically significant when compared with controls.

### 3.3 Results

#### 3.3.1 BCI

The BCI results of all organosilaned and poly-SIBS nano-coated substrates are shown in figure 3-7.
Figure 3-7. BCI of all organosilaned and poly-SIBS nano-coated substrates (n=12 per condition in 4 repeats).

The ‘-OH’ and 6000 rpm spin coated poly-SIBS samples showed significantly higher BCI values comparing to all other samples (P < 0.05), while the ePTFE controls showed the lowest BCI values comparing to all other samples (P < 0.05). We knew from the previous chapter that ePTFE has a strong hydrophobic surface with extremely low surface energy, that surface could have reacted strongly with the RBC membrane protein and causing it to rupture, resulting in the low BCI.

3.3.2 PAI

The PAI results of all organosilaned and poly-SIBS nano-coated substrates are
shown in figure 3-8.

![Bar chart showing PAI values for different conditions.](chart.png)

**Figure 3-8.** PAI of all organosilanised and poly-SIBS nano-coated substrates (n=9 per condition in 3 repeats).

All organosilanised samples, as well as, ePTFE controls showed increased PAI values, exceeding the suggested range of general biomaterials used for implantable devices. Among them, ePTFE showed the highest PAI values. The 6000 rpm spin-coated samples showed a significant decrease in PAI values comparing to all other samples (P < 0.05), and lower than the suggested range for biomaterials used for implantable devices. FE-SEM images of all samples after platelet adhesion tests are shown in figure 3-9 and 3-10 to highlight platelet morphology on each of the test substrates.
Figure 3-9. FE-SEM images of organosilanised samples and on untreated glass control after platelet adhesion tests.
Figure 3-10. FE-SEM images of plates on poly-SIBS nanosurface coated samples and using collagen type 1 and ePTFE as positive controls.
It could be seen that FE-SEM images of all treated and coated samples are consistent with the PAI value obtained in this study. All organosilanised samples showed a large amount of platelets adhered on their surfaces. Higher magnification images show platelets with different morphologies such as rounded, spread or dendritic phenotypes, or extensively spreading with smooth edges. Moreover, we can see many platelets adhered and overlaying each other trying to form a more complex structure on the surface.

The UC and the collagen type 1 control show a similar densely packed platelet distribution. The higher magnification images suggest that the platelets are mostly spreading with dendritic shaped morphology. In the ePTFE controls, we can see an extensive amount of platelets adhered on the surface and are all extensively spreading with smooth edges observed in the higher magnification images. All platelets on ePTFE controls were adhered and overlaying over each other forming a web-like scaffold structure and dense mesh of adhered and activated platelets.

On the poly-SIBS nanosurface coating, we observed very few platelets adhered to the surface compared with all other samples. The platelets were mostly rounded with some spreading with dendritic shaped morphology under higher magnification images. On the background of the dip coated samples, we observed poly-SIBS nano-surface features forming irregular nanopores and pits, which are consistent with the topographic findings in chapter 2, section
2.3.2.1. While on the poly-SIBS coated samples set at different speeds, we also noted the poly-SIBS nano-island surface features distribute further away from each other as the spin speed increased, and was also consistent with previous findings. However, the morphology of those poly-SIBS nano-surface features was only just about barely visible (if any) at this magnification as it is at the limit of resolution of the FE-SEM.

3.3.3 TEG® analysis of organosilanised and pol-SIBS nanosurface coatings

All sample groups tested for TEG® analysis generated a typical cigar-shaped profile. The TEG® results of all samples were shown in figure 3-11.
Figure 3-11. TEG results of all samples (n=9 per condition in 3 repeats).
R values (figure 3-11, upper left) for most of the organosilanised glass substrate, as well as, ePTFE controls were shortened compared to normal reference values (9~27 mins). All of the poly-SIBS nanosurface coatings, as well as, plain glass controls (-OH), and fluorinated (-F) treated glass demonstrated normal R values. Spin-coated samples using poly-SIBS at 6000 rpm, and 3000 rpm, as well as, -F treated glass show significantly prolonged R time values compared to all other samples (P < 0.05). Among them, we observed that the 3000 rpm spin coated sample had the longest R time than all other samples.

K values (figure 3-11, upper middle) on all of the samples fell within the normal range (2~9 mins). Among them, 6000 rpm spin poly-SIBS coated substrates, and -F treated glass samples showed significant prolonged K time values comparing to all other samples (P < 0.05).

We found that α-angle values (figure 3-11, upper right) for most of the samples, as well as, ePTFE controls fell into the normal range (22~58°). The -CH₃ treated glass samples showed increased α-angle values exceeding the normal range. It also had the highest α-angle values among all other samples. However, the difference was not significant compared to samples with higher α-angle values (P > 0.05). We also observed that the 6000 rpm poly-SIBS spin coated, and -F treated glass substrates showed a significant decrease in α values compared to all other samples (P < 0.05).

MA values (figure 3-11, middle left) for most samples fall into the normal
range (44~64 mm) except for 3000 rpm poly-SIBS spin-coated samples and ePTFE controls, which showed increased MA values exceeding the normal range. There were not any significant differences in MA values among all samples (P > 0.05).

The -\(\text{CH}_2\text{O}=\text{COCH}_3\) and -\(\text{CH}_3\) treated samples, poly-SIBS dip coated and 3000rpm spin-coated samples, and ePTFE controls show increased TPI values (figure 3-11, central), exceeding the normal range (3~20 sec\(^{-1}\)). Among them, ePTFE showed the highest TPI value. The rest of the samples displayed TPI values, which falls into the normal range. We also observed that 6000 rpm spin-coated samples, and -F treated glass substrates show a significant decrease in TPI values compared to all other samples (P < 0.05).

The -\(\text{CH}_2\text{O}=\text{COCH}_3\) and -\(\text{CH}_3\) treated samples, as well as, ePTFE controls showed increased CI values (figure 3-11, middle right), exceeding the normal range (-3~3). Among them, ePTFE showed the highest CI value. Poly-SIBS coated at 6000 rpm samples, and -F treated glass substrates show a significant decrease in CI values compared to all other samples (P < 0.05), falling slightly below the normal lower range. The rest of the sample displayed CI values, which fell within the normal range.

There was no normal reference range for TMA values (figure 3-11, lower left). -F treated samples demonstrated the highest TMA when compared to all other samples, and 3000rpm spin coated substrates show a significant increase in TMA values than all other samples (P < 0.05).
Fibrinolysis and LY30 results (figure 3-11, lower middle) suggest that there was no thrombolysis on all samples after 30 mins of achieving the maximum amplitude (MA). This is a normal phenomenon according to suggested reference values (0~8%). It is unlikely that 8% of thrombolysis effects can be expected at this point. LY60 results (figure 3-11, lower right), according to the suggested reference values (0~15%) was less than 15% of thrombolysis effects can be expected at this point. The untreated glass controls (-OH) -F treated glass, and the 3000rpm spin coated poly-SIBS samples demonstrate significantly increased LY60 values compared to all other samples (P < 0.05). Among them, untreated glass controls showed the highest percentage of thrombolysis comparing to all other samples. However, the results also suggest that there were no thrombolysis effects observed on -CH₃ treated samples, as well as, ePTFE controls after 60 mins of achieving MA.

3.4 Discussion

For all endovascular devices, the in vivo contact activation process is often complicated by various factors besides contact activation of active blood components. The regional vascular structure could interfere with the coagulation process by affecting the hemodynamic of local circulation, for example, turbulence flow is one of the known factors that cause emboli to build up in atrial walls. Secondly, in situ endothelial function also increase the risk of coagulation activation by defecting the intrinsic pathway, this is also important
as most endovascular devices tend to damage local ECs. Finally, WBC would play a more prominent role during the *in vivo* coagulation as they would not only complete the coagulation pathway by secreting various of cytokines and coagulation-related factors, they would also recruit and mobilizing more WBCs to the site to clear out and breakdown foreign bodies, magnifying the effect of cellular signal pathway, as well as taking active role in the wound healing process, all these effects we won’t be able to duplicate in our *in vitro* experiments, but would be explored into indirectly in our *in vivo* pilot study in chapter 7.

### 3.4.1 BCI

BCI is an index measuring hemolysis and the consumption of RBCs after the biomaterials have made contact with whole blood. After contacting whole blood, the extrinsic coagulation pathway is activated, and platelets start to adhere on the surface of the biomaterial. If platelets become activated they release PF-4 granules to initiate platelet aggregation and bind to fibrin to form a mesh-like network forming a stable blood clot. RBCs and WBCs are also captured and trapped inside to blood clot. Therefore, the lower the residual RBC levels suggest that more extensive clot formation as well as low efficiency of thrombolysis to free trapped RBCs. The BCI is measuring the combined effects of both thrombosis and thrombolysis aspects of the materials. Therefore, the higher the BCI, fewer RBCs were consumed after contact releasing less
haemoglobin, and less prone to induce thrombosis, and vice versa.

In our studies, we found that ePTFE films had significantly lower BCI compared to all samples, and suggests that ePTFE is more prone to induce thrombosis, which is in agreement with TEG® and PAI results. Untreated plain glass controls and poly-SIBS spin coated at 6000 rpm show a significantly higher BCI value when compared to all other samples, which suggests that they show anti-thrombogenic like properties.

3.4.2 PAI

PAI is another parameter to describe how platelet adhered, activate and aggregate on the material surface when in contact with platelets in the blood. When the blood makes intimate contact with the implanted biomaterial platelet adhere to the material, and depending on the level of surface interactions, they change morphology and become activated through the blood coagulation cascade. Depending on the level of activation more platelets are recruited, and begin to aggregate to form a fibrin mesh-like platelet plug resulting in a blood clot. In about 6~8 hours after the blood clot has formed if the thrombolytic pathways have not been activated, fibroblasts grow in and harden the clots to form a white blood clot, which are irreversible clots, therefore in the clinical situation thrombolysis therapy would no longer be an option. In this study, we found the poly-SIBS nanosurface coating, especially the 6000 rpm spin-coated samples show significantly lower PAI values than all of the other samples tested.
in this study. This suggested that the Poly-SIBS nanosurface coatings show reduced platelet adhesion when compared than all the other samples and controls. Collagen type 1 is known to act as a positive control and attract and activate platelets (Farndale, 2006). Whenever the integrity of a diseased blood vessel is compromised, the endothelial cell (EC) layer exposes collagen within the vessel wall to initiate the intrinsic coagulation cascade in response to injury to release tissue factor to form small blood clots to prevent blood loss (Roberts et al., 2004).

At rest, platelets can be described as non-adhesive or 'non-sticky,' however, when activated (as seen on highly thrombogenic materials such as ePTFE), are more susceptible to adhere on to foreign body surfaces. Platelet phenotype and change in morphology usually occurs after initial adhesion and result in an early dendritic or platelet spreading phase. This continuous change in shape and switch in morphology can be attributed to dynamic changes in the platelet cytoskeleton, as described previously. A subsequent degranulation and release insoluble factors (e.g. PF-4) associated with platelet activation result in platelet aggregation, which is a largely irreversible process (Rao and Chandy, 1999). However, it is also important to note that platelet adhesion does not necessarily lead to activation, and non-activated platelets can form a passive layer of cells around the prosthetic surface (Ratner et al., 2004). Nevertheless, the entire process is known as surface-induced platelet activation and is an integral part of the thromboembolic phenomenon, so often seen in blood-contacting devices
and related applications (Rao and Chandy, 1999). FE-SEM image analysis revealed the difference in platelet morphology when adhered to organosilanised and poly-SIBS nanosurface coatings. The stages of platelet activation can be determined via their phenotypic appearance as shown by Cooper’s platelet index in figure 3-12 (Ko and Cooper, 1993).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Type</th>
<th>Morphology</th>
<th>Morphology description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Round</td>
<td><img src="image1" alt="Image" /></td>
<td>Round or discoid, with no pseudopodia present</td>
</tr>
<tr>
<td>II</td>
<td>Dendritic</td>
<td><img src="image2" alt="Image" /></td>
<td>Rounded with early pseudopodia formation. No flattening evident.</td>
</tr>
<tr>
<td>III</td>
<td>Spread-dendritic</td>
<td><img src="image3" alt="Image" /></td>
<td>Intermediate pseudopodia, one or more pseudopodia flattened, hyaloplasms not spread between pseudopodia</td>
</tr>
<tr>
<td>IV</td>
<td>Spreading</td>
<td><img src="image4" alt="Image" /></td>
<td>Prominent pseudopodial formation, hyaloplasms spreading.</td>
</tr>
<tr>
<td>V</td>
<td>Fully-Spread</td>
<td><img src="image5" alt="Image" /></td>
<td>Hyaloplasms fully spread, no distinct pseudopodia. Central bodies have flattened into mounds or disappeared entirely</td>
</tr>
</tbody>
</table>

**Figure 3-12.** Cooper’s platelet index highlighting five key stages of platelet morphology upon contact with biomaterials.

It can be seen that on the FE-SEM images of platelets on poly-SIBS nanosurface coatings display very few platelets with a spread and dendritic shaped phenotype resembling the morphology of stage II activated platelets. While on all the organosilanised samples, as well as, untreated and collagen treated controls, we noticed platelets at various stages of development ranging from stage I to V. On ePTFE controls, we see an increased amount of adhered and activated platelets mostly at the stage V level of activation, and form a dense network aggregated platelets. It was apparent that on the poly-SIBS nanosurface coatings did not result in widespread platelet adhesion under the
same conditions or when to compare to all other samples.

3.4.3 TEG analysis®

TEG was one of the most commonly used tests to measure in vitro contact activation for blood coagulation, it measured and quantified the entire coagulation process from reaction at first contact to initiation of coagulation to the process of clot building up and finally to the thrombolysis pathway being initiated. It is still commonly being used in clinical practice, especially in surgery and during general anaesthesia, as monitoring of patients’ coagulation status. For endovascular devices, when they are freshly implanted into active circulation, they are expected to be challenged by active blood component continuously until they achieve local tissue enveloping. In physiology, all foreign body surface would activate coagulation to certain extents, where proteins would start to accumulate on the device surface, and clot start to build up, but at the same time, thrombolysis pathway would be inactive as well. By achieving the balance between the thrombosis and thrombolysis in the acute implanting stage, is the key to avoid acute thrombus and clot occlusion. This is why the measuring of how these biomaterials affect the coagulation pathway and the thrombolysis pathway is important to prove the suitability of using these biomaterials to improve the thrombogenicity of endovascular devices.

TEG® was used to analyse the organosilanised and poly-SIBS treated samples in citrated whole blood with all of the blood components present. Four values that represent clot formation was determined by this test known as reaction time, K value, α-angle and maximum amplitude (MA). R-value represents the time until the first evidence of a clot is detected as a result of contact activation and enzymatic reactions. The K-value is the time from the end of R until the clot reaches 20mm and is a measure of the speed of clot formation. The α-angle is the tangent of the curve made as K and offers complimentary information. MA values are a reflection of overall clot strength,
and TMA is time to reach maximum amplitude, which is a parameter used to measure how fast the blood clot develops. TPI and CI are two values determined using mathematical formulas. The TPI suggests the speed of blood clot formation, which usually can be associated with a hypercoagulable state, while CI is the overall assessment of blood coagulation. TEG® also measures clot lysis, which is reported as both the estimated per cent lysis (EPL) and the percentage of the clot which has lysed after 30 minutes (LY 30,%). Although a normal EPL can be as high as 15% and a normal LY 30 can have as high as 8%, some studies in the trauma population suggest that a LY30 greater than 3% is associated with risk of haemorrhage (Olsson et al., 2000, Amiji and Park, 1993).

In this study, we found that in all of the treated samples, especially poly-SIBS nanosurface coatings at 6000 rpm showed anti-thrombogenic properties in all TEG® parameters tested, and when compared with ePTFE controls. This suggests that the poly-SIBS nano-surface coating does not trigger the blood coagulation cascade as easily as positive controls, and also highlights improved thrombolysis effects. Most of the organosilanised samples displayed TEG® parameters with middle range values, except for -F treated glass substrates. However, -F treated glass substrates had higher α-angle and MA values, which suggest that the coagulation cascade was activated, blood clot formation was induced at an increased rate.

Overall, the findings in this study suggest that the poly-SIBS nanosurface coatings delay contact activation and blood coagulation events in vitro, and possess some degree of anti-thrombogenic properties with low platelet adhesion and is less prone to induce thrombosis (Fittipaldi et al., 2014, Pinchuk et al., 2008).
Chapter 4  Cell viability on the nano-surface coating

Having investigated the hemocompatibility of poly-SIBS nanosurface coatings, we investigate other aspects of biocompatibility to test the cytotoxicity of novel surfaces towards vascular cell types. In this chapter, we investigate the influence of poly-SIBS nanocoatings towards human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (hASMCs) \textit{in vitro} and study their interactions to model cellular events at the vascular interface.

4.1 Introduction

All living cells and tissues interact with the one another, and to the physical or biochemical cues within their micro-environment (Dvir et al., 2011). The micro-environment influences cell fate by providing biological, chemical and mechanical stimuli, which activates or deactivates cellular signal pathways (Dalby et al., 2014, Solomonov et al., 2014). The ability to control cell fate or direct cell behaviour is the key to improving the biocompatibility of biomaterials and the long-term performance of implantable devices (Son et al., 2015). Therefore, many studies have explored different strategies to unveil the mechanism that interferes with cell fate such as cell adhesion, proliferation, growth, migration, differentiation, and apoptosis. The mechanical properties of
biomaterials, as well as, the physicochemical properties such as the surface chemistry (wettability, energy, charge) and topography at the micro- and nanometer scale are known to influence the adhesion proteins, microorganisms and mammalian cells to influence their biological activity (Lima and Mano, 2015, Nguyen et al., 2016a, Curtis et al., 2006, Han et al., 2015, Dalby et al., 2002, Buttiglieri et al., 2003, Ranjan and Webster, 2009, Biela et al., 2009, Bettinger et al., 2006, Carpenter et al., 2008).

In this chapter, we explore the cellular response to the poly-SIBS nanosurface coatings using different vascular cell types: 1) HUVECs; and 2) hASMCs. We evaluate cell metabolic activity and proliferation and growth on a range of surfaces and compare cell morphology under light microscopy over short and prolonged periods in cell culture to determine optimal cell growth conditions for applications for ICS (Ivanova et al., 2014a, Adiga et al., 2009, Ordikhani et al., 2017).

4.2 Material and methods

All chemicals were obtained from Sigma-Aldrich UK unless otherwise specified. All poly-SIBS nanosurface coatings were synthesised according to the same protocols as mentioned previously in chapter 2, section 2.2.1—2.2.2.
4.2.1 Cell culture and maintenance

4.2.1.1 Human umbilical vein endothelial cells (HUVECs)

Human umbilical vein endothelial cells (HUVECs) were purchased from Gibco, UK and cultured in Medium 200 with low serum growth supplements (LSGS, Invitrogen, UK). HUVECs were seeded onto the 24-well TCPs loaded with modified and coated glass substrates at a density of 50,000 cells/ml. Seeded plates were cultured for ten days in at 37°C in 5%CO₂/95% humidified air, and complete cell feeds were carried out every 48 h. All HUVECs used in this study was used between passage 3~6 for experimentation with organosilanised substrates and poly-SIBS nanocoatings.

4.2.1.2 Human aortic smooth muscle cells (hASMCs)

hASMCs were purchased from PromoCell, UK and cultured in Smooth Muscle Cell Growth Medium 2 with Smooth Muscle Cell Growth Medium 2 Supplement Mix (PromoCell, UK). hASMCs were seeded onto the 24-well TCPs loaded with modified and coated samples at a density of 50,000 cells/ml. Seeded plates were cultured for ten days in at 37°C in 5%CO₂/95% humidified air, and complete cell feeds were carried out every 72 h. All SMCs used in this study was used between passage 3~6 for experimentation with organosilanised substrates and poly-SIBS nanocoatings.
4.2.2 Cell viability tests

4.2.2.1 Sample sterilisation and cell seeding

Samples were placed in a 24-well TCP and irradiated in a biological safety cabinet using a 254nm ultraviolet (UV) germicidal lamp (Osram, UK) at 30W for 3 h in a tissue culture hood then equilibrated overnight (18 h) in 2 ml sterile PBS at 37°C in 5%CO₂/95% humidified air before being used for experimentation. The cell suspension was prepared following previously mentioned protocols and was seeded into 24-well plates at a density of 1 X 10⁶ cells/ml (figure 4-1).

![Figure 4-1. Schematic diagram of cell viability tests on to different modified substrates and on poly-SIBS nanosurface coatings.](image)
4.2.2.2 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay for metabolic activities

There are many cell assays can be used to measure the cell viability as well as cytotoxicity test indirectly by measuring certain biochemical reaction inside the cell, such as the MTT assay and Alamar blue assay, etc. In our study, we chose to use MTT assay to quantify the viability of cell at the very beginning of our project, and for repeatability and consistency, we had repeated and duplicated the same experiments for all our cellular viability and cytotoxicity tests.

The MTT assay is a colourimetric assay for assessing cell metabolic activity. Cellular oxidoreductase enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple colour. The measurement of the density of the colour after incubating the MTT reagent with live cells reflect the number of viable cells present, or how intense their oxidation activities were. Hence the MTT dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials.

HUVEC metabolic activity was evaluated using the MTT assay (Invitrogen, UK) on day 1, 7 and 10 in culture. Cell media was removed from each well, and the cells were washed three times with 1ml sterile PBS on the plate at designated time points. 1 ml 0.5mg/ml MTT solution was added to each well and incubated at 37°C in 5%CO₂/95% humidified air for 1 hr. After incubation, 0.5ml dimethylsulfoxide (DMSO) was added to each well to dissolve the
granules and returned for incubation for further 5min. 100µl of each solution was transferred from each well into a transparent 96-well TCP plate. The Anthos 2010 Absorbance Microplate Reader machine (Biochrom Ltd, MA, USA) and ADAP (Version: 2.0 Plus) software were used to measure absorbance at 490nm (n = 12 samples per condition in 4 repeats).

### 4.2.2.3 Total deoxyribonucleic acid (DNA) assay for cell proliferation

HUVECs cell proliferation was measured using a total DNA quantitation kit (Sigma-Aldrich, UK) on day 1, 7 and 10. All media was removed from each well, and the samples were washed three times with 1ml PBS. 400µl dH20 (molecular grade) was added into each well. 100µl of each solution was transferred from each well into a black 96-well TCP plate and mixed with a bisbenzimide stock solution (1 Hoescht 33298: 100 assay buffer). The Fluoroskan Ascent™ FL fluorescent plate reader (ThermoScientific, UK) and Ascent software v.2.6 software were used to measure the absorbance with excitation was set at 360nm and emission at 460nm.

100µl calf thymus DNA (Sigma-Aldrich, UK) stock (1 DNA standard, calf thymus DNA (1mg/ml): 1 assay buffer) was added into one well of the black 98-well, then half diluting it using sterilised PBS into a descending gradient of DNA standard solutions. Mixed well with the bisbenzimide stock solution and read plate following the same protocol mentioned above. A standard curve using calf thymus DNA was generated, and the DNA concentrations of all samples were
calculated according to the standard curve (n=12 per condition in 4 repeats).

4.2.3 Cell morphology using light microscopy

After 10 days all samples were retained, removed supernatants and rinsed with PBS three times before fixing with 4% (v/v) glutaraldehyde for 30 mins, followed by dehydration with an ascending ethanol series (20, 40, 60, 80, 100% v/v) with a duration of 30 min each step. Fixed samples were kept at 80% v/v ethanol in the fridge for further processing. Selected fixed samples were stained with 0.04% (v/v) methylene blue (40 mg/L) for 3 mins, then rinsed with copious amounts of dH2O to wash off the residual dye. The stained samples were subsequently air-dried in the laminar flow hood for overnight before imaging. Optical micrographs were taken using the EVOS™ LX Core microscopy (ThermoScientific, UK).

4.2.4 Long-term studies of poly-SIBS nanosurface coating

Both HUVECs and hASMCs were cultured on poly-SIBS nanosurface coatings on plain glass controls, CDTFS (-F) modified glass and TCP as controls, following the protocol mentioned in the previous section for a prolonged period of 12 weeks. Conventional prolonged culture for ECs as described in the literature is generally over a period of 20 days, however, we intentionally extend our culturing time to explore the biology of cells under the
influence of poly-SIBS nanosurface coatings (Butler et al., 2009, Burton et al., 2011, Butler et al., 2005). Selected samples were fixed at week 6, 8, 10 and 12 and stained with methylene blue following the protocol mentioned previously.

4.2.5 Statistical analysis

Parametric data had been expressed as mean and standard deviation. SPSS 21.0 (IBM) was used to perform one-way ANOVA with post-hoc Bonferroni’s test for analysis of statistical significance in between groups. A $P<0.05$ was considered statistically significant.

4.3 Results

4.3.1 HUVEC viability

The total DNA standard curve was generate using calf thymus DNA and is in figure 4-2, all total DNA assay values were calculated using the subsequent formula.
HUVEC viability was accessed by MTT and total DNA assays, and the results are presented in figure 4-3. No significant results were obtained in both assays between all cultured samples on day 1. HUVECs cultured on poly-SIBS samples spin coated at 6000 rpm (nano-island surfaces) started to show significantly higher MTT and total DNA values than all other samples by day 7 in cell culture (P<0.0001). By day 10, cells cultured on both CDTFS (-F) treated and spin-coated samples show a significantly higher MTT value of metabolic activity, and total DNA values than on controls (P<0.0001), while the DCDMS (-CH₃) and TMPM (-CH₂O=COCH₃) treated samples showed significant lower MTT and total DNA assay values than all other samples (P<0.0001).
Figure 4-3. MTT and total DNA assays with HUVECs culturing on different modified and nano-surface coating glass samples on day 1, day 7 and day 10 (n=12 per condition in 4 repeats).

4.3.2 HUVECs morphology

HUVEC morphology was characterised via methylene blue staining under light microscopy, and the result is presented in figure 4-4. Due to a large number of samples, modified glass substrates result and UC were shown in figure 4-4, while all the poly-SIBS nanosurface coatings, as well as, TCP controls were
shown in figure 4-5. The cell density on different samples evaluated by microscopy corroborates with cell-based assay results, and are representative of the cellular response as described in the previous section.

In figure 4-4, we noticed that on image 4-4 A on the UC (plain glass, smooth flat surface control), cells were proliferating and migrating on the surface, and after ten days of culture, we see that the cells are nearing confluence. Image 4-4 B, the TMPM (-CH₂O=COCH₃) modified samples; cells showed significantly reduced proliferation resulting in a reduced surface coverage and appear sparse throughout the surface. Images 4-4 C and 4-4 D, show organosilanised MPTS (-SH) and APTS (-NH₂) modified glass substrates, we can see cells growing in small clusters and with poorer surface coverage than the UC. Image 4-4 E, the CDTFS (-F) modified glass show enhanced HUVECs proliferation and migration, resulting in increased surface coverage and near confluent with only a few small spaces on the surface by day 10. Higher magnification images of 4-4 A~E revealed that the HUVECs had a typical cobblestone morphology, and spread phenotype and in the normal size range. However, the DCDMS (-CH₃) modified samples, image 4-4 F, had almost no HUVECs adhered on to the surface. Higher magnification images of 4-4 F showed that few HUVECs were non-spreading and in a rounded cell phenotype.
Figure 4-4. HUVECs on organosilanised glass substrates after ten days of culture and stained with methylene blue.
Figure 4-5. HUVECs on poly-SIBS nanosurface coatings after ten days of culture stained with methylene blue.
HUVECs cultured on poly-SIBS nanosurface coatings had demonstrated greater surface coverage over ten days compared with all modified samples, as well as, controls, UC (4-4 A) and TCP (4-5 E). Poly-SIBS samples spin coated at 1000 rpm (4-5 A) and 3000 rpm (4-5 B) had similar HUVEC coverage as CDTFS (-F) modified samples (4-4 E), and were almost confluent by the end of day 10. The 6000 rpm spin coated substrates (4-5 C) and dip coated (4-5 D) showed complete surface coverage and confluent samples with HUVECs forming an intact cell monolayer. High magnification images of 4-5 A~E suggest that HUVECs had predominantly healthy cell phenotypes with a spread morphology within the normal size range (20-50 μm). Moreover, most of the HUVECs on the 6000rpm spin coated poly-SIBS nanocoating (4-5 C) samples appeared to have more spindle-shaped and seemingly aligned with each other towards the same direction.

4.3.3 hASMCs morphology

hASMCs morphology was also characterised using light microscopy and methylene blue staining, and results are shown in the following figures. hASMCs on all the modified glass samples results and UC controls were shown in figure 4-6, while all the poly-SIBS nanosurface coatings, as well as, TCP controls were shown in figure 4-7. The hASMCs grew relatively poorly on the UC (4-6 A), cells showed reduced proliferation and were sparsely distributed
throughout the surface. hASMCs show a similar behaviour on TMPM (-CH₂O=COCH₃) modified samples (4-6 B), as well as, MPTS (-SH) modified glass samples (4-6 C) and appeared to be denser than those on the UC (4-6 A). Image 4-6 D show APTS (-NH₂) modified glass samples with cells growing in clusters with improved surface coverage than on untreated controls (UC). Image 4-6 E, similar to the reaction of HUVECs, CDTFS (-F) modified glass substrates show enhanced hASMCs proliferation and migration than on all other modified glass samples. Higher magnification images revealed that the hASMCs on 4-6 A~E samples were all spreading, suggesting they were in a healthy state despite some cells attached that were poorly distributed. Also, we noticed that hASMCs on CDTFS (-F) modified glass substrates in image 4-6 E appeared to be slightly bigger, and more spread compared to hASMCs on other modified glass samples. Finally, the DCDMS (-CH₃) modified samples (image 4-6 F) also showed only a few hASMCs adhered and growing on their surface. The higher magnification images of 4-6 F show that few anchored hASMCs were not spreading and seemingly too rounded and small showing poor proliferation.
Figure 4-6. hASMCs on organosilanised glass samples after ten days of culture and stained with methylene blue.
Figure 4-7. hASMCs on nano-surface coating samples after ten days of culturing and stained with methylene blue.
hASMCs growth on all of the poly-SIBS nanosurface coating (4-7 A~D) was enhanced, as well as, TCP (4-7 E) after ten days compared with all modified glass samples. The 1000 rpm spin coated (4-7 A) and 3000 rpm spin coated (4-7 B) poly-SIBS samples had similar hASMCs coverage as the CDTFS (-F) modified samples (4-6 E). The 6000 rpm poly-SIBS spin-coated samples (4-7 C) showed slightly less dense hASMCs surface coverage when compared with other poly-SIBS nanosurface coatings. The poly-SIBS dip coated substrates (4-7 D), as well as, TCP (4-7 E) showed almost complete surface coverage of hASMCs. Higher magnification images revealed that the hASMCs on all poly-SIBS nanosurface coatings (4-7 A~D), as well as, TCP (4-7 E) were in a healthy state showing a spread morphology. The hASMCs on 3000 rpm spin coated (4-7 B) and 6000 rpm poly-SIBS spin-coated samples (4-7 C) were more spindle-shaped, while cells on the 1000 rpm spin-coated samples (4-7 A) and TCP (4-7 E) was more spread and rounded in comparison. Moreover, all hASMCs on poly-SIBS dip coated samples (4-7 D) were allied with each other in a similar direction and what appeared to be thick tissue. The cells on TCP, on the other hand, even though there was complete surface coverage, they were observed in high magnification images and appeared to be no longer forming cell monolayer structures.
4.3.4 Long-term culture studies

To test the long-term stability of the poly-SIBS nanosurface coating, as well as, evaluating the long-term interactions between cells and poly-SIBS nanosurface coating, we conducted a 12 weeks prolonged culture of HUVECs and hASMCs on all the treated samples and UC as a control.

4.3.4.1 HUVECs prolonged culture

The results of prolonged cell culture of HUVECs at week 6, 8, 10 and 12 are shown in figure 4-8. We can see from the result that HUVECs on the UC (4-8 E) maintain their confluence till around week 6 and begin to die out gradually. However, HUVECs grown on poly-SIBS nanocoatings spin coated at 6000rpm (4-8 C) and dip coated (4-8 D) samples demonstrated enhanced cell growth and maintained their confluence for the full twelve-week period and by the end of the study demonstrate cell viability and proliferative capacity. The 3000rpm spin coated (4-8 B) and 1000rpm spin coated (4-8 A) samples also reached confluence by week 6 and maintained their confluence until week 10, before the HUVECs gradually started to die off.
**Figure 4-8.** HUVECs on nano-surface coating samples for prolonged culture and stained with methylene blue.
4.3.4.2 hASMCs prolonged culture

The results of hASMCs prolonged cell culture after week 6, 8, 10 and 12 in cell culture are shown in figure 4-9. The hASMCs on UC (4-9 E) maintained their confluence throughout the entire study and appeared to be viable throughout. hASMCs on the poly-SIBS dip coated samples (4-9 D) grew and proliferated through the entire culturing period, and we can see layers of hASMCs aligned to the sample surface resembling healthy tissue. However, we also observed that the stability of the coating was compromised with small holes and imperfections in various places of the sample surface after week 8 and from week ten, we can see the coating detaching from the substrate. hASMCs also reached confluence by week six on 1000rpm (4-9 A), 3000rpm (4-9 B) and 6000rpm spin coated (4-9 C) samples, however, compared with the cells on the dip coated samples (4-9 D), the cells, appeared to be sparse and form monolayer structures. We first observed the coating detaching from the edge of the glass surface on 6000rpm spin-coated samples (4-9 C) on week eight, and the same observed on 3000rpm (4-9 B) and 1000rpm (4-9 A) spin-coated samples on week 10 and week 12, respectively. On 3000rpm (4-9 B) and 6000 rpm (4-9 C) spin-coated samples, we observed the coating started to detach from the surface presumably as a result of the interactions with the hASMC monolayer.
**Figure 4-9.** hASMCs on the poly-SIBS nanosurface coated samples after prolonged studies and stained with methylene blue.
4.4 Discussion

It is well known that interaction between ECs and the underlying substrate nanotopography lead to changes in the cellular response. ECs have been known to increase the level of cellular adhesion and spreading behaviour on nanofeatures with a profile of around 13 nm in height (Han et al., 2015, Dalby et al., 2002, Buttiglieri et al., 2003); ECs have also been shown to align to nanogrooves with depth profile of 100~300 nm, which enhance their orientation and migration along the grooves (Ranjan and Webster, 2009, Biela et al., 2009, Bettinger et al., 2006); ECs on surfaces modified with nanocones with a height profile of 300 nm show enhanced cellular adhesion (Carpenter et al., 2008). In our study, we see similarities in the HUVEC response to poly-SIBS nanosurface coatings. For example, poly-SIBS nanoislands had a height profile around 20nm and were found to enhance the ECs metabolic, and proliferation activity, as well as, changes in alignment characteristic of cell migration.

Moreover, we also found that CDTFS (-F) modified glass, which acts as a negatively charged base layer to adhere cationic poly-SIBS also enhances EC behaviour to produce a non-cytotoxic electrostatic coating and appears to promote endothelialisation. Also, comparing the difference between HUVECs on CDTFS modified glass and poly-SIBS nanosurfaces spin coated at 6000 rpm was found to favour HUVEC adhesion, spreading and proliferating throughout the surface to form a uniform monolayer of HUVECs. The cellular morphology of HUVECs on the poly-SIBS appeared to be arranged in a
cobblestone fashion but appeared more stellate and spindle-shaped like and aligned head-to-end with each other, this presentation is similar to the presentation of ECs seen in the native endothelium of blood vessels. HUVECs on organosilanised and control substrates appeared to be more spread and rounded in cell morphology with varying degrees of cell proliferation. This also suggests that the poly-SIBS nanosurface coating promotes cell alignment and guidance of the cells, which favours cell proliferation. It is unknown at present what the ECs are responding too, but presumably altered patterns of adsorbed proteins from the culture media or change in surface chemistry as a result of the arrangement of nanofeatures.

hASMCs was found to remain active, spread and proliferate on most of the samples under investigation. However, their proliferation was enhanced on poly-SIBS nanosurface coatings on dip coated samples composed of nanopits and nanopores. We observed that on smoother surfaces, hASMCs grow in a sheet-like structure of striated cells without confluence. However hASMCs cultured on dip coated nanoporous surfaces appeared to influence the growth of more aligned tissue structures across the sample surface. One explanation could be in that the nanopits promote hASMCs cell growth and orientation by promoting cell alignment, which is observed in all dip coated samples from day 10 of culture and maintain this structure throughout prolonged periods leading to tissue formation although few studies report hASMCs interactions with a surface nanotopography and warrants further investigation.
Prolonged cell cultures are designed to test the durability and longevity of the nanosurface coatings suggesting that are stable towards vascular cell types such as HUVECs. However, we found some interesting observations with the coatings when used in cultures of hASMCs. We observed some imperfections and damage to the poly-SIBS coating layer after spin coating in the prolonged cultures. Perhaps the damage maybe caused by the strength of hASMC adhesion or migration and/or contraction and relaxation circles of the cell monolayer causing micromotion or micromovement on the coating layer causing the electrostatic coating to detach from the substrate. However, hASMCs cultured on the poly-SIBS dip coated was found to not detach from the surface and had resistance to this phenomenon, which warrants further investigation to assess the peel strength of the coatings. Further evidence suggests that the higher the spin coating speeds, the weaker the coating layer as coatings on 6000 rpm peeled away much earlier that 1000 rpm spin-coated samples, and were found to roll up into a cell sheet-like structures composed of poly-SIBS and hASMCs.

Nonetheless, we found that CDTFS treated samples with a negatively charged -F group, as well as, the cationic poly-SIBS substrates coated at 6000 rpm promote endothelization and enhance the metabolic and proliferative activity of HUVECs \textit{in vitro}. Precisely how such surface nanocues influence HUVEC fate remains unknown at present, and by exploring further cell-material interactions and their gross cell morphology and functional biomarkers may
reveal how HUVECs are influenced by surface nanotopography. Understanding their long-term stability of the nanosurface coatings, which also prolong the endothelial monolayer to retain endothelialisation, and how they fail when cultured with hASMCs also warrants further investigation.
Chapter 5  Gross morphology of HUVECs and their functions on the nano-surface coating

In previous chapters, we found that the poly-SIBS nanosurface coatings possess anti-thrombogenic surface properties, and enhance metabolic activity and growth of HUVECs to promote endothelization. In this chapter, we explore the interactions between HUVECs and poly-SIBS nanosurface coating and investigate the gross morphology of ECs using FE-SEM image analysis, as well as, immunofluorescent staining to view functional biomarkers (e.g. von Willebrand factor, VEGFR-2 and vinculin) to investigate the cellular response to nanofeatures.

5.1 Introduction

5.1.1 Imaging and gross morphology of HUVECs

Many studies have demonstrated that a surface topography on the nanometer scale (1-100nm) can influence the cellular response such as changes in the level of cell adhesion, migration, growth, proliferation and differentiation of cells (Lima and Mano, 2015). For example, research exploring interactions between ECs and nanotopography of the underlying substrate has shown increased levels of cell proliferation, elongation and enhanced filopodial
protrusions formed on titanium nanotubes (Han et al., 2015); ECs on nanobulges with a height of 13 nm show increased cell adhesion and spreading (Dalby et al., 2002, Buttiglieri et al., 2003); ECs on nanogrooves with a depth of 100 to 300 nm show enhanced cell elongation, increased orientation, alignment and migration along the grooves (Ranjan and Webster, 2009, Biela et al., 2009, Bettinger et al., 2006); and ECs on nanocones with a height of 300 nm show enhanced cellular adhesion (Carpenter et al., 2008).

Previously, we have developed a series of poly-SIBS nanosurface coatings, which form nano-islands with a height of around 20 nm, as well as substrates forming nano-pores and nano-pits with depth and width ranging from 20 to 200 nm, as shown in chapter 2, section 2.3.2. Among these nanosurfaces, we found that poly-SIBS nano-island surface coatings produced with 6000 rpm spin coating techniques with an average spacing of 650 nm in between the nano-islands (chapter 4, section 4.3.1.) were the most influential in promoting endothelization. Such substrates represent ideal cell culture conditions to explore EC interactions with the underlying nanotopography on the material surface.

5.1.2 HUVEC functional biomarkers

There are many types of functional biomarkers for ECs, and we selected biomarkers associated with cell function, viability and adhesion on material substrates, which are described in the following sections:
5.1.2.1 Von Willebrand Factor (vWF)

vWF is a glycoprotein present in blood, which is involved in haemostasis. It is a large multimeric glycoprotein present in plasma and is produced constitutively in ECs. Its primary function is to bind other proteins, such as coagulation factor VIII and is essential in wound healing as it regulates platelet adhesion to the site of wounds. The presence of vWF is an indication of active endothelial cell function in mature, healthy ECs (Hartholt et al., 2017, Michiels et al., 2017, Morici et al., 2017, Selvam and James, 2017).

5.1.2.2 Vascular endothelial growth factor receptor 2 (VEGFR-2)

VEGFR-2 is an important cell-signalling protein involved in vasculogenesis and angiogenesis. VEGFR-2 activity is restricted to native ECs. In vitro, VEGF-2 has been shown to stimulate mitogenesis and migration, and also enhances microvascular permeability and is sometimes referred to as vascular permeability factor, which allows the passage of small molecules (e.g. nutrients, water, ions, drugs) and whole cells (e.g. lymphocytes) in and out of blood vessel. There are 3 types of VEGF receptors on the surface of ECs, and VEGFR-2 on the cell surface is both a biomarker of ECs and an indicator of vascularisation and angiogenesis (Zafar et al., 2017, Cao and Zhang, 2017, Peng et al., 2017, Karimian et al., 2017, Corti and Simons, 2017).

The potential effect of prompting angiogenesis was generally undermined for ICS systems as the purpose of ICSs were to restore vessel patent, not generating new vessels to supply the area. However, in this study, we think it
is important to look into VEGFR-2 as it is one of the markers that indicate the ECs were actively proliferating as per pushing surface coverage on the implanting device. Secondly, this also hints the potential of this coating being used in other endovascular implanting devices that promote vascularisation, and they could be applied in multiple neuro-degenerative conditions such as vascular dementia, moyamoya disease etc.

5.1.2.3 **Vinculin**

In mammalian cells, vinculin is a membrane-bound cytoskeletal protein present in focal adhesion plaques that provides a link to integrin receptors with a complex of adhesion proteins and molecules linked to the actin cytoskeleton. Vinculin is a cytoskeletal protein associated with cell-cell and cell-matrix junctions, where it is thought to function as one of several proteins involved in anchoring F-actin to the cell membrane. Cell spreading, migration and movement occur through binding of cell surface integrin receptors to extracellular matrix (ECM) adhesion molecules such as fibronectin or vitronectin on the surface of the substrate. Vinculin is associated with focal adhesion sites or plaques, and adherens junctions, which are complexes that nucleate actin filaments plasma membrane, and the actin cytoskeleton. The complex at the focal adhesion site consists of several proteins such as vinculin, α-actin, paxillin, and talin at the intracellular face of the plasma membrane. Vinculin's ability to interact with integrins on the cytoskeleton at the focal adhesion site appears to be critical for control of cytoskeletal mechanics, cell...
spreading, and lamellipodia formation. Thus, vinculin appears to play a key role in cell adhesion, and cell morphology and shape control based on its ability to modulate focal adhesion structure and function (Goldmann et al., 2013, Leerberg and Yap, 2013, Thompson et al., 2013, Atherton et al., 2016, Goldmann, 2016, Bays and DeMali, 2017). In the following chapter, we explore the interactions of HUVECs using FE-SEM to observe the morphological appearance of ECs and functional biomarkers on ECs in contact with a substrate nanosurface topography.

5.2 Material and methods

All chemicals were obtained from Sigma-Aldrich, UK unless otherwise specified. All poly-SIBS nanosurface coatings were synthesised according to the same protocol as mentioned previously in chapter 2, section 2.2.1-2.2.2), and sterilised before being used for experimentation (chapter 4, section 4.2.2.1). We selected poly-SIBS nano-islands spin coated at 6000 rpm as it represented optimised surface coatings to investigate changes in endothelialisation when compared with other nanosurface coatings. Similarly, CDTFS treated samples (-F) was selected as a highly negatively charged substrate, which was found to produce a uniform thin layer of poly-SIBS in the formation of electrostatic coatings when compared with other organosilane precursors. We also selected dip coated samples as the nanosurface coatings with nano-pits and nano-pores to observe any changes in cell surface interactions due to the spacing and size
of the nanofeatures alongside untreated controls (UC).

5.2.1 Cell migration studies

Glass substrates were coated with CDTFS, and poly-SIBS dip coated samples on half of the glass slide only to expose the leave the other portion exposed as the untreated control. 100 µl HUVEC suspension at a density of \(1 \times 10^6\) cells/ml was seeded on to the centre of the treated slide of the samples to allow the cells to migrate up to the untreated glass portion. The samples were placed in a humidified incubator at 37°C, 5% CO\(_2\) and 95% humidified air for 1 hr. After incubation, 1 ml fresh media was added into each well and cultured for ten days.

5.2.2 High-resolution FE-SEM image analysis

HUVEC samples were removed after ten days in culture, and the supernatant was discarded and rinsed with PBS three times before fixing with 4% (v/v) glutaraldehyde for 30 min. A dehydration step with an ascending ethanol series (20, 40, 60, 80, 100% v/v) was carried out with a duration of 30 min each step. Fixed samples were stored in 100% (v/v) ethanol in the refrigerator for 24 h before FE-SEM examination. One the day of FE-SEM image analysis, the solvent was removed from the samples and was left in a laminar flow hood overnight (18h) for air-drying before imaging. FE-SEM analysis was performed following the protocol mentioned in chapter 2, section 2.2.3.2.
5.2.3 Immunofluorescent staining

5.2.3.1 vWF

The HUVEC samples mentioned previously was washed three times with warmed sterile PBS, and blocked with 1% (w/v) bovine serum albumin (BSA) for 1 h. 300 µl primary mouse anti-human vWF antibody (1:400 dilution) was purchased from Abcam, UK was added into each well and incubated at 37°C/5% CO₂/95% humidified air for 1 h. The samples were rinsed with PBS, and a secondary antibody was added composed of goat anti-rat IgG antibody (1:1000 dilution) conjugated with Alexa Fluor® 594 (Abcam, UK), and was incubated for a further 1 h. After another cycle of washing and rinsing with PBS, the samples were incubated with DAPI (1 µg/mL) for 15 min for nucleic acid staining to visualise the cell nucleus. Finally, the samples were permeabilised with 0.5% (v/v) Tween for 5 min before incubating with Alexa Fluor® 488 phallotoxin (1:99 dilution) purchased from Invitrogen, UK for 1 h at room temperature to visualise F-actin. The samples were then mounted on to glass microscope slides using Vectashield fluorescent mounting medium (Vector Laboratories, USA), and imaged using an LSM 880 confocal microscope (Carl Zeiss Ltd, Germany).

5.2.3.2 VEGFR-2 and vinculin

Immunostaining HUVEC samples for VEGFR-2 and vinculin, respectively was performed following the above mentioned protocol as in the previous section with exception of the dilution factor used for primary mouse anti-human VEGFR-2 antibody, which was diluted 1:100, and primary mouse anti-human
vinculin antibody, which was diluted 1:250 (Abcam, UK).

5.3 Results

5.3.1 Cell migration studies

The result of HUVEC cell migration studies is shown in figure 5-1. HUVECs demonstrate enhanced migration and proliferation on the half treated CDTFS (fluorinated organosilane, -F) portion of the sample, but HUVECs did not migrate and spread on to the untreated portion to the same extent during ten days of cell culture. Similarly, HUVECs spread and proliferated throughout the dip coated poly-SiBS sample after ten days of culture, however, few cells were present and were less spread on untreated portions of the side.
5.3.2 Gross morphology of HUVECs on different substrates

Cell morphology was characterised in detail using a high-resolution FE-SEM as shown in figure 5-2A-D. It can be seen that HUVECs cultured on untreated controls (UC) display a smaller cell size with a flat shape morphology (figure 5-2A). The lamellipodium is apparent and has small filopodial protrusions characteristic if migratory cells that sense the environment...
highlighted in yellow.

Figure 5-2. FE-SEM images of HUVECs on A. UC; B. CDTFS (-F) modified; C. poly-SIBS nanoislands; and D. dip coated nanopits and nanopores.
HUVECs cultured on CDTFS or fluorinated organosilanes (-F) appear to have a much larger cell size, as well as, spreading behaviour throughout the surface (figure 5-2B). We also observed HUVECs cultured on fluorinated surfaces had significantly more filopodial protrusions on the cell membrane presumably as a change in surface chemistry and energy as described previously. The cell shape morphology is highlighted in yellow, and high magnification images highlight some small filopodial protrusions similar to control samples. However, the lamellipodia also had much longer and thicker filopodial protrusions (highlighted in yellow) when compared with UCs.

HUVECs cultured on surfaces with poly-SIBS nanoislands surface coatings (6000 rpm spin coated substrates) appeared to be similar to fluorinated surfaces (-F) as shown in figure 5-2C). They also had a larger cell size only this time with significantly more filopodial protrusions radiating in all directions from the cell membrane, indicating that the cells were actively migrating, sensing the surface and cell-cell interactions. Higher magnification images of the filopodia show that they appear to be making contact with the poly-SIBS nanoislands as if acting as stepping stones or cell guidance cues.

HUVECs cultured on poly-SIBS dip coated substrates show a predominantly rounded cell phenotype, which was less spread on the surface when contacting nanopits and nanopores (figure 5-2D). The lamellipodia at the leading edge of the cell had a notable increase in filopodial protrusions that were also thicker and longer to those observed on UCs. However, they were also shorter and thicker to filopodia observed on HUVECs cultured on poly-SIBS nanoislands surface coatings. We also quantified the number of filopodial protrusions on each substrate from cell images using ImageJ.
software. We found that on average ten filopodial protrusions emerge from each cell on UCs, while there the average number of filopodial protrusions from each cell on -F, poly-SIBS nanoisland coatings and dip coated samples was 96, 129 and 78, respectively. This suggests that over ten times more filopodial protrusions appear on HUVECs cultured on poly-SIBS nanoislands surface coatings than on UCs, and about 50% more than -F modified and poly-SIBS dip coated substrates.

5.3.3 Immunofluorescent staining

5.3.3.1 vWF immunostaining

EC function was characterised by immunostaining of vWF, and the results are shown in figure 5-3A-D. HUVECs cultured on all samples stained positive for vWF with composite images appearing yellow after co-localization of both green and red channels, which is indicative of healthy EC phenotypes. HUVECs cultured on untreated controls (UC) proliferated and grew slowly and had a lower cell density with poor surface coverage at day 10 (figure 5-3A). HUVECs cultured on organosilanised fluorinated samples (-F) show significantly higher cell growth and proliferation in comparison to UC (figure 5-3B). High magnification images revealed predominantly spread cell phenotypes forming a cobblestone monolayer of ECs. HUVECs cultured on poly-SIBS nanoisland coatings displayed a significantly greater cell number and cell density, which formed a confluent monolayer of ECs (figure 5-3C).

Moreover, the poly-SIBS nano-islands act as cell guidance cues as shown earlier and the presence correlates with enhanced cell activity and growth, and
some cells appeared to show an aligned configuration within the cell monolayer. HUVECs cultured on dip coated poly-SIBS coatings also show dense cell growth when compared with UC, however, surface coverage was patchy in areas and significantly decreased in comparison with -F treated samples and poly-SIBS nano-island coatings (figure 5-3D). These results were consistent with the cell viability and proliferation data as shown in chapter 4, section 4.3.1-4.3.2.
Figure 5-3. HUVEC growth on model glass substrates: A. UC; B. (-F) organosilane; C. poly-SIBS nano-islands (6000 rpm); D. poly-SIBS dip coated sample. Color indicator: Blue: DAPI (DNA); Green: Phalloidin (F-actin); Red: vWF.
5.3.3.2 VEGFR-2 immunostaining

Immunostaining of VEGFR-2 in HUVECs cultured on different model glass substrates is shown in figure 5-4A-D. Generally, HUVECs stained positive for VEGFR-2 with the composite images appearing yellow, which is indicative of widespread expression. HUVECs show enhanced growth, proliferation and migration on -F modified substrates (figure 5B) and poly-SIBS nano-island coatings (figure 5C) when compared with untreated controls UC (figure 5A). HUVECs on poly-SIBS dip coated samples (figure 5D) demonstrate show increased cell growth and adhesion when compared with UC. However, surface coverage and cell migration were patchy in comparison to other samples. Overall, the levels of VEGFR-2 with HUVECs on the model samples resemble the results as seen previously and is consistent with the cell viability and proliferation data as shown in chapter 4, section 4.3.1-4.3.2.
Figure 5-4. HUVEC growth on model glass substrates: A. UC; B. (-F) organosilane; C. poly-SIBS nano-islands (6000 rpm); D. poly-SIBS dip coated sample. Color indicator: Blue: DAPI (DNA); Green: Phalloidin (F-actin); Red: VEGFR-2.
5.3.3.3 Vinculin immunostaining

To explore HUVEC adhesion further, we selected vinculin as a marker for focal adhesion sites of the cells to the underlying substrate as shown in figure 5-5A-D. The presence of vinculin (red) highlights the co-localization of cytoskeletal F-actin (yellow) and focal adhesion sites as a result of cell-surface interactions. It can be seen that HUVECs cultured on UC show poor cell growth and surface coverage (figure 5-5A). Vinculin was mainly present in the cytoplasm (co-localised in yellow), but not surrounding the periphery of the cell lamellipodia or filopodia suggesting poor cell adhesion and migration. HUVECs cultured on organosilanised (-F) samples show a random distribution of vinculin co-localised within the cytoplasm and surrounding the periphery of the cells (figure 5-5B).

High magnification images revealed that cells were spread with more filopodial protrusions. HUVECs on the poly-SIBS nano-island coatings demonstrate dense cell growth and great surface coverage, as well as, cell alignment. Moreover, high magnification images between HUVECs revealed that the cells were contacting each other, but also contacting the underlying nanofeatures with filopodial protrusions making contact with the nano-islands (figure 5-5C). Higher magnification images show distinctive red spots in the background of the poly-SIBS nano-island surface and localised in some regions contacting the cells as the filopodial protrusions reach out to the red spots, which is in agreement with FE-SEM image analysis that filopodial protrusions
appear to contact the nano-islands for sensing and guidance.

HUVEC growth on dip coated poly-SIBS with nanopits and nanopores revealed poor surface coverage (figure 5-5D). However, due to the quality of the samples at higher magnification, we could not locate the level of vinculin surrounding the cell periphery and was predominately present in the cytoplasm and required higher magnification or alternative imaging techniques. The samples were difficult to focus and were likely due to the film thickness of the substrates. However, HUVEC growth on different samples resembled data from previous findings and was consistent with cell growth with other immunofluorescent stains as mentioned earlier in chapter 4, section 4.3.1-4.3.2.
Figure 5-5. HUVEC growth on model glass substrates: A. UC; B. (-F) organosilane; C. poly-SIBS nano-islands (6000 rpm); D. poly-SIBS dip coated sample. Color indicator: Blue: DAPI (DNA); Green: Phalloidin (F-actin); Red: Vinculin.
5.4 Discussion

In this chapter, we explored the level of cell function and expression of functional markers and the level of adhesion and interaction of the cells to the underlying substrate. HUVECs cultured on all of the samples revealed a positive EC functional marker for vWF, VEGFR-2 and vinculin at various levels and was indicative of healthy EC function. The key differences in cell morphology and level of expression occur as a result of the extent of cell growth and proliferation. For example, HUVECs had poor surface growth, coverage and migration on UC. On -F modified samples, the cells were almost confluent at day 10 with enhanced growth, proliferation and migration. The change in surface chemistry to a more negatively charged, hydrophobic surface presumably influenced the level of protein adsorption and cell adhesion on the surface to increase growth and proliferation by activation of biochemical pathways and warrants further studies. HUVECs on poly-SIBS nano-island demonstrated a fast rate of cell proliferation, growth and migration resulting in a confluent cell monolayer and coverage on the sample surface.

The level of enhanced growth and proliferation of ECs was induced by the presence of the poly-SIBS nano-surface coatings resulting in morphological
differences in the level of lamellipodia and filopodial protrusions. To explore cell-surface interactions with the poly-SIBS nano-islands and level of cell adhesion, we investigated the formation and localisation of focal adhesion sites and plaques. Where the level of cell-surface interaction could be seen, filopodial protrusions seemed to reach out towards the nano-islands to make contact with them and appeared to act as a cell surface guidance cue to sense the surrounding environment, e.g. contain adsorbed proteins on the top of the nano-island or differences in chemistry in between the islands. This finding is consistent with similar studies that revealed that fibroblasts react to polymer demixed nano-islands with a height range of 20 nm on the substrate surface, which was also found to promote enhanced metabolic activity and cell proliferation of fibroblasts with pseudopodial protrusions that make contact with nano-islands as acting as stepping stones and guidance cues (Dalby et al., 2002, Dalby et al., 2004, Curtis et al., 2006, Dalby et al., 2006, Biggs et al., 2010, Chrzanowski et al., 2012, Dalby et al., 2014). This study is in agreement with our findings that nano-surface features can influence cell behaviour to promote endothelization.

When considering the results of this study and others, the level of interactions
appears to be linked to cell adhesion and growth as most observations occur as a result of cells making contact with nano-islands. Vinculin is a component of the integrin receptors that drive cell fate through mechanotransduction events through sensing biochemical and physical cues that guide cell fate. Perhaps the nano-islands provide guidance cues that signal to the nucleus or through changes in distortion of the cytoskeleton cause changes in the level of protein or gene expression, as well as, the level of filopodial protrusions? This is most probably due to the accumulation of adhesive matrix proteins such as fibronectin accumulating on the top of the nano-islands to bind to integrin receptors (via vinculin) to guide cell adhesion and migration. Filopodia sense the nano-islands, which act as surface cues so that ECs can explore the surrounding environment and guide migration as a result of the level of the underlying substrate, and the level of focal adhesions on the surface of the nano-islands warrants further investigation with high-resolution imaging techniques to investigate if the cells are responding to changes in nanotopography or chemistry (e.g. differences in adsorbed proteins). This could be due to the non-homogeneous surface coverage of nano-island coatings, which promote different protein absorption patterns on the substrate. Poly-SIBS nano-islands are more hydrophobic (hard
polystyrene segments) and favour protein absorption, which is known to inhibit cell adhesion; while the soft-segment of the poly-SIBS in between the nano-islands on the substrate surface may favour cell adhesion, hence the patterning effects (e.g. cobblestone morphology and alignment) observed with the cell adhesion and growth, and warrants future investigation.

Nonetheless, we have demonstrated that the poly-SIBS nano-islands promote cell adhesion, migration, growth and proliferation by providing contact guidance cues that enhance endothelialisation, which may enhance wound healing at the damaged vascular cell wall when used as a surface coating for vascular stents or ICS leading to in situ endothelialisation.
Chapter 6  Translation of the nano-surface coating platform onto other biomaterials

In this chapter, we have developed and optimised a standard coating procedures to apply the electrostatic coatings on to a range of cardiovascular biomaterials, e.g. intracranial stents (ICS), bare metal stents (BMS) and polymeric material used for bypass graft and covered stent materials. We explore the feasibility of organosilane treatments and transferring the poly-SIBS nano-island surface coatings on to metallic and polymeric biomaterials composed of shape memory alloy (SMA) Nitinol®, platinum-chromium (PtCr) alloy, as well as, expanded polytetrafluoroethylene (ePFTE) to evaluate the coating feasibility before in vivo studies.

6.1 Introduction

6.1.1 What are SMA and Nitinol®?

SMA is derived from nickel-titanium and was first discovered by the US Naval Ordnance Laboratory (Silver Spring, Maryland, USA) and called Nitinol® by
Buehler and co-workers in the 1960s (BUEHLER et al., 1963). Nitinol® is manufactured by forging under extreme heat and passed through a series of cold phases, and eventually finalised by heat treatment at 450-550°C for 1-5 mins to form the desired shape or structural form. Zijderfeld and co-workers first described shape-memory effects of Nitinol® in the 1960s as a result of temperature changes and/or application of stress induced during the crystalline transition (ZIJDERVE.JA et al., 1966, WASILEWSKI, 1975). When heated above a certain temperature range, typically 20-50°C, Nitinol® recovers to a 'memorised shape and structure' (Jani et al., 2014). Currently, all commercial ICSs are made with a single type of SMA – Nitinol®. However, there are known problems with the use of Nitinol® as a biomaterial for ICS. First of all, Nitinol® is high in thrombogenicity, the incidence of thromboembolic events shortly after implantation with Nitinol® ICS is between 5~10% (Zhao et al., 2014a). This is undesirable since the standard guidelines set for cardiovascular stenting is <1% according to the American Heart Association (Hussain et al., 2012). In clinical practice, intracranial stenting is regularly followed by prolonged DAPT therapy, which can cause unwanted side effects, and an increased risk of bleeding complications and haemorrhage in some individuals. Secondly, in-situ neointimal
hyperplasia (NIH) is a long-term complication after stenting procedures. Nitinol® ICS are also prone to induce NIH due to the poor endothelization on the implant surface. In a longer time-span, NIH would eventually cause over-proliferation of SMCs, resulting in thickening of the vessel lumen. This is known as re-stenosis or narrowing of the blood vessels, and it is one of the principal modes of stent failure in clinical practice (Froelich et al., 1999, Palmaz et al., 1999, KAMBIC et al., 1988, WAKHLOO et al., 1994, HAGEN et al., 1993, Verheyte et al., 1999). Finally, nickel (Ni) ion sensitivity is also observed in all Nitinol® based implants. Ni hypersensitivity in ICS is more common in female than in males with a population ratio of 5:1 (Ulus et al., 2012), and typical symptoms including reversible brain parenchymal oedema occurring within 4 wks of stent placement, which can also develop from ipsilateral to contralateral positioning. Migraines are also very commonly observed with an increase in both frequency and severity over several weeks post-stenting. However, it is usually self-limited and requires no specific management (Darlenksi et al., 2012).
6.1.2 PtCr BMS

There have been many types of metallic biomaterials used for cardiovascular stents. Over the years, 316L stainless steel (316L), cobalt-chromium (Co-Cr) alloys such as MP35N® and L605, and gold coated alloys have been used to developed previous generations of vascular stents (Slodownik et al., 2018, Aoki et al., 2018, Di Santo et al., 2017, Ivanova et al., 2014b, Fishbein et al., 2006, Fedorov and Mandler, 2016, Song et al., 2016, Fort et al., 2007). However, due to poor mechanical properties and difficulty in visualising under X-ray analysis, 316L stainless steel stents have been removed from the market; while gold coated stents display poor clinical performance, such as high rates of restenosis (Kastrati et al., 2000, Reifart et al., 2004). In recent years, the medical device industry favoured the use of Pt alloys, which had better tensile properties and density compared to 316L, MP35N® and L605 alloy. The yield strength of the Pt-enhanced materials are superior to 316L and MP35N® and is comparable to L605 alloy. The elastic modulus of the Pt-enhanced alloy is higher than stainless steel, while lower than the Co-Cr materials. The material is microstructurally similar to stainless steel and has a chromium-rich oxide on the surface, which imparts a high resistance to pitting corrosion. This oxide layer also contains a small amount
of Pt dispersed through the structure, and results in a very good balance from a stent design perspective as excessive stiffness can lead to challenges with optimising the devices flexibility for imagining and tracking the deployment site, and with maximising conformity against the vessel wall. (O'Brien et al., 2010). Pt-Cr alloys have already been used in some cardiovascular stents including the Rebel™ coronary stent system (Boston Scientific, USA). Pt-Cr alloys used in the Rebel™ system, which nominally consists of the following elemental composition: Iron – (Fe, 37%), Pt (33%), Cr (18%), Ni (9%), Molybdenum – Mo (3%) and Manganese – Mg in trace amounts (Scientific, May 2014).

6.1.3 ePTFE for small diameter vascular bypassing grafts

ePFTE mentioned in chapter 1, section 1.5.4.1. used as a polymeric biomaterial for coronary artery bypass grafts with diameters over 6 mm, as well as, coatings or covered stent materials (McMahon et al., 2013). Currently, it is being used as a cover material for the Willis covered stent (a form of ICS) and most AAA stents. However, this materials demonstrate poor biocompatibility and have a high rate thrombogenicity in smaller diameter grafts, e.g. the patency rate
of ePTFE bypass graft for lower limbs is only 25% in 5 yr (Willigendael et al., 2005).

In this chapter, we optimised the poly-SIBS nano-island coatings on to a range of commercially available cardiovascular and neurosurgical biomaterials to test the feasibility of the coating platform on these materials. We applied the poly-SIBS nano-island surface coating on to Nitinol®, PtCr alloy, as well as, ePTFE to investigate the coating uniformity and surface morphology and roughness. By creating an additional base layer on the surface of biomaterials, we hope to improve their biocompatibility and enhance and promote in situ endothelialisation as well as, minimise their cytotoxicity and improve blood compatibility.

6.2 Material and methods

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. Surgical grade Nitinol® samples were manufactured into a rectangular triangular shape with dimensions of 1cm X 1cm X 1.414cm and were obtained from (Memry Saes Group, GmbH, Germany). Rectangular Pt-Cr samples with dimensions of 1cm X 1cm were purchased from American Elements (Salt Lake City, Utah, USA).
ePTFE sheets were purchased from Goodfells UK and manufactured into circular discs (diameter 1cm) following the same protocols as mentioned in chapter 3, section 3.2.2. All poly-SIBS nano-island coatings were synthesised according to the protocol for 6000 rpm spin coated glass substrates as mentioned in chapter 2, section 2.2.1–2.2.2.

6.2.1 Surface characterisation of coated samples

6.2.1.1 Contact Angle ($\theta^\circ$) and Surface Energy ($\gamma$) Measurements

6.2.1.2 AFM

Surface characterisation 6.2.1.1-6.2.1.2 was carried out on samples following protocols described in chapter 2, section 2.2.3.1 and 2.2.3.3.

6.2.2 Haemocompatibility tests

6.2.2.1 BCI

6.2.2.2 PAI

6.2.2.3 TEG

Haemocompatibility tests 6.2.2.1–6.2.2.3 was carried out on samples following the protocols described in chapter 3, section 3.2.2.
6.2.3 HUVECs culture, maintenance and cell viability tests

6.2.3.1 MTT
6.2.3.2 Total DNA

HUVECs culture, maintenance and cell viability tests 6.2.3–6.2.3.2 were carried out on samples following the same protocols as described in chapter 4, section 4.2.1–4.2.2.

6.2.4 Statistical analysis

Parametric data were expressed as mean and standard deviations in all experiments performed with 6 samples per condition (n = 12 in 4 repeats). SPSS 21.0 (IBM) was used to perform one-way ANOVA with post-hoc Bonferroni’s test for analysis of statistical significance between groups. A p value of <0.05 was considered to be statistically significant.

6.3 Results

6.3.1 Contact Angle (θ°) and Surface Energy (γ) Measurements

Nitinol®, Pt-Cr and ePTFE exhibited contact angles (θ°) of 80.0 ± 1.05°, 74.7 ± 3.09° and 143.87 ± 5.10° respectively, compared to θ for an untreated glass of 65.5 ± 6.22°. The θ° of poly-SIBS spin coated at 6000 rpm spin-coated on Nitinol®,
Pt-Cr and ePTFE samples were $92.7 \pm 2.07^\circ$, $90.9 \pm 4.16^\circ$ and $98.9 \pm 4.77^\circ$ respectively, comparing to $\theta^\circ$ for poly-SIBS spin coated at 6000 rpm on glass substrates at $77.1 \pm 2.34^\circ$. By comparing the contact angle results revealed that all samples’ surface wettability altered significantly after applying the poly-SIBS nano-island coating ($P < 0.001$).

The Owens-Wendt equation was applied to estimate the $\gamma_S$ of the samples. The $\gamma_S$ for Nitinol®, Pt-Cr and ePTFE were $31.73 \pm 0.29$, $36.48 \pm 0.39$ and $2.03 \pm 0.55$ mN/m respectively, compared to untreated glass $37.51 \pm 1.25$ mN/m. It was also found that the $\gamma_S$ altered significantly after applying the poly-SIBS nano-island coatings ($P < 0.001$). The $\gamma_S$ for 6000 rpm poly-SIBS spin coated on to Nitinol®, Pt-Cr and ePTFE samples was $24.96 \pm 0.53$, $25.48 \pm 0.36$ and $23.98 \pm 1.52$ mN/m respectively, comparing to coated glass samples $34.18 \pm 0.44$ mN/m. The results indicated that the surfaces of all the substrates were successfully modified. WCA and surface energy results of all samples are shown below in figure 6-1.
Figure 6-1. Contact angle (θ°) and surface energy (γ) results on to biomaterials before and after coating with Poly-SIBS nano-islands. Left: WCA (n=12 per condition in 4 repeats); right: Surface energy (n=12 per condition in 4 repeats).
6.3.2 AFM

The surface morphology and topography of Nitinol®, Pt-Cr and ePTFE samples before and after applying the poly-SIBS nano-island coating, along with plain glass and model glass substrates coated with poly-SIBS for comparison in figure 6-2 and 6-3. We found that surgical grade SMA Nitinol® samples displayed an overall smooth surface profile under AFM besides some minor surface defects observable as small pits (figure 6-2C). This was expected as the samples went through electropolishing to passivate their surfaces. The Pt-Cr substrate demonstrated a rough surface profile with multiple parallel grooves resembling scratch marks presumable after polishing (figure 6-3 E). AFM measurement revealed that the grooves on the Pt-Cr samples had the dimensions of 7.2 ± 0.31 µm in width and 0.39 ± 0.082 µm in depth. Their surface feature might be the result of sandblasted surface treatment. Moreover, the ePTFE substrate displayed an extremely rough surface with randomly stretching grooves (figure 6-3 G). AFM measurement revealed that the grooves on the ePTFE samples had the dimensions of 1.5 ± 1.56µm in width and 0.86 ± 0.973µm in depth.

After applying spin coating at 6000rpm with the poly-SIBS nano-island
coating, the Nitinol®, Pt-Cr and ePTFE samples demonstrated similar nano-island features on their surface (figure 6-2 D, figure 6-3 F and H) as the ones we found on the 6000rpm spin coated glass samples (figure 6-2 B). Compared to the untreated glass and the 6000rpm spin coated glass controls, the nano-island coating produced on the Nitinol®, Pt-Cr and ePTFE samples showed very similar heights while slight variations in the other dimensions as well as the surface distributions. The AFM measurements of the nano-islands on the Nitinol® samples revealed the dimension of 287.2 ± 36.1nm in radius, 23.6 ± 6.2nm in height and 389.9 ± 100.2nm in spacing between them. While the nano-islands on the Pt-Cr samples were found with a radius of 71.6 ± 8.1nm, a height of 12.4 ± 3.0nm and spacing of 720.1 ± 112.2nm, and the ones on the ePTFE samples were measured with a radius of 366.1 ± 62.9nm, a height of 10.9 ± 1.2 nm and spacing of 1106.1 ± 252.9nm.
Figure 6-2. AFM images of poly-SIBS nano-island coatings on different samples (Part one). A. Untreated glass; B. 6000 rpm spin coated glass; C. Nitinol®; D. 6000rpm spin coated Nitinol®.
Figure 6-3. AFM images of poly-SIBS nano-island coatings on different samples (Part Two).
E. Plain Pt-Cr; F. 6000 rpm spin coated Pt-Cr; G. ePTFE; H. 6000 rpm spin coated ePTFE.

Summaries of AFM surface measurement for different poly-SIBS coated samples were shown in table 6-1 below.
Table 6-1. AFM surface measurement of different spinning speed poly-SIBS coated substrates (n=12 per condition in 4 repeats).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Surface feature</th>
<th>Island Radius (nm)</th>
<th>Island Height (nm)</th>
<th>Spacing (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated glass</td>
<td>Flat</td>
<td>Overall flat, with few minor surface defects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6000rpm spin coated</td>
<td>Nano-island</td>
<td>117.2 ± 12.3</td>
<td>16.7 ± 4.2</td>
<td>658.8 ± 59.0</td>
</tr>
<tr>
<td>glass</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plain Nitinol®</td>
<td>Smooth with dented spots</td>
<td>Smooth, with few dented spots as surface defects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6000rpm spin coated</td>
<td>Nano-island</td>
<td>287.2 ± 36.1</td>
<td>23.6 ± 6.2</td>
<td>389.9 ± 100.2</td>
</tr>
<tr>
<td>Nitinol®</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plain Pt-Cr</td>
<td>Rough with grooves</td>
<td>Rough surface with multiple parallel grooves with width of 7.2 ± 0.31 µm and depth of 0.39 ± 0.082 µm, resembling scratch marks.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6000rpm spin coated</td>
<td>Nano-island</td>
<td>71.6 ± 8.1</td>
<td>12.4 ± 3.0</td>
<td>720.1 ± 112.2</td>
</tr>
<tr>
<td>Pt-Cr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ePTFE</td>
<td>Extreme rough with randomly stretching grooves</td>
<td>Extremely rough surface with randomly stretching grooves with a width of 1.5 ± 1.56 µm and depth of 0.86 ± 0.973 µm.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6000rpm spin coated</td>
<td>Nano-island</td>
<td>366.1 ± 62.9</td>
<td>10.9 ± 1.2</td>
<td>1106.1 ± 252.9</td>
</tr>
<tr>
<td>ePTFE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Surface Roughness of different samples was also measured and described.
using Ra and Rq measurements. They are shown below in figure 6-4. We noted there was a significant decrease in both Ra and Rq values before and after we applied the poly-SIBS nano-island coatings on to Nitinol®, Pt-Cr and ePTFE (P < 0.001). The Ra values for Nitinol®, 6000 rpm spin coated Nitinol®, Pt-Cr and 6000 rpm spin coated Pt-Cr, ePTFE and 6000 rpm spin coated ePTFE samples was 20.76 ± 2.460, 10.04 ± 0.3682, 119.2 ± 36.42, 21.85 ± 3.725 nm, 271.0 ± 45.98 and 38.31 ± 3.400 nm, respectively, and the Rq for those samples were 28.02 ± 3.652, 12.95 ± 0.2646, 150.0 ± 40.45, 27.77 ± 4.653, 399.0 ± 59.00 and 40.97 ± 5.200 nm, respectively.
Figure 6-4. AFM surface roughness results of nano-island coatings on different samples (n=12 per condition in 4 repeats).

6.3.3 Hemocompatibility tests

6.3.3.1 Blood compatibility test

The BCI results of the poly-SIBS nano-island coatings on different samples were shown in figure 6-5 as follows:
Pt-Cr samples showed significant higher BCI values after applying the poly-SIBS nano-island coating (P < 0.001), while the ePTFE control showed the lowest BCI values comparing to all other samples (P < 0.05). There was no significant difference detected among all other samples.

### 6.3.3.2 Platelet adhesion test

The PAI results of poly-SIBS nano-island coatings on different samples were shown in figure 6-6 as follows:
Figure 6-6. PAI of poly-SIBS nano-island coatings on different samples (n=12 per condition in 4 repeats).

Plain Pt-Cr control samples, as well as, ePTFE controls show increased PAI values, exceeding the suggested range of general biomaterials for implantable devices, and all the poly-SIBS nano-island coatings show low PAI values, slightly below the suggested range. While all the other samples fell into the normal PAI value range, we also noted that the PAI value of all samples decreased after applying the poly-SIBS nano-island coating. However, none of the differences...
was significant (P > 0.05).

6.3.3.3 TEG

All sample groups applied for TEG generated typical cigar-shaped profiles.

The TEG results of poly-SIBS nano-island coating on different samples were shown in figure 6-7 as follows:
Figure 6-7. TEG results of poly-SIBS nano-island coatings on different samples (n=12 per condition in 4 repeats).
In the results, we found the R values (figure 6-7, upper left) for all the plain and poly-SIBS nano-island coated samples were within the normal range (9~27 mins), while ePTFE controls demonstrate a shortened R time compared to the normal reference range. R values for ePTFE were significantly shortened compared to all other samples (P < 0.001). We also observed slight elevations in R time after we apply the poly-SIBS nano-island coatings on to different biomaterials. However, none of the differences were significant (P > 0.05). The K values (figure 6-7, upper middle) of all samples fell on to the normal range (2~9 min). ePTFE controls also demonstrate a significantly shortened K time than all other samples (P > 0.001). Both glass and Pt-Cr samples demonstrate slightly prolonged K times after applying the poly-SIBS nano-island coating, while the coated Nitinol® samples showed a decrease in K time compared with plain Nitinol®. However, none of these differences were significant (P > 0.05).

The α values (figure 6-7, upper right) for all samples also fell into the normal range (22~58°). ePTFE controls showed the highest α-values than all other samples. However, there was no significant difference among all samples (P > 0.05). Similarly, MA values (figure 6-7, middle left) for most samples fell in the normal range (44~64 mm) except for ePTFE controls, which showed increased MA values exceeding the normal range. There were no significant differences in MA values among all sample (P > 0.05).

All the samples showed normal TPI values (figure 6-7, centre) except for
ePTFE controls, which displayed increased TPI exceeding the normal range (3~20 sec\(^{-1}\)). Both glass and Pt-Cr samples demonstrate slightly decreased TPI values after applying the poly-SIBS nano-island coating, while the coated Nitinol® samples showed increased TPI values comparing with plain Nitinol®. ePTFE controls had the highest TPI value, significantly higher than all the other samples (P < 0.001), while the plain Nitinol® had the lowest TPI value, however, there was no significant difference detected among all the samples (P > 0.05).

Similarly, all samples except ePTFE controls had negative CI values (figure 6-7, middle right), ePTFE controls show significantly higher CI values (P < 0.001), exceeding the normal range (-3~3). While all the poly-SIBS nano-island coated samples, as well as, plain Nitinol® samples had low CI values, falling below the normal lower range. Both glass and Pt-Cr samples demonstrated slightly decreased CI values after applying the poly-SIBS nano-island coating, while the coated Nitinol® samples showed increased CI values comparing with the plain Nitinol®. However, there wasn’t any significant difference detected among all the comparing samples (P > 0.05). There was no normal reference range for TMA (figure 6-7, lower left). The ePTFE controls showed the shortest TMA than all other samples; the difference was of significance (P < 0.001). There were no significant differences detected among all other samples (P > 0.05).

The results of LY30 (figure 6-7, lower middle) suggested that there was no thrombolysis on all samples 30 mins after achieving maximum amplitude. This
is a normal phenomenon according to the suggested reference values, and it is unlikely that more than 8% of thrombolysis effects can be expected at this point. As for the LY60 (figure 6-7, lower right), according to the suggested reference, less than 15% of thrombolysis effect can be expected at this point. All samples showed normal LY60 values. Both plain glass and Nitinol® samples demonstrate higher LY60 values compared to poly-SIBS nano-surface coated samples, the difference with plain Nitinol® and coated Nitinol® was significant (P < 0.001). The Pt-Cr samples showed significant lower LY60 values comparing to the Pt-Cr samples with poly-SIBS nano-island coatings (P < 0.001). However, the results also suggested that there were no thrombolysis effects observed on the ePTFE controls 60 mins after achieving the maximum amplitude.

6.3.4 HUVECs viability tests.

HUVECs cell viability was accessed by MTT and total DNA assays; results were shown in figure 6-8. No significant results from both assays between all culturing samples on day 1. By day 7, we noted both the MTT and total DNA values of the samples with poly-SIBS nano-island coatings were elevated compared to plain untreated samples. However, the differences were not significant (P > 0.05). By day 10, MTT and total DNA data of the poly-SIBS nano-island coatings continued to increase and were now significantly higher.
than their plain samples (P < 0.001). All poly-SIBS nano-island coated samples showed significant higher MTT values than TCP control (P < 0.001).

**Figure 6-8.** MTT and total DNA assays with HUVECs cultured on different poly-SIBS nano-island coatings on day 1, 7 and 10 (n=12 per condition in 4 repeats).
6.4 Discussion

Optimisation of the coating procedure using poly-SIBS nano-island coatings was found to be easily applicable on to biomaterial substrates with surface passivation producing a native oxide layer resulting in the presence of hydroxyl (-OH) groups on the surface. We demonstrate that the successful translation of the poly-SIBS nano-island surface coating on to metallic and polymeric biomaterials, all three of which are widely used as neurovascular and endovascular implantable devices. By following the same coating procedures, we managed to reproduce similar dimensions of nano-islands on to biomaterial substrates after optimisation of standard methods, and besides ePTFE, we have proven that the nano-island coatings on metallic biomaterials could enhance their hemocompatibility, as well as, promote in-situ endothelization compared to untreated biomaterials. This strengthens our origin hypothesis, that the poly-SIBS nano-islands are the key for promoting endothelization (Dalby et al., 2014, Chrzanowski et al., 2012, Biggs et al., 2010, Curtis et al., 2006).

We have successfully transferred the nano-surface coating platform on to both surgical grade Nitinol® and Pt-Cr alloy, which are used to develop ICS and BMS, respectively. We demonstrated successful uniform coatings applied to other biomaterial substrates. Post-coating characterisation suggest that reproducible and identical surface properties such as the wettability, contact
angles, surface energy, and nano-islands revealed by AFM on the surface similar to model glass substrates described in chapter 2, section 2.3.2.

The poly-SIBS nano-surface coated biomaterials showed no higher thrombogenicity values as with the original biomaterial. Moreover, HUVECs viability tests continue to demonstrate enhanced metabolic activities and cell growth and proliferation on the surface of poly-SIBS nano-islands despite the differences in biomaterials. These findings are consistent with the results from previous chapters regarding blood compatibility and promotion of endothelization on the treated biomaterial surfaces.
Chapter 7  Optimisation of poly-SIBS nano-island coatings using ultrasonic spraying techniques on BMS: Steps towards Translation

In previous chapters, we have successfully developed a non-toxic, poly-SIBS nano-surface coating that promotes endothelialisation on a range of 2D metallic biomaterial substrates. In this chapter, we have optimised standard coating procedures to reproduce the same coatings for 3D biomaterials and dimensions of poly-SIBS nano-islands on bare metal stents (BMS). Before implanting new biomaterials and medical devices in humans, they first must be evaluated *in vivo* for their safety and efficacy in animal models. Therefore, we performed a pilot study to evaluate poly-SIBS nano-island coatings on to commercially available BMS (Rebel™ PtCr stents) to evaluate their safety profile in preparation for pre-clinical studies.

7.1  Introduction

7.1.1  Ultrasonic atomization

The key to reproducing the exact same electrostatic coatings on 3D biomaterial substrates using poly-SIBS nanosurface coatings platform is to find
the right coating equipment to allow us to apply optimal conditions to transfer the coatings on to stent devices. The current industrial gold standard for coating medical device and stents utilizes ultrasonic atomization vapour spray technology. This approach applies energy in to liquid materials via a conducting ultrasonic energy. The traditional concept of ultrasonic atomization is where small droplets (10-30 µm) are formed on the surface of the material to produce a thin film when sonicated via ultrasonication. By applying different energy (kJ) and frequencies (Hz), this technique can generate different surface tensions on the liquid surface. Computational fluid dynamics (CFD) can visualise the capillary waves created by different surface tension, as shown in figure 7-1. With CFD stimuli, as the capillary wavelength increase, the surface tension also increases. The phase distribution indicates the increase of critical amplitude, which also causes the increase in surface tension (Yi et al., 2011).
Figure 7-1. CFD stimuli induced ultrasonic atomization. A. Atomization phenomenon creates different surface tensions generated on the liquid surface resulting in capillary waves; B. The capillary waves cause changes in amplitude.

By suspending the liquid droplets and applying ultrasonic waves, it can conduct similar energy on to liquid droplets surface and generate differences in surface tension to stretch the liquid droplet (Indolfi et al., 2011, Choi et al., 2014). A schematic diagram of the effects of ultrasonic atomization is shown in figure 7-2.
Figure 7-2. Ultrasonic atomization of liquids. A: Ultrasonic waves conduct energy on to the surface of the liquid droplets and generate different surface tensions resulting in the stretching of the droplets; B: by applying ultrasonic frequency and atomization power results in different surface tensions on the liquid droplet cause stretching and deformation.

7.1.2 Ultrasonic atomisation spray coating techniques (UASC)

UASC is one of the most widely used spray coating techniques in the medical device sector, which applies energy on to the materials via ultrasonic techniques to create thin film coatings in the range of 10-30 µm (Kim et al., 2017, Liu et al., 2017a, Marchal et al., 2017, Qiao et al., 2017, Williams et al., 2017, Choi et al., 2014, Yi et al., 2011). For example, polymers can be diluted in suitable solvents and then the nozzle can be used to spray fine particles, and droplets on to the surface of any 3D object, e.g. coatings tablets, stents and
other devices. Fine particles can become attracted to the material based on their surface chemistry (energy, charge) and wettability to create a splash spot on the surface of the target, as more particles are sprayed and applied on to the object, more splashed spots would merge in to a thin film layer, which eventually covers the surface of the 3D object to form a uniform coating. At this point, applying different surface tensions on to the polymer coating layer via high-frequency vibration can be used to serve a variety of platforms on 2D or 3D substrates. To control the surface tension applied via CFD stimuli by induced ultrasonic atomization, the parameters that require safe and effective control of the droplets include 1) particle size, 2) ultrasonic frequency, 3) ultrasonic atomization power, and 4) viscosity of the polymer solution.

7.1.3 Animal models for testing endovascular stents

Over the years, various animal models have been developed for testing biomaterials and medical devices from small to large animals, e.g. mice, rats, guinea pigs, rabbits, pigs, dogs and sheep. Different animals and different approaches are often chosen depending on very specific needs for testing, e.g. metabolic activity. For endovascular implants due to their unique requirements, as well as, their mode of delivery, larger animal models such as pigs, sheep and horses. The logic is simple, most of the endovascular implantable devices come down to two categories: 1) vascular stents or 2) heart valves. For small diameter stents between 3~6mm, and the desired implantation site would be
similar diameter blood vessel; for heart valves, they usually require to be placed into the animal hearts with similar structures to the size of the human heart.

Moreover, standard radiological interventional delivery techniques for stents and heart valves require peripheral arterial access, which is preferred to be far away from the designated implantation site to avoid interference of the puncture entry, and with a diameter no less than 1.5mm to introduce the entrance sheath. With this in mind, all small animal models were excluded as the largest vessel in all species was less than 3mm in diameter (Chan et al., 2011). Ovine models had been a preferred option for testing endovascular implants and medical devices due to their similarity to the human vascular system. This allowed most studies to design the implant site and location to match the intended application of their medical device and ensure that the entry point would be large enough for the peripheral arteries. This application has been used to test tri-leaflet heart valves, and bypass grafts, as well as, drugs and various bio-active coatings (Wang et al., 2010, Kannan et al., 2007, Aldenhoff et al., 2001). Porcine models were another popular choice, especially for endovascular stents. The reason why they were more favourable for testing stents is mostly due to the unique anatomy of their cardiovascular system, and arterial system from the brain to the lower extremities was almost in one straight line without any major curves. This makes the delivery procedures much easier as it does not require complex radiological interventional navigation techniques.
to get to the desired implantation site. Much work had been developed on porcine models over the years, besides the normal healthy physiological model, multiple pathological models such as an aneurysm and arteriosclerosis models were developed and optimised for testing various stents. Porcine models was one of the preferred biological models for testing ICS (Swindle et al., 2012). Over the years, it had been selected to test polymeric biomaterials or hydrogel-coated stents, DES, and stents with bio-active coatings such as antibodies and therapeutic agents such as Persantin® (Matsuda et al., 2013, Kealey et al., 2012, Zhu et al., 2012, Meng et al., 2009, Cilingiroglu et al., 2006). Besides large animal models as the ones mentioned previously, canine as a medium size animal was also selected for testing stents due to the need for advanced veterinary care in canine models. There are very few studies for using canine models for testing novel stents, as well as, flow diverters - FDS (Darsaut et al., 2012, WAKHLOO et al., 1994).

7.1.4 Small animal models for testing the efficiency of small diameter stents

Currently, there are no well-established small animal models for testing small diameter stents. Most of the popular stent testing models, as mentioned previously, comes with a high financial cost, as well as, technical difficulties in animal husbandry. In order to meet the needs of this project, we require a more economic alternative to assess the safety and efficacy of the poly-SIBS nano-
island surface coatings on BMS, which is a small diameter stent between 2.5-4.5 mm. This would obviate the need for a large animal study, therefore reducing the number of large animals during pre-clinical studies and following the 3Rs principles or refinement, reduction and replacement of animals. In 2011, Ding and co-workers used a rabbit model to test endovascular aneurysmal embolizing devices (Ding et al., 2011). Here, they selected a rabbit model as a choice of small animal to test small diameter stents. The diameter of the rabbit aorta is approximately 3.5mm in diameter, which can vary from 2.8 to 5.5mm, and is ideal to test the safety and efficacy of poly-SIBS coatings on BMS. In this chapter, we explore the optimisation and translation of poly-SIBS nano-island coatings on to BMS using UASC techniques to explore coating uniformity and morphology. With this approach, we aim to reproduce the same dimensions and surface nanotopography as seen previously with poly-SIBS coatings on the surface of BMS, as well as, maintaining the stability of the final thin film structure after sterilisation, and cycles of crimping, expansion and stent deployment to test coated stents with conventional interventional procedures. Currently, there is no standard small animal model used for aortic stenting or any protocols available in the literature. Therefore, we aim to develop a small animal rabbit model and design a pilot study for testing small diameter stents and monitor their safety and in vivo performance of poly-SIBS nano-island coated BMS.
7.2 Material and methods

All chemicals were obtained from Sigma-Aldrich, UK unless otherwise specified.

7.2.1 Manufacturing of poly-SIBS nano-surface coatings

The Rebel™ Pt-Cr coronary stent systems (diameter: 3.5 mm and length: 8 mm) was kindly donated by Boston Scientific Inc. (Galway, Ireland). BMS was carefully dismounted from the attached balloon catheters after removing the packages. They were stored in clean, ambient conditions before surface treatments, and the balloon catheters were stored in clean, ambient conditions, ready for use following optimisation of standard coating procedures.

7.2.1.1 Stent surface treatments

BMS were cleaned ultrasonically with 70% v/v ethanol and 100% v/v acetone for 30 mins, respectively, before being placed in a laminar flow hood for drying. After drying, the BMSs were transferred to a warm alkaline cleaning solution (7.5% v/v NaOH) at 65°C and was placed inside an air circulated oven for 5 mins. The samples were rinsed with deionised water (dH₂O) and allowed to dry in air. After cleaning, the samples were treated with fluorinated (-F) organosilanes using the same procedures as described in chapter 2, section 2.2.1.
7.2.1.2 UASC of BMS

The UASC system (MediCoat DES 1000, Sono-Tek Corp. USA) was selected for coating BMS, and the poly-SIBS solution was diluted in tetrahydrofuran (THF) to control the viscosity of the solution ready for spray coating BMS. The key parameters for controlling UASC techniques include; 1) ultrasonic power, 2) polymer solution advancing speed, 3) focusing gas pressure, and 4) drying gas pressure. All parameters were adjusted to generate a stable flow and constant nozzle. Clean BMS was mounted on to a mandrill and placed inside the ultrasonic coater. The mounted BMS was then subjected to a pre-programmed spray coating cycle with the distance from the nozzle set at 15 mm with a translational movement of 2.0 mm/s, and rotational movement set to 120 rpm. The programmed cycle involved two sets of four back-and-forth spraying cycles with one drying step in between the cycles. The mounted BMS was set to endure a single-programme cycle, two programmed cycles with a 5min break in between and then four programmed cycles with the same break period. When the spray coating cycles had completed, all stents were transferred into an air circulating oven to dry at 65°C for 24 h before removal. Digital images and an outline schematic of spray coating procedures is shown in figure 7-3.
Figure 7-3. Spray coating procedure of BMS. A: MediCoat DES 1000 ultrasonic atomising spray coating system; B. Stents being cleaned and mounted on to a mandrill and loaded into a programmed spray cycle; C. Stents were transferred to an oven after spray coating.

7.2.2 Characterisation of the coated stents

7.2.2.1 FE-SEM

7.2.2.2 AFM

Surface characterisation (see 7.2.2.1-7.2.2.2) was carried out on samples following protocols described previously in chapter 2, section 2.2.3.2–2.2.3.3.

7.2.3 Assembly of the coated BMS system and dry run

After the poly-SIBS nano-surface coating was applied on to BMS and allowed to dry in the oven, the BMS was removed and assembled back on to the matching balloon catheters ready to perform an ex vivo dry run of stent deployment procedures. The purpose of the dry run was to repeat standard interventional deployment procedures in an ex vivo setting to make sure that
balloon inflation and stent placement could be easily achieved as planned.

7.2.3.1 Crimping of coated BMS

The coated BMS was re-mounted back on to their corresponding balloon catheters and was crimped using the stent crimper (SC model no. F033828) series purchased from Machine Solution Inc (MSI), USA. Digital images of the crimping machine are shown in figure 7-4.

![Digital images of the MSI SC](image)

**Figure 7-4.** Digital images of the MSI SC. A. Front view, shows the crimping chamber on the left and push quill on the right; B. and C. Side views; and D. Shows the dial on the pressure handle of the crimping chamber to adjust stent crimping to a designated size from 0-5 mm.

The MSI SC is designed to precisely and accurately reduce the diameter of BMS. It features a spring-loaded locking mechanism to hold the SC at the desired diameter, and a custom push quill to hold the delivery sheath and load
the product. This design comes with different models, and a variety of diameter and length ranges to crimp balloon-expandable stents (BES) and self-expandable stents (SES). Three BMS was coated using optimised spray coated procedures and re-assembled on to the balloon delivery system. The coated BMS and delivery systems were placed inside the SC, and the pressure was applied to the maximum strength. The machine was held at that pressure for 60 mins before releasing to ensure that coated stents were crimped on to the balloon catheter.

7.2.3.2 Dry run tests for stent deployment ex vitro

Three coated BMS was removed from the delivery sheath and placed on a clean bench. The coated BMS system was introduced through a 5F Guider Softtip XF guiding catheter (Boston Scientific, USA) until the entire balloon tip was exposed via the distal end of the catheter. The coated BMS system was connected to the Encore™ balloon Inflator using a three-headed hydra connection provided, and normal saline (0.9% NaCl) was infused into the system. Stent deployment was commenced by initiating the inflation device attached to the balloon catheter. By slow rotations of the inflator’s handle, the pressure increased until the gauge reached 10 ATM of pressure, which was held for 10 secs before deflating the balloon by applying maximum negative pressure. Finally, the balloon was deflated, and the catheter was removed from the guiding catheter following the successful deployment of coated BMS.
7.2.4 Poly-SIBS coating delamination study

Under current ISO test standards (109934/25539) for endovascular stents, all BMS must undergo three cycles of crimping and expansion procedures without causing any unwanted damage to the integrity of their overall stent structure (Chen et al., 2014, Kealey et al., 2012, Wang et al., 2010). We performed three crimping and expansion cycles with optimised spray coated stents, following the same procedures as mentioned previously. FE-SEM image analysis was used to examine the coating integrity and overall structure in detail.

7.2.5 Sterilisation of poly-SIBS nano-island coatings on BMS

Poly-SIBS nano-island coatings on BMS following satisfactory crimping and expansion cycles was mounted on to the delivery system, and was re-introduced back into its original sheath, and repackaged and sent to H.W. Andersen Products (Andersen Products Ltd, UK) for ethylene oxide (EtO) gas sterilisation. A 12 h EtO sterilisation cycle was run on each delivery system, as indicated by both a positive surface indicator and negative microbiological tests included inside each sterilising patch to give a certificate of conformity.

7.2.6 Development of rabbit model for testing small diameter stents

All animal work used in this project was approved by the UCL research ethics committee under project ethical guidelines, project ID 9215/001, and all animal work was carried out in compliance with UK animal act 1986. Personal
certificates were obtained for module 1, 2, 3, and 4 for competency working with rats, mice and rabbits: UCL/15/24 and a personal certificate was obtained for the PIL A (theory & skills), and PIL B, K (theory) tests to work with pigs and sheep from the Royal College of Veterinary Surgeons (RVC/16/138. PIL: IB2C99B8B).

7.2.6.1 Surgical procedures

A rabbit model for testing small diameter stents was developed in collaboration with Northwick Park Institute of Medical Research (NPIMR), and linked with two related research projects working on small diameter stents within the Division of Surgery & Interventional Science funded by Action Medical Research (AMR), and the Engineering and Physical Sciences Research Council (EPSRC). Healthy New Zealand White (NZW) rabbits were purchased and delivered to NPIMR and acclimatised in the designated dormitory for a minimal of 6 weeks before surgical procedures. All rabbits were given a high energy diet to ensure growth to their optimal size by the end of the acclimatisation period. The optimal size for rabbits was between 4.0 to 6.0kg, thus to ensure that the aorta had reached their largest diameter, while the body fat ratio remained within the healthy range. All animals were inspected daily to monitor growth and health. Animals were given 37.5mg Clopidogrel and 37.5mg Aspirin on the day of surgery. Oral sedatives (40mg meshed diazepam tables mixed with banana) was given before initiating general anaesthesia. The skin
area on the abdominal and lower limbs was shaved and prepared as the operating area during the induction period. Buprenorphine 0.03mg/kg IM, ketamine 35mg/kg/xylazine 5mg/kg SC (3 mins after) and carprofen 0.1mL/kg SC was given for analgesia before a face mask was placed on the rabbit to maintain anaesthesia with isoflurane and oxygen. The rabbit was allowed to breathe spontaneously, and isotonic saline IV was continuously given via the ear vein through the procedure. The depth of anaesthesia was accessed by withdrawal reflexes by pinching the ear of the rabbit and oxygen (O₂) saturation, and heart rate was assessed via a pulse oximeter probe in peripheral tissue. Operators were equipped with X-ray protective gear before scrubbed hands and put on aseptic surgical gowns. Lower abdominal and lower limb skin regions of the animal was scrubbed followed by a straight incision on the animal’s upper right leg to dissect and expose the femoral artery. Once the artery was exposed, a surgical microscope was applied to assist the procedures that followed. The distal end was tied off, and an untied suture loop was placed at the proximal end of the exposed artery, procaine was given locally, and allowed to rest to dilate the vessel. The proximal end was clamped, and a transverse incision was made on the artery where the wire and a sheath was inserted, and then slowly the clamp was removed as the sheath was fully inserted. Once successful, the wire was withdrawn, and the sheath was fixed by suturing it on the skin. 20ml of fresh whole blood was collected via the sheath
before 100 unit/kg of heparin was administered via IV channel in the ear vein.

A detailed diagram of the procedures mentioned above were illustrated in figure 7-5, 7-6 and 7-7 as follows:
Figure 7-5. NZW Rabbit model for testing small diameter stents (step one): skin preparation and position for general anaesthesia.

Step One

a. Skin area on the abdominal and lower limbs was shaved and prepared during induction

b. Face mask being placed on the rabbit to maintain anesthesia with isoflurane and oxygen (O₂)
Figure 7-6. NZW Rabbit model for testing small diameter stent (step two): incision made and exposing the femoral artery.

Step Two

a. Straight incision was made on the animal’s upper right leg

b. The femoral artery was dissected and exposed
Step Three

a. The sheath was gently introduce the sheath into the exposed femoral artery

b. The sheath was sutured and fixed in place, ready for radiological intervention

Figure 7-7. NZW Rabbit model for testing small diameter stent (step three): arterial sheath insertion.
A Radifocus Guild Wire M and a 5F Boston Guider Softtip™ XF delivery catheter sheath was used to navigate to the designated section of aorta according to Innova 3100 DSA (General Electricity, USA) and an initial angiogram was performed. The BMS catheter was connected to the Boston Encore™ balloon inflating device before being infused with saline. The stent delivery system was then navigated under the guidance of initial angiogram to position the stent in the level of the second vertebra beneath the opening of the left renal artery. After the stent catheter was in place, the balloon was inflated for 10 sec (under optimal pressure suggested by the balloon pressure guide, as shown in the previous section of crimping and expansion tests) for stent deployment. Then balloon deflated, and negative pressure was maintained for approximately 10 sec. The balloon catheter was gently withdrawn, leaving the stent in place. Aortic angiography and a 3D CTA were performed using DSA to check the position of the stent. Once satisfactory imagine results were obtained, the guide catheter was then removed along with the arterial sheath. The femoral artery was ligated, and the wound closed up before the animal was resuscitated. An image of the Innova 3100 DSA machine is shown in figure 7-8.
7.2.6.2 Post-surgery aftercare

Carprofen (0.1mL/kg) SC per day was given for three days after surgery to each animal and daily medication of 37.5 mg Aspirin and 37.5mg Clopidogrel dissolved in 2 ml water and added to mashed banana or bread added to their diet. Animals were inspected daily to monitor wound healing and possible post-surgery complications. Animals were kept for 28 days and enrolled into the harvesting procedures. Anaesthesia and surgery protocol remained the same with an incision made on the left leg on harvesting. 20ml of fresh whole blood was collected after sheath administration and an angiogram and a 3D CTA were performed using DSA. 100 mg/kg Pentobarbital Sodium IV was given for termination. The aorta segment containing the stent was harvest with a minimum of 1cm surplus aorta on both ends. Both ends of the vessel were wet by saline or PBS solution to avoid tissue drying and the whole tissue was placed
in a labelled pot containing 4% v/v formalin with the proximal end of the vessel marked. Representative samples of the local lymph nodes, spleen, kidney, heart, lung and liver were taken and stored in 10% v/v formalin for toxicity studies. Altogether, 9 BMS were implanted in to 9 different NZW rabbits. A detailed diagram of the procedures mentioned above is illustrated in figure 7-9.

Figure 7-9. Tissue harvesting after animal termination: A. Postmortem incision; B Locating the stent inside the abdominal aorta.

7.2.6.3 Histology analysis

Histological analysis was performed on the enveloping stent aorta. PMMA resin H&E and Masson’s trichrome (MT) stained sections were microscopically screened for histopathological changes. The luminal occlusion percentage of treated test or control sites was quantitatively evaluated from H&E-stained
PMMA sections of aortas. The following parameters were measured by morphometry on one section of each test or control site from each animal: 1). Residual lumen area (RLA), including the stent strut area (SSA); 2). Theoretical lumen area (TLA) including the stent strut area (SSA). All morphometric measurements are shown in the following figure 7-10:

![Diagram](image)

**Figure 7-10.** Schematic drawing of the morphometric measurements of the vessel lumen.

The patency ratio was calculated with equation 1, as the following:

\[
\text{Patency ratio (\%) = } \frac{\text{RLA}}{\text{TLA}} \times 100
\]

Eq 1.

The luminal occlusion percentage (stenosis) was calculated with equation 2 as follows:

\[
\text{Occlusion ratio (\%) = } \frac{\text{TLA} - \text{RLA}}{\text{TLA}} \times 100
\]

Eq 2.
7.2.7 Evaluation of the nano-island coating ICS in the pilot rabbit model

Altogether, two sterilised poly-SIBS nano-island coated Pt-Cr BMS was implanted into two healthy NZW Rabbits following the exact protocol as described previously. DSA graphic and post-mortem histological analysis was carried out and compared with BMS as a model control.

7.3 Results

7.3.1 Manufacturing of poly-SIBS nano-surface coated stents

After adjusting the main parameters, we found that we can generate stable and constant nozzle flow using 1% poly-SIBS solution to advance at 0.03 ml/min with the ultrasonic power set at 0.83 W, and focused gas at 1.5 psi, and a drying gas at 3.0 psi to stabilize the settings of the UASC machine.

7.3.2 FE-SEM

FE-SEM image analysis of ultrasonic spray coatings on BMSs are shown in figure 7-11.
Figure 7-11. Representative images of control and sprayed coated BMS. A. BMS (control); B. BMS coating after a single spray cycle; C. two spray cycles; and D. four spray cycles.

FE-SEM image analysis revealed that after the coating steps, there are layers of poly-SiBS nano-island coatings on the BMS compared with the plain BMS. However, the integrity of the coating varied under different coating conditions and was dependent upon the number of coating cycles. For example, BMS had a smooth and flat surface profile with almost no identifiable surface features with some surface imperfections. BMS with a single spray coating cycle had polymer coatings that show incomplete surface coverage, and small holes that exposed the underlying BMS and stent structure. Higher
magnification images show very thin layers of polymer detaching from the surface as a result of a non-uniform coating that was delaminating from the surface due to incomplete spraying. When the number of cycles was doubled, we see a consistent, uniform thin film coating on the stent structures indicating that coating procedure was successful on to the 3D stent structure. Higher magnification images revealed a relatively smooth coating and surface profile. Finally, by applying four spray coating cycles to BMS was found to have complete surface coverage with an intact layer of polymer film on the surface compared to single cycle coatings. Higher magnification images show that with four cycles of UASC, there were distinctive differences in the surface morphology and topography compared with two cycles of spray coated BMSs. Overall, this surface appeared to be rough, and corrugated with more surface groves and pits, as a result of the accumulation of excess polymers on the stent surface, which produced a thicker coating layer and more variable surface profile.

7.3.3 AFM

AFM images of ultrasonic electrostatic spray coated BMS revealed similar findings to images obtained with FE-SEM, as shown in figure 7-12. Due to the incomplete coverage of the single spray cycle coated BMS, AFM imagining revealed very few surface features on the sample surface, showing that the coating coverage was non-uniform throughout the sample surface.
Figure 7-12. Control and sprayed coated BMS. A. BMS (control); B. BMS after two cycles; C, and four cycles.
It can be seen that sharp edge effects and a predominantly flat surface on Pt-Cr BMS during AFM analysis presumably due to electropolishing. After two spray coating cycles on BMS, AFM revealed nano-islands were present on the BMS surface. The morphology of the poly-SIBS nano-islands was similar to spin-coated samples, as shown previously in chapter 2, section 2.3.2.2 and chapter 6, section 6.3.2. AFM measurements of the nano-islands revealed the dimension of 85.1 ± 11.0 nm in radius, 18.9 ± 6.1 nm in height, and 590.1 ± 180.8 nm in spacing. BMS samples with four spray cycles revealed a surface profile with deep grooves and pits resembling a similar topography to dip coated and 1000 rpm spin-coated samples described earlier in chapter 2, section 2.3.2.2. AFM measurements revealed surface grooves and pits with dimensions of 200 to 950 nm in width and between 50 to 180 nm in depth.

Coating uniformity and delamination tests

7.3.3.1 Crimping spray coated Pt-Cr BMS

A schematic diagram of stent crimping procedures is shown in figure 7-13.
Figure 7-13. Stent crimping procedures. A. Structure of the stent delivery catheter and positioning of the stent; B. Spray coated stent loaded on to a balloon catheter, before crimping; C. Stent crimped under maximum pressure; D. Spray coated BMS crimped down on to the balloon catheter; E. The spray coated stent delivery system installed in the protective sheath prior to sterilization with EtO.
It can be seen that crimping and expansion tests was successfully carried out on to the spray coated BMS without any noticeable surface imperfections or defects on the thin film coating, which remained intact on the surface of BMS.

7.3.3.2 Deployment test

All three-spray coated BMS went through the dry run tests, and the results are presented in figure 7-14. We can see that the spray coated BMS was successfully delivered and deployed in an ex-vivo setting following the same procedures following standard stenting protocols in vivo.
Figure 7-14. A diagram describing a dry run of stent deployment. Left: Schematic diagram of balloon expansion and deployment in vivo; Right. The ex vivo dry run of spray coated BMS matching the in vivo stenting protocol. A. The stent catheter is passed through a 5F guiding catheter; B. Balloon inflated to deploy the spray coated BMS; C. Balloon deflation; D. Balloon catheter is withdrawn leaving behind the spray coated BMS.
7.3.4 Delamination study

FE-SEM images of the three UASC BMS, which underwent three cycles of crimping and expansion are shown in figure 7-15. We can see from the high-resolution images that all of the three stents maintained their structural integrity after three cycles of crimping and expansion. The coating of stent 02 is almost perfectly intact, while coatings on stent 01 and 03 have minor stretch or fatigue marks, which can be observed in singular locations, presumably due to the detachment of the coating layers. The higher magnification images revealed a more detailed surface morphology of the coated surface of stents, and they were found to remain smooth and intact covering almost the entire surface of the stent. Generally, the coatings on the stents remained intact, and unaffected after the three cycles of crimping and expansion testing.
Figure 7-15. FE-SEM images of the spray coated stents after three cycles of crimping and expansion. A. Stent 01; B. Stent 02; and C. Stent 03.
7.3.5 *In vivo* NZW rabbit model for testing small diameter stents

All 9 BMS were successfully implanted into the rabbits following the surgical protocol, and all of them survived through the 28 days trial. The rabbit model for testing small diameter stents demonstrate a 100% successful rate. Among the 9 BMS, the data from 6 NZW rabbits could not be disclosed due to the time or reporting and disclosure of new IP from collaborative research projects. Histological results describe the remaining 3 controls of BMS. The BMS structure remained integrated within the histological sections, and after being implanted for 28 days *in vivo*, we noticed the presence of early-stage endothelial progenitor cells in the lumen of the BMS. We also found multifocal neointimal hyperplasia (NIH) formation on various spots in the BMS, however, very few neutrophils and white blood cells were present indicating low levels of inflammation. Representative images of the BMS histological analysis is presented in figure 7-16 as follows.
Figure 7-16. Representative images of BMS histological samples. Upper: H&E staining of the stent deployed in the aorta forming an enveloped segment; Lower: MT staining.

The TLA was measured in all of the BMS control from H&E stained histological specimens and was found to be 1.02 ± 0.22 mm² while the RLA was 0.92 ± 0.08 mm². The patency ratio was 92.79 ± 3.61 %, and the occlusion ratio was 7.21 ± 3.61 %.
7.3.6 In vivo testing of poly-SIBS nano-island surface coatings

Poly-SIBS nano-island coatings on BMS was successfully sterilised through EtO sterilisation and was successfully implanted into NZW rabbits. Both rabbits survived 28 days of implantation and were terminated with the stent and enveloped aorta harvested. Digital subtraction angiography (DSA) findings show that during the implantation poly-SIBS nano-island coatings had good wall apposition against the implant site inside the blood vessel. Vessel patency immediately after surgery was satisfactory. Selected images of the DSA analysis of poly-SIBS nano-island coated BMS are shown in figure 7-17.

![Representative images of DSA analysis of poly-SIBS nano-island coated BMS: Left – before stent deployment; Right – after stent deployment.](image)

We are currently waiting for the harvest date and DSA graphic analysis (at the time of writing this thesis), as well as, post-mortem histological analysis for
the poly-SIBS nano-island coated BMS. We intend to compare the vessel patency after 28 days *in vivo*. We have shown that by using coating optimisation with UASC can successfully translate the poly-SIBS nano-island coatings on to 3D biomaterials such as BMS. We also found that we could successfully reproduce the poly-SIBS nano-island coatings on to BMS. We also demonstrate the feasibility of applying the spray coating on to the stent delivery system, and that they can be deployed under standard interventional methods by crimping and expansion studies before *in vivo* studies. Moreover, we have also demonstrated the feasibility of using a small animal, rabbit aorta stenting model for testing small diameter stents, and prove that by carrying out this procedure can potentially obviate the need for large animal studies during pre-clinical testing. We have successfully implanted two poly-SIBS nano-island coated BMS into two NZW rabbits as a pioneer study to evaluate theirs *in vivo*.
Chapter 8  Conclusion & Future Work

In this thesis, we have successfully developed a novel coating platform composed of poly-SIBS tri-block copolymers to create electrostatic surface coatings that create nano-pits, nano-grooves and nano-island surface topographies on to a range of model and biomaterial substrates to influence the cellular response. In vitro tests demonstrate both haemocompatible and biocompatible properties, as well as, the ability to promote and enhance endothelization. We optimised the coating procedure using ultrasonic vapour atomization spray coating (UASC) technology and applied the coatings on to commercially available bare metal stents (BMS). Following coating optimisation and stability of the coating layer, we also tested its safety and efficacy in vivo using a small animal rabbit model. This proof of concept study will test the development of next-generation stents incorporating nanotechnology to improve the performance of current BMS and ICS devices used for cardiovascular and neurovascular patient management, respectively.

The development of CVD and sudden onset of stroke is a devastating condition affecting over 100,000 people annually in the UK alone. For those who survive are highly prone to disabilities resulting in a poor quality of life. ICS are essential devices used for stroke management. However, the materials can become problematic. They are highly thrombogenic and have a poor tissue response due to limited endothelization and overproliferation of SMC, leading to re-stenosis and Ni sensitivity, which contributes to inflammation and immune responses leading to their failure. It is well known that the surface topography
of biomaterials at the nanometer scale (1-100 nm) has been shown to influence cell fate such as adhesion, migration, growth, proliferation, differentiation and apoptosis.

Moreover, ECs have been shown to increase their level of cell orientation and elongation, adhesion, spreading and migratory behaviour, and enhanced filopodia formation when in a substrate nanotopography. Our original hypothesis is agreement with the findings in this thesis, as we have developed a range of electrostatic coatings using poly-SIBS and produced a variety of nanoscale surface features by applying different coating methods, and when suitable optimised was found to enhance endothelialisation. The novel coating procedures that we had developed in this thesis was the ability to manipulate the packing density and distribution of the hard and soft segment chemistry to create a variety of poly-SIBS nanoisland surface coatings ranging from 10 nm in height with a radius of 200-750 nm in radius and was dependent upon the speed of deposition of the cationic polymer layer on to the negatively charged fluorinated organosilanes base layer on the substrate surface. When the speed of deposition was significantly reduced, the nanoisland features became densely packed together to form irregular nanopores and nanopits on the substrate’s surface, followed by nonporous structures on the underlying base substrate. This resulted in a change in surface properties (e.g. wettability, contact angle, surface energy and was a highlight of this thesis.

Next, we explored the biological response and investigated their interactions with components of the blood. RBC interactions were studied using the BCI, and platelets via PAI, as well as, whole blood experiments using TEG. We found that the poly-SIBS nanosurface coatings were generally superior to
all other samples, as well as, ePTFE controls. Especially, poly-SIBS nano-islands, which were processed at 6000 rpm from spin-coated samples, which showed prolonged contact activation times, less potential to form blood clots, better thrombolysis effects after clot formation, and significantly less (activated) platelets. Overall, the coatings were less prone to induce thrombosis and confer some level of anti-thrombogenicity when compared with controls.

Biocompatibility and cytotoxicity tests on the poly-SIBS nano-surface coatings were carried out to evaluate the cellular response. We found that the fluorinated (-F) organosilanes (CDTFS) treated samples, as well as, samples that were spin coated at 6000rpm could promote endothelization by enhancing both the metabolic and proliferation activities of HUVECs. Long-term stability and durability tests of the poly-SIBS nanosurface coatings demonstrate that the coatings have relatively longer stability and prolonged shelf-life when placed in culture with HUVECs. However, they are prone to detachment and remove themselves when cultured with hASMCs for the long term.

In this thesis, we have demonstrated that the poly-SIBS nano-island coatings enhance cell behaviour, metabolic activity, growth and proliferation of HUVECs, which suggests that these substrates are key to promoting in-situ endothelization when the device is coated and placed in vivo. Migration studies, FE-SEM and immunofluorescent staining have revealed further details about their gross morphology and cell function when contacting the nanoisland coatings. The nanoisland coatings were found to promote HUVEC adhesion, migration, and proliferation, and also induced the formation of filopodia at the edge of the cells, presumably through cell-signaling from biochemical or physical changes on the substrates to sense the nanostructures and initiate
contact guidance cues through filopodial interactions to cause cell alignment and patterning on the nanostructures to induce a fast growth rate over a 10-day period. This suggests that the tissue was being regenerated at a faster rate, which promotes the formation of newly formed tissue at the vascular wall and warrants further investigation. Understanding cell-material interactions with poly-SIBS nano-islands provides the key to promoting endothelization, and the next step was to transfer this coating technology onto current stent biomaterials used for blood-contacting device applications. After further optimisation, we successfully transferred the poly-SIBS nano-island surface coatings onto surgical grade Nitinol® and Pt-Cr alloy, which are used to develop ICS and coronary artery stents. We demonstrated the feasibility and ease of transfer of the nano-island coatings onto metallic biomaterials with similar surface properties as seen on model glass substrates rather than those on polymeric biomaterials such as ePTFE, which are used for bypass grafts. Moreover, metallic biomaterials also showed no higher anti-thrombogenicity after applying the poly-SIBS nano-island coatings, and HUVECs was also found to enhanced metabolic activity, cell growth and proliferation on the nano-island coatings despite the biomaterial. This finding strengthens our original hypothesis that the nano-islands influence the cellular response at the tissue-implant interface.

At this stage, further optimisation was necessary to transfer the poly-SIBS nano-island coatings onto 3D substrates to turn this feasibility study into a technology towards clinical applications, e.g. BMS. UASC techniques were fully optimised to translate the poly-SIBS nano-island coatings, as seen on model substrates, as functional thin films on the surface of BMS structures, e.g. 3D stent struts. Moreover, poly-SIBS nano-island coated BMS
was mounted on to the Rebel™ stent delivery system, and in vitro deployment
tests was carried out and deployed under conventional interventional methods,
and crimping and expansion studies proved to be positive with no delamination
of the surface coating ready for in vivo pioneer studies. Their overall safety
profile of the poly-SIBS nano-island coated BMS was tested further in a small
animal rabbit aorta stenting model in vivo, and three control BMS was carried
out previously, and two poly-SIBS nanoisland coated BMS was implanted in
two NZW rabbits to evaluate theirs in vivo performance. The scheduled harvest
date was May 2018 when reports will be available shortly after outlining the
tissue response through histopathology of explanted stents and will explore the
extent of inflammation, NIH and endothelialisation occurring at the tissue-
implant interface.

Future work and limitations

In this study, we conclude in our findings that by manipulating the rate and
speed of deposition of poly-SIBS nano-islands on biomaterial substrates
promote endothelization by providing contact guidance cues for ECs. However,
the exact mechanism of how the nanomaterials induce cell-surface interactions
either through changes in surface chemistry or topography or vice versa
remains unknown at present. Distinctive changes in cell morphology and cell
phenotypes were noted on different surfaces, and hints towards changes in
phenotype (via cell differentiation) or genotype as a result of changes in protein
expression (e.g. extracellular matrix proteins) or gene expression may have
occurred under the influence of nano-islands and warrant future investigations.
Cellular signal pathways may have also become activated or deactivated by
interactions with the nanoislands, as well as, surface chemical or physical/mechanical cues to reveal the underlying mechanism behind cell-material interactions at the nanoscale.

Even though we have demonstrated the safety and efficacy of poly-SIBS nano-island coatings on BMS through in vivo pilot studies and developed an animal model, full post-mortem analysis of histological data is unavailable at present but expected to some similar finding as that seen in vitro.

However, the pilot study is only proof of the practicality and feasibility of the nano-surface coating ICS system. The number of testing subjects can hardly have enough power to prove any findings significant statistically. Moreover, we only collect data on the patency of the ICS at day 0 and at termination, as well as histology analysis at postmortem due to lack of expertise under a very strict and demanding timeline of the study setting. We did not manage to get more data from the serology of animal, like in vivo coagulation studies, systemic cytokine monitoring as well as other biomarkers like platelet activation factor etc.; this is because we were unable to secure species specified ELISA kits to detect all those markers and antibodies. We do have all the serum samples collected at the designated time point and kept in minus 80 fridges frozen waiting to be analysed when the appropriate kit arrives in future.

To advance this research further, more work is needed to optimise the UASC techniques for poly-SIBS nano-island coatings for other types of BMS and ICS. Better control over surface nanotopography on the stent surface will ensure quality and consistency and further bioengineering tests are needed such as peel strength tests and deployment tests to explore the coatings effects on radial elasticity. This would be followed by a full GLP pre-clinical study to
provide us with more data, and sufficient statistical power to demonstrate the superiority of the nanoisland coating for ICS compared to plain ICS or FDS. This would place the research into a strong position when liaising with regulatory bodies (e.g. MHRA), and eventually on to clinical trials.

With the support of MRC and the approval of MHRA, we would initiate a few first in man trials in well-established neuro-radiological interventional centres in London, such as UCLH, Charing Cross Hospital or St George’s Hospital. We will be setting up the case by case series and arrange for follow up for up to two years after the stenting procedure. Following guideline from the MHRA, we would submit our proposal for multiple centre stage 1 clinical trials to be audited if all data from the first in man trials were good. Moreover, we would start recruiting once the proposal is approved, trial coordinators would be appointed to manage all the records as well as keeping track of data collection. We will report our progress in the major medical and academic conference to update clinicians and scientists of our progress, to get more feedbacks and added back onto the trials protocols. When the trials concluded, more retrospective clinical studies can be conducted at a later stage to compare the efficacy of our nano-surface coating ICS and other ICS; and the efficacy of our nano-surface coating ICS and best medical therapy etc.

We intend to develop this technology further to explore controlled drug delivery, which would also be desirable for neurosurgical implants to promote wound healing across the blood-brain barrier, devices that promote angiogenesis, devices with anti-inflammatory effect to prevent in situ scar formation, as well as, their potential application in nerve regeneration. Our ultimate goal would be to develop the coating platform to improve the
performance of a number of stent devices including current ICS under GLP, and through clinical studies translate them directly in to the clinic without any unwanted side effects such as prolonged inflammation, NIH or thrombogenicity for neurovascular stenting in stroke patients, and take them directly to market through commercialization of this technology.

A flow chart of future trend of work is shown in the following:
Figure 8-1. A flow chart of future work
All references were last updated via EndNote at the time when this work is completed.


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Appendix

A Nano-surface Coating for Neurosurgical Implants

In the previous chapters, we already demonstrated the nanotopography could influence the cell fate of ECs and by controlling and manipulating the nanotopography on the poly-SIBS nano-surface coating we can promote in-site endothelization.

Similarly, we expect to achieve the same influence on the material-cellular interface with other cells of CNS origin. By providing combined biological moieties with nanotopographical cues, as well as, adding chemical structure and composition on the biomaterial surface, and other physiological stimuli, we can to influence cell behaviour and improve the neuro-interface, so as to promote nerve regeneration. Therefore, the we believe the potential of this poly-SIBS nano-surface coating platform to be applyed onto neurosurgical implantable devices.

Moreover, taking advantageous of the nano-scale surface feature would allow us to further exploit the possibly of using this coating platform to incorporate controlled delivery of therapeutic agents or enhance the surface electrical conductivity of the material.
Studies arise from the result this research - 1

Nano-surface Coating for Eye Prothesis

**Introduction**: Acute macular degeneration (AMD) and Retinitis pigmentosa (RP) are common causes of degenerative retinal disease and a major contributor of blindness worldwide. It is estimated that 1 in 3500–5000 persons are affected with RP worldwide and AMD results in 5-6% of all cause of blindness. Currently there is no cure for either of these illnesses. Several approaches to treating these diseases are being investigated. One approach seeks to capitalize on the relative preservation of functionality of the cells within the inner segment of the retina and their responsiveness to electrical stimulation. This backdrop has engendered the development of the bionic eye to restore vision to these patients. In spite of the promise of these implants, there are still challenges with performance stemming from poor integration of these implants at the neural interface. This study aims to focus on the use of the formerly mentioned polymer as a coating for electrodes in the bionic eye which shows selectivity to neuron cells.

**Material & methods**: Materials were characterized using contact angle ($\theta$) and AFM. The viability of Pheochromocytoma cells (PC-12) and
Human retinal pigment epithelial cells (ARPE-19) on each coating was assessed with Almar blue, total DNA and immunofluorescence. The ability to induce neuronal differentiation was also assessed for each polymer coating.

**Results:** This fabrication technique created unique nanotopography similar with previous studies. Characterisation of the surface topography revealed increase contact angle (θ) of the substrate with both 5% poly-SIBS (100 ± 2.23) and 5% poly-SIBS-MPS (93.4 ± 5.47) both with p<0.001. 5% poly-SIBS formed nanois-land with height of around 9nm and radius of around 200nm. In contrast, the 5% poly-SIBS-MPS formed a surface with ridges and grooves with depth of 113 ± 46nm and height around 150nm. Finally, our material show a significant impaired ARPE-19 cell metabolic activity and proliferation (p<0.05). Inconclusive finding for PC-12 Cells.

**Conclusion:** We have successfully applied another functional layer on top of our nano-surface coating while maintaining its nano-surface features. This proved the concept of the possibility of applying further functionalization techniques based on our nano-surface coating platform. We noticed there are distinctive morphological and behavioural difference of both PC-12 and ARPE-19 cells on the surface with nano-features than on flat surfaces. Further investigations are warranted to unveil the interactions between cells and those nano-features in detail.
Studies arising from the result this research - 2

Nano-surface Drug-Eluting Coating

Introduction: Currently, there is no commercially available DES for intracranial applications. The DESs can be used to treat neurodegenerative conditions such as Parkinson’s or Alzheimer’s disease via achieving highly selective up or downstream drug targeting, and release of anti-inflammatory cytokines, growth factors and antibodies. By infusing the drug into our poly-SIBS block copolymer, we hope to incorporate a thin layer of bio-active drugs on top of our nano-surface coating where it can elute in a controlled manner, while maintaining its nano-topography. In this study, we blend aspirin and clopidogrel into our nano-surface coating, and we test if this combination affects the thrombogenicity of the coating and the reaction of HUVECs towards this coating. Were this concept to be found successful, it would contribute to the future generation of ICSs where life-long or long-term systematic administration of DAPT or even anti-coagulation therapy, e.g. warfarin can be avoided to minimise unwanted side-effects.

Material & methods: Materials were characterised using contact angle (θ) and AFM. Drug-eluting profiles of the coatings were evaluated using
UV vision (UVV) spectrometry. Haemocompatibility of the coatings were evaluated via BCI, PAI and TEG. Moreover, finally, the viability of HUVECs were assessed with MTT and total DNA essays as well as immunofluorescence.

Results: We successfully blend the two different drugs into the poly-SIBS co-polymer. Characterisation with FE-SEM and AFM revealed the drug crystals on the surface of the coating. Drug-eluting curves confirmed the release of aspirin from poly-SIBS-ASP over six days. The concentration of aspirin increases from 0 to 18 and 93 mg/dl for 1, 2% poly-SIBS-ASP on day 6, whilst the 4% increase in concentration from 0 to 12 (mg/dl) on day 6 (P < 0.01). Similarly, with the clopidogrel drug-eluting curves, the concentration of clopidogrel (ug/dl) increases from 0 to 141 for 0.1 % poly-SIBS-CLO on day 6. Drug elution concentration for 0.2 and 0.4 % poly-SIBS-CLO increases from 0 on day 0 to 146 and 224 (ug/dl) on day 5 (P < 0.01). The haemocompatibility studies had also revealed increased BCI, deceased PAI, as well as improved TEG results comparing with controls (P < 0.01). There is no significant difference between plain poly-SIBS controls and the drug infused polymer with MTT and total DNA assay.

Conclusion: We have successfully infused two drugs, aspirin and clopidogrel into the nano-surface coating platform. Our finding suggested our nano-surface coating platform is suitable as a vector to be incorporated
a drug-eluting layer on. Moreover, drugs can be embedded in the reservoir between the nano-islands on the coating surface. We found both drugs we infused into our nano-surface platform being eluting into the contacting medium and demonstrated platelet inhibiting effect when in contact with active blood components. However, to achieve better control releasing effect and longer lasting drug-eluting period, more sophisticated drug processing technique or further modification of the coating platform is required to improve its property as an eluting vector.
Awards, qualifications, conference presentation and publications arising from this research

Awards:

1. Chinese National Scholarship for Outstanding Students Studying Abroad
   Awarded by the Chinese Scholarship Council in 2016

2. Robert Brown Travel Award
   Awarded by UCL in 2017

3. SLMS Graduate Student Conference Fund Award
   Awarded by UCL in 2017

Qualifications:

1. PIL (Personal license for animal research in the UK), specified species: rats, mice, rabbits, pigs and sheep
   Awarded by UK Home office in 2015

2. Certificate of attending a microsurgical skill training workshop
   Awarded by Blackrock Microsystems Europe GmbH in 2016

3. UCL Arena Associate Fellowship for teaching
   Awarded by UCL CALT in 2016

4. Certificate of GLP “Good Laboratory Practice” training
   Awarded by Northwick Park Institute of Medical Research in 2016
Conference presentations:

1. The next generation of Nitinol stent for intracranial applications
   
   Eleanor Davies-Colley Prize PhD presentations in 2015

2. A novel electrostatic nano-surface coating for neuro-implants
   
   UCL Departmental Research Seminar in 2016

3. A nano-surface patterned coating promote in-situ endothelization
   
   ESVS (European Society of Vascular Surgeons) spring conference 2016

4. Polymer nano-island surface coatings for intracranial stents
   
   ESB (European Society of Biomaterial) conference 2017

5. A nano-surface coating promotes endothelization for intracranial stents
   
   MRS (Material Research Society) fall meeting 2017

6. A Nano-Island Coatings Promotes Endothelization for Endovascular Stents
   
   ISABC Bordeaux meeting 2018

Publications:


2. The influence of porosity on the hemocompatibility of polyhedral oligomeric silsesquioxane poly (caprolactone-urea) urethane (2015). The international journal of biochemistry & cell biology(68), 176-186. doi:
