Thrombogenicity studies on micro/nano topographic surfaces

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Thrombogenicity Studies on Micro/Nano Topographic Surfaces

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Abstract

Platelet adhesion and activation resulting from the interaction between the flowing blood and the surface of foreign material of a medical device or implant are adverse events that lead to the formation of thrombus. The modulation of platelet-surface interactions to increase the thromboresistance of foreign surfaces is therefore a vital aspect in the design of blood-compatible surfaces. In this thesis, the effects of fibrinogen adsorption and subsequent platelet interaction on a variety of surfaces with chemical and topographical structures are investigated.

Carbon nanotubes are initially evaluated due to their promising applications in a variety of biological systems. They are employed as means of topographical features to investigate on the influence topographical effect on platelet interaction with surfaces. Additionally, the influence of surface chemistry was also investigated. Specifically, two different types CNT orientations, namely tip-wise and diameter-wise, are incorporated separately within the polymer matrix and compared to that of a pristine graphite surface with similar surface chemistry. The effect of surface chemistry was evaluated by comparing the results from two material surfaces; graphite and a biodegradable polymer. The results highlight that in addition to chemistry, appropriate topographical surface modifications can be a powerful approach to elicit a low level of platelet response.

It is demonstrated that size, density, and aspect ratio have a decisive effect on the degree of fibrinogen, platelet adhesion and activation of a particular surface, as

Abstract

quantified by the enzyme linked immunosorbent assay (ELISA), lactate dehydrogenase assay (LDH) and flow cytometry, respectively. The results indicate that by merely changing the physical topographic parameters of a surface with the appropriated dimensions, platelet adhesion and activation can be reduced to much lower levels than that observed on the “minimally thrombogenic” surfaces such as pyrolytic carbon. The topographical parameters found to induce low levels of fibrinogen adsorption and platelet response were high aspect ratio structures ($> 3:1$) with reduced interspacing (< 200 nm).

To understand the mechanisms behind the topographical effects, systematic studies of fibrinogen adsorption and platelet activation were carried out. The conformational changes of adsorbed fibrinogen were examined on the topographical surfaces and compared to that of a pristine surface by the Attenuated Total Reflectance – Fourier Transform Infrared (ATR-FTIR). The results showed that on thrombogenic surfaces, the conformation of fibrinogen deviates from that of the native fibrinogen. The results highlight that by tailoring the surfaces with the appropriate topographical features it is possible to influence the conformation adopted by fibrinogen on a surface and hence, the related platelet interaction with a surface.

The study carried out in this thesis demonstrates that in addition to the chemical characteristics of a surface, specific topographical parameters can impact greatly the thrombogenicity of a surface to a comparable extent. Hence, controlled topographic modifications to a surface could be a practical tool in the development of blood-contacting materials with low thrombogenicity.

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To this end, I would like to dedicate an image of my research work along with a poem in the following page to all my loved ones as well as those who have loved me.

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“LOVE WILL OVERCOME ALL OBSTACLES”

**Sometimes when we encounter roadblocks at certain point in our lives,
they can appear difficult, complicated and unachievable.
Just remember that these impossible hurdles can be overcome by love.
With words of encouragement and praises by our loved ones,
it will definitely bring us a step closer to the realization of our dreams.**

About:

*FESEM micrograph showing the nanostructures (obstacles) on a polycarbonate film
by nanoimprint lithography technique, exquisitely displaying an artifact of
the fallen nanostructures in the form of a heart shape (love).*

Publications arising from this work

Patent

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Journal articles

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3. L.B. Koh, I. Rodriguez, S.S. Venkatraman. *A Novel Nanostructured Poly (lactic-co-glycolic-acid) - Multi-walled Nanotube Composite for Blood-contacting Applications: Thrombogenicity Studies*. **Acta Biomater** 2009;5:3411-22.
4. L.B. Koh, I. Rodriguez, J.J. Zhou. *Platelet adhesion studies on nanostructured poly(lactic-co-glycolic-acid)-carbon nanotube composite*. **J Biomed Mater Res A**. 2008;86A:394-401.

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1. L.B. Koh, I. Rodriguez, S.S. Venkatraman. *Surface Nano-Patterning to Regulate Platelet Response on Polymeric Surfaces*. **ICMAT**, 28 June - 3 July 2009, Singapore (Poster).
2. L.B. Koh, I. Rodriguez, S.S. Venkatraman. *Platelet Response on Poly(D,L - lactide-co-glycolide) (PLGA) Film with Nano-structured Fillers*, **MRS fall**, 1 - 5 December 2008, Boston, USA (Poster)
3. L.B. Koh, I. Rodriguez, J.J. Zhou, S.S. Venkatraman. *Platelet Adhesion Studies on Poly (lactic-co-glycolic-acid)–Vertically Aligned Carbon Nanotube Composite Film*, **ICCMB**, 10 - 12 December 2007, Singapore (Oral Contribution).
4. L.B. Koh, J.J. Zhou, I. Rodriguez. *Platelet Adhesion Studies on Nanostructured Poly (lactic-co-glycolic-acid) – Carbon Nanotube Composite*, **ISTH**, 6 - 12 July 2007, Geneva, Switzerland (Poster).
5. L.B. Koh, J.J. Zhou, I. Rodriguez. *Preparation of Poly (lactic-co-glycolic-acid) – Carbon Nanotube Multilayer Film for Platelet Adhesion Studies*, **ICMAT**, 1 - 6 July 2007, Singapore (Oral Contribution).

Table of Contents

Abstract	<i>i</i>
Acknowledgements	<i>iii</i>
Publications arising from this work	<i>v</i>
Table of Contents	<i>vii</i>
List of Figures	<i>x</i>
List of Tables	<i>xiv</i>
List of Abbreviations	<i>xv</i>
Chapter 1	<i>1</i>
Introduction	<i>1</i>
1.1 Background	<i>1</i>
1.2 Development and Challenges	<i>2</i>
1.3 Scope of Study and Specific Aims	<i>4</i>
1.4 Significance of Research	<i>6</i>
Chapter 2	<i>8</i>
Literature Review	<i>8</i>
2.1 Evaluation on the blood compatibility biomaterials	<i>8</i>
2.1.1 Hemocompatibility of Biopolymers	<i>10</i>
2.2 Proteins	<i>12</i>
2.2.1 Protein adsorption at interfaces	<i>12</i>
2.2.2 Role of fibrinogen in thrombus formation	<i>14</i>
2.2.3 Adsorption properties of fibrinogen	<i>14</i>
2.2.4 Structure of fibrinogen and conformation	<i>16</i>
2.2.5 Fibrinogen binding sites to platelet	<i>17</i>
2.2.6 Quantification of adsorbed proteins	<i>19</i>
2.3 Platelets	<i>25</i>
2.3.1 Platelet biology	<i>25</i>
2.3.2 GP IIb/IIIa Receptor	<i>26</i>
2.3.3 Role of platelet in thrombus formation	<i>27</i>
2.3.4 Platelet characterization techniques	<i>31</i>
2.4 Carbon-based materials in biomedical systems and devices	<i>36</i>
2.4.1 Carbon films	<i>36</i>
2.4.2 Carbon nanotubes as artificial prostheses	<i>38</i>
2.4.2.1 Background on CNTs	<i>39</i>
2.4.2.2 Adsorption of plasma proteins onto CNTs	<i>40</i>
2.4.2.3 Carbon nanotube polymer composites for biomedical applications	<i>42</i>
2.4.2.4 Techniques for dispersing CNTs	<i>43</i>
2.4.2.5 Toxicity and Biocompatibility Issues of CNTs	<i>45</i>

Table of Contents

2.5 Surface modifications	48
2.5.1 Surface modifications on polymeric surfaces	48
2.5.1.1 Chemically modified polymer surfaces	49
2.5.1.1.1 Fibrinogen and platelet interaction with varying surface chemistries	50
2.5.1.2 Topographical structures on polymeric surfaces	53
2.5.1.3 Fabrication of Polymer Nanocomposite with CNTs	53
2.5.1.4 Fabrication of topographic surfaces in polymers with physical attributes	56
2.5.1.4.1 Fibrinogen and platelet interaction on topographic surfaces	58
Chapter 3	62
<i>Platelet Adhesion Studies on Nanostructured Polymer Carbon</i>	62
<i>Nanotube Composite</i>	62
3.1 Introduction	62
3.2 Materials and Methods	64
3.2.1 Materials	64
3.2.2 Functionalization of MWCNTs	65
3.2.3 Preparation of PLGA-MWCNT composite by LbL technique	65
3.2.4 Plasma protein adsorption for platelet studies	66
3.3 Results and Discussion	68
3.3.1 Analysis of functionalized groups on MWCNTs	68
3.3.2 Adsorption of MWCNTs onto PLGA film by LbL technique	70
3.3.2.1 Stability of PLGA film adsorbed with polyelectrolytes	72
3.3.3 Platelet studies on PLGA-MWCNT composite	76
3.4 Summary	80
Chapter 4	83
<i>Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled</i>	83
<i>Nanotube Composites with Different Nanotube Orientations</i>	83
4.1 Introduction	83
4.2 Materials and Methods	85
4.2.1 Materials	85
4.2.2 Fabrication of randomly dispersed PLGA-MWCNT composite	85
4.2.3 Fabrication of vertically aligned PLGA-MWCNT composite	86
4.2.4 Fibrinogen adsorption quantification by ELISA	86
4.2.5 Platelet adhesion studies	87
4.2.6 Characterization of PLGA-MWCNT composites	87
4.2.7 Statistical analysis	88
4.3 Results and Discussion	88
4.3.1 Functionalized MWCNTs in randomly dispersed PLGA-MWCNT composite	88
4.3.2 PLGA-MWCNT composites with oxygen plasma treatment	93
4.3.3 Analysis and quantification for adsorbed fibrinogen on substrates	98
4.3.4 Platelet adhesion analysis on substrates and response to nanostructured surfaces	103
4.4 Summary	108

Table of Contents

Chapter 5	110
<i>Platelet Interactions on Surfaces with Topographically Controlled Features</i>	110
5.1 Introduction	110
5.2 Materials and Methods	112
5.2.1 Materials	112
5.2.1.1 Substrates	112
5.2.1.2 Buffers, biochemicals and antibodies	112
5.2.2 Fabrication of nano porous anodized alumina (NPAA) template	113
5.2.3 Fabrication of master template in silicon	113
5.2.4 Fabrication of topographical structured features in PLGA film	114
5.2.5 Fibrinogen adsorption determined by ELISA	114
5.2.6 Platelet adhesion determined by LDH assay	114
5.2.7 Platelet activation determined by flow cytometry	115
5.2.8 Observation of adhered platelet on substrates by FESEM	116
5.2.9 Statistical analysis	116
5.3 Results and Discussion	117
5.3.1 Topographical dimensions of substrates	117
5.3.2 Adsorbed fibrinogen and platelet activity	118
5.3.3 Platelet adhesion and activation on topographically modified substrates	124
5.3.3.1 Interspacing effect	125
5.3.3.2 Aspect ratio effect	129
5.3.3.3 Width effect	133
5.3.3.4 Micron scale dimensions	135
5.3.3.5 Chemical versus topographical effect	137
5.3.4 Morphology of activated platelets	140
5.4 Summary	142
Chapter 6	144
<i>Surface Induced Conformational Changes of Adsorbed Fibrinogen on Surfaces</i>	144
6.1 Introduction	144
6.2 Materials and Methods	147
6.2.1 Materials	147
6.2.2 Conformational assessment of fibrinogen by Attenuated Total Reflection – Fourier Transform Infrared Spectroscopy (ATR-FTIR)	147
6.2.3 Fibrinogen adsorption quantification by ELISA	148
6.2.4 Statistical analysis	149
6.3 Results and Discussion	149
6.3.1 Conformational assessment of fibrinogen by ATR-FTIR	149
6.3.2 Adsorption kinetics of Hfg	159
6.4 Summary	162
Chapter 7	163
<i>Conclusions and Perspectives</i>	163
<i>References</i>	168
<i>Appendix</i>	187

List of Figures

Fig. 2.1: Chemical structure of poly(lactide-co-glycolide)(PLGA).	12
Fig. 2.2: Schematic illustration of a fibrinogen molecule showing the different domains and tertiary structure of the protein.	17
Fig. 2.3: Structure of platelet GP IIb/IIIa receptor. [149]	27
Fig. 2.4: Overview of blood coagulation cascade showing both the intrinsic and extrinsic pathways [151].	31
Fig. 2.5: Schematic drawing showing the effect of platelet activation on monoclonal antibody binding [168].	35
Fig. 2.6: Idealized representations of different structures of defect-free and opened carbon nanotubes showing a) concentric MWCNTs and SWCNTs with different chiralities displaying the b) armchair structure, c) chiral structure and d) zigzag structure [206].	40
Fig. 2.7: Schematic illustrating the sequential dipping of alternating polyelectrolytes with different charges in the electrostatic layer-by-layer self-assembly.	55
Fig 3.1: Schematic diagram on the fabrication process for the PLGA-MWCNT composite by employing the LbL technique.	67
Fig. 3.2: TEM micrograph on a segment of MWCNT showing (a) the presence of dark spots from carboxylic acid treatment and (b) magnified view of the dark spots on the walls of the MWCNTs indicated the possibility of defects induced as lattice distortion from the aggressive acid treatment.	68
Fig. 3.3: ATR-FTIR spectra of a) MWCNTs (control) and b) functionalized MWCNTs with the attached carboxylic acid groups at 1217cm^{-1} and 1738cm^{-1} .	69
Fig. 3.4: PLGA-MWCNT composite with an enhanced MWCNTs coverage with 0.08 wt% of PSS treatment.	71
Fig. 3.5: (a) PLGA-MWCNT composite showing a homogeneous MWCNTs adsorption with 15 mins and 30 mins dip in polyelectrolyte and MWCNTs solutions respectively and (b) a non homogeneous MWCNTs coverage due to the presence of local charges with a short dipping time of 1 min.	72
Fig. 3.6: Regions with no MWCNTs adsorbed on the PLGA-MWCNT composite showed signs of polymer degradation with the presence of pit-holes and uneven surface.	74

List of Figures

Fig. 3.7: (a) Reduced platelet adhesion showing no signs of activation on the PLGA-MWCNT composite, (b) increase platelet adhesion on the PLGA-MWCNT composite at regions with no adsorbed MWCNTs, (c) distinct platelet adhesion preferential on regions without any MWCNTs adsorbed and (d) platelet adhesion and activation observed on the pristine PLGA. 79

Fig. 4.1: Illustration on the orientations and dimensions of the multi-walled carbon nanotubes in the PLGA-MWCNT composite for a) randomly dispersed and b) vertically aligned. 89

Fig. 4.2: ATR-FTIR spectra on (a) acid treated MWCNTs and HOPG, (b) PLGA, (c) vertically aligned PLGA-MWCNT composite and (d) randomly dispersed PLGA-MWCNT composites, plasma treated PLGA and aged PLGA. The peaks at 1092 and 899cm^{-1} indicate the presence of the $-\text{COOH}$ groups. 91

Fig. 4.3: TEM image of the randomly dispersed PLGA-MWCNT composite showing (a) a well dispersed MWCNTs throughout the PLGA and (b) a magnified view of a single MWCNT well blended into the PLGA matrix from inset (a). 93

Fig. 4.4: AFM images (amplitude) showing the well-dispersed functionalized MWCNTs within the PLGA matrix with little agglomerations. (a) Before O_2 plasma treatment with an average $R_a \sim 13\text{nm}$ and (b) when a partial layer of PLGA surface was etched after 1 min of O_2 plasma treatment with an average $R_a \sim 39\text{nm}$. 95

Fig. 4.5: Contact angle variation on randomly dispersed PLGA- MWCNT composite with different O_2 plasma treatment times. An increase in hydrophilicity with treatment times can be observed. Contact angle readings ($n=3$) were taken 24 hrs after O_2 plasma treatment. 95

Fig. 4.6: ATR-FTIR spectra of the aged plasma treated PLGA films showing the disappearance of the $-\text{COOH}$ peak ($\sim 899\text{cm}^{-1}$) after a period of 20 days. 96

Fig. 4.7: FESEM images showing a) the original vertically grown MWCNTs, vertically aligned PLGA-MWCNT composites b) with 2 mins of O_2 plasma treatment revealing an enhanced PLGA infiltration, c) without O_2 plasma treatment prior to PLGA infiltration and d) the cross-sectional view of the nanotubes within the PLGA matrix (arrows refer to the protruding MWCNTs). 97

Fig. 4.8: Raman spectroscopy of the vertically aligned MWCNTs a) before PLGA infiltration treatment and b) after PLGA infiltration (PLGA-MWCNT composite) showing the presence of D ($\sim 1351\text{cm}^{-1}$) and G ($\sim 1577\text{cm}^{-1}$) peaks. 98

Fig. 4.9: Calibration curve showing the amounts of adsorbed Hfg on the microtiter plate against different concentrations of Hfg measured by ELISA. Data are expressed as the means $\pm$ SD of three independent measurements. 99

Fig. 4.10: Graphs showing a) contact angle measurements in which readings were taken 20 days after O_2 plasma treatment on the randomly dispersed PLGA- MWCNT composite, b) amount of adsorbed Hfg determined by ELISA and c) amount of adherent platelets. Data were run in triplicate ($n=3$) and expressed as mean $\pm$ standard deviation of 3 independent experiments. 101

Fig. 4.11: High platelet adhesion amount with discoid platelet morphology were observed with connecting membrane links that fuse platelets from one to another to form elongated (string like) aggregates on a) randomly dispersed pristine PLGA-MWCNT composite, b) magnified view from inset (a), and c) HOPG, d) magnified view from inset (c). 105

List of Figures

Fig. 4.12: FESEM images showing the morphology of adhered platelets on a) pristine PLGA film with large amount of platelet adhesion forming elongated (string like) aggregates and b) randomly dispersed etched PLGA-MWCNT composite with modest platelet adhesion and low spreading. 107

Fig. 4.13: Vertically aligned PLGA-MWCNT composite displayed a) the least platelet adhesion amount with low spreading as compared with other substrates and b) magnified view from inset (a). 108

Fig. 5.1: Quantification graphs showing the a) amount of fibrinogen adsorbed on substrates determined by ELISA, b) number of adherent platelets determined by LDH assay and c) PAC -1 expression on platelet activation derived from flow cytometric analysis. Data were run in triplicate ($n = 3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments. 121

Fig. 5.2: Forward versus side light scatter dot plots with mean intensities for stained platelet-bound fibrinogen obtained through gating displaying a) platelets only (positive control), b) higher platelet activation on the pristine PLGA as compared to c) P(1,2,5,8) with a lower mean intensity. All events were gated for platelet specific surface marker using PAC-1 FITC. 121

Fig.5.3: Scatter diagrams tabulated from Fig. 5.1 showing a strong correlation on the a) amount of adsorbed fibrinogen by ELISA with the number of adherent platelets by the LDH assay ($r = 0.88876$) and the b) number of adherent platelets against the PAC-1 expression for activated platelets determined by flow cytometric analysis ($r = 0.84492$). 124

Fig. 5.4: Platelet response on the substrates a) P(14,6,0.4) and b) P(8,10,0.4) showing similar amounts of adhered platelets, magnified view showing the morphologies of platelets adopting the c) spread-dendritic morphology where a high level of platelet activation is observed with increased interspacing on P(14,6,0.4) as compared to d) a reduced interspacing on P(8,10,0.4), adopting a dendritic morphology e) a supplement study comparing platelet adhesion on a topographical surface with interspaces of $\sim 0.15 \mu\text{m}$ versus a pristine surface showing a much higher adhesion level on the pristine surface, f) a supplement study on the effect of interspacing on platelet adhesion across the topographic PLGA substrate (constant aspect ratio with varied interspacing) displayed a higher amount of platelet adhesion with interspaces $\sim 1 \mu\text{m}$ (right) as compared to $\sim 0.15 \mu\text{m}$ (left) and g) tabulated data from (f) obtained by visually counting platelets over an average of 6 images, ($n = 3$). 128

Fig. 5.5: Plot of platelet adhesion per area versus aspect ratio of structures on the substrates tested. Substrates with increasing aspect ratio demonstrated reduced level of adherent platelets. An aspect ratio of 3 - 5 (arrow) appears sufficient to achieve a low level of adherent platelets. Dotted lines refer to the controls. Data are tabulated from Fig. 5.1b. 130

Fig.5.6: a) P(1,1,8) showing the clustering of pillars after drying, b) an unactivated platelet with a round shape morphology and c) platelet adhesion on P(1,1,8) displaying the lowest amount of adherent platelets (circled) as attributed by their high aspect ratio. 131

Schematic 5.1: Illustration on the effect of aspect ratio on platelet adhesion. a) The bending movements of the high aspect ratio flexible pillars prevent the platelets from making a successful contact on the topographic surfaces compared to b) the low aspect ratio pillars where they remain stiff and upright in liquid media even under agitation, thereby permitting platelet attachment at a greater ease. Dimensions are not drawn to scale. 132

Fig. 5.7: Platelet adhesion on P(1,1,2) showing a) moderate amount of adherent platelets and b) magnified inset from (a) displaying an activated platelet with spiky pseudopods. 132

List of Figures

Fig. 5.8: Platelet adhesion on P(1,2.5,8) demonstrating the a) preferential adhesion on the planar region in significant quantity as compared to the region with nanotopographical features and b) platelets with a dendritic morphology. 134

Fig. 5.9: Platelet adhesion on P(1,5,8) displaying a) the slight increase of adhered platelets (circled) observed on the 500 nm (width) pillars, b) magnified view showing a platelet with a spread-dendritic morphology on the tips of the pillars. 135

Fig. 5.10: a) Platelet aggregation and adhesion observed on micron-sized PLGA pillars, P(9,30,150) and b) an overlaid of fibrinogen on the periphery of pillars stretching from pillar-to-pillar. 136

Fig. 5.11: a) Platelet adhesion on pristine PLGA observed with the highest adherent platelet amount where this morphology increase the potential for multivalent adhesive interactions by enabling maximal surface contact area with the substratum surface and is evidenced in, b) showing the platelet spreading at its advanced stage, c) HOPG surface showing a moderately high level of adherent platelets and d) vertically aligned CNT C(~,1.5,190) displaying much reduced platelet adhesion level when compared to (c), implying the influential role of surface topography in the regulation for platelet activity. 139

Fig. 6.1: Native Hfg dissolved in PBS displaying a) FTIR spectrum with Amide bands I and II, b) fitted FTIR spectra of the Amide I band into its resolved component bands of (i) β -sheets, (ii) β -turns, (iii) α -helix, (iv) random chains, (v) extended chains and (vi) side chain moieties. 151

Fig. 6.2: Fitted FTIR spectra of the Amide I band into its resolved component bands of (i) β -sheets, (ii) β -turns, (iii) α -helix, (iv) random chains, (v) extended chains and (vi) side chain moieties on a-c) pristine PLGA and d-f) P(1,2.5,8), respectively at adsorption times of a) & d) 5 mins, b) & e) 30 mins, c) & f) 60 mins. 152

Fig. 6.3: Comparison plot showing the changes induced in the secondary structure of Hfg with adsorption times for a) pristine PLGA film and b) P(1,2.5,8). 154

Fig. 6.4: Comparison of the induced changes in the secondary structure content of the adsorbed Hfg showing the band components of a) β -sheet, b) side chain moieties, c) extended chains, d) random chains, e) α -helix and f) β -turns on the pristine PLGA and P(1,2.5,8) substrates with increasing adsorption times to the corresponding content in the native Hfg. * denotes significance for ($p < 0.05$). 157

Fig. 6.5: Ratios comparison of a) α -helix to β -turn structures and b) β -sheet to β -turn structures on the pristine PLGA and P(1,2.5,8), with respect to the native Hfg. * denotes significance for ($p < 0.05$). 158

Fig. 6.6: Adsorption kinetics of Hfg onto the various substrates at various adsorption times quantified by ELISA. Data were run in triplicate ($n = 3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments. 160

Fig. 6.7: Correlation plot on the amount of adsorbed Hfg to the induced changes in the secondary structure content of the adsorbed Hfg on the 3 major band components; a) β -sheet, b) β -turn and c) α -helix at various time intervals of 5, 30 and 60 mins, with their respective corresponding r values. Data are tabulated from Fig. 6.6 (amount of adsorbed Hfg) and Fig. 6.2 (secondary structure content). 161

List of Tables

Table 4.1: Wavenumber and assignment of ATR-FTIR spectra for PLGA, HOPG, randomly dispersed and vertically aligned PLGA-MWCNT composites. 92

Table 4.2: Tabulated data from Fig. 8 for the comparison between substrates for their statistical significant differences in a) contact angle measurement, b) amount of adsorbed Hfg determined by ELISA and c) amount of adherent platelets. The statistical significance ($p < 0.05$) is indicated by asterisks and $\neq$ denotes non-significance. Data were run in triplicate ($n=3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments. 102

Table 5.1: Summarized table showing an overview on the topographical dimensions and aspect ratio for all substrates. 118

Table 5.2: Comparisons between the statistical significance performed by ANOVA and tabulated from Fig. 5.1 for a) the amount of adsorbed fibrinogen, b) the number of adherent platelets and c) platelet activation intensity. Data were run in triplicate ($n=3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments. 127

List of Abbreviations

AAO	<i>Anodic aluminum oxide</i>
AFM	<i>Atomic force microscopy</i>
ATR-FTIR	<i>Attenuated Total Reflectance – Fourier Transform Infrared</i>
C₆₀	<i>Fullerene</i>
CA	<i>Contact angle</i>
CD 62P	<i>P-selectin</i>
CNTs	<i>Carbon nanotubes</i>
DCM	<i>Dichloromethane</i>
DLC	<i>Diamond-like carbon</i>
ELISA	<i>Enzyme linked immunosorbent assay</i>
FESEM	<i>Field Emission Scanning Electron Microscope</i>
FITC	<i>Fluorescence-isothiocyanate</i>
GP	<i>Glycoprotein</i>
HEMAPC	<i>Hydroxyethylmethacrylate phosphatidylcholine</i>
Hfg	<i>Human fibrinogen</i>
HOPG	<i>Highly ordered pyrolytic graphite</i>
HRP	<i>Horseradish peroxidase</i>
KQAGDV	<i>Lys-Gln-Ala-Gly-Asp-Val</i>
LBL	<i>Layer-by-layer</i>
LDH	<i>Lactate dehydrogenase</i>
LTI	<i>Low-temperature isotropic</i>
MWCNTs	<i>Multi-walled carbon nanotubes</i>
NPAA	<i>Nano porous anodized alumina</i>
PAC-1	<i>Procaspase-activating compound 1</i>
PBS	<i>Phosphate buffer saline</i>
PDDA	<i>Poly(diallyldimethylammonium chloride)</i>
PDMS	<i>Polydimethylsiloxane</i>
PEI	<i>Poly(ethyleneimine)</i>
PLGA	<i>Poly(lactic-co-glycolic acid)</i>
PRP	<i>Platelet-rich plasma</i>
PSS	<i>Poly(sodium-p-styrenesulfonate)</i>
RGD	<i>Arg-Gly-Asp</i>
SAMs	<i>Self-assembled monolayers</i>
SWCNTs	<i>Single-walled nanotubes</i>
TEM	<i>Transmission Electron Microscopy</i>
TMB	<i>3,3',5,5' – tetramethyl-benzidine</i>

Chapter 1

Introduction

1.1 Background

Biomaterials have played a critical role in the life-saving treatment of patients requiring implants and are becoming an important aspect in the practice of modern medicine. Biodegradable polymers, in particular have been used widely in the administration of pharmaceuticals and biomedical devices over the past decade. Some of the important applications of these biodegradable polymers are in areas of controlled drug delivery systems, artificial organ implants, scaffolds or other blood-contacting medical devices. Despite the wide use of biomaterials in the clinical environment, the biocompatibility of these materials is still far from ideal because a variety of foreign body reactions may be triggered in a living body when the medical device comes into direct contact with the blood. Typically, upon the contact with blood, foreign materials cause a rapid adsorption of plasma proteins on the biomaterial surface leading to platelet adhesion, activation, aggregation, and finally thrombosis formation through a cascade of reactions mediated by the nonspecific binding of plasma proteins [1]. The current control of thrombus formation by

systemic anticoagulant therapy is clearly not ideal due to the number of side effects associated such as thrombocytopenia, neutropenia and hemorrhage. Thus, the minimization of thrombosis through the control of platelet-surface interactions is without doubt an important criterion for biomaterials for uses either as a temporary support for organs or as permanent implants in a human body in order to minimize or eliminate the need for anti-coagulation therapy. However, till date, all blood-contacting devices that are being used currently are to some degree incompatible with blood.

1.2 Development and Challenges

In order to improve the compatibility between blood and biomaterials, research has been done extensively on the reduction of protein adsorption and platelet adhesion, which are the two main factors that lead to blood coagulation. In the last decade, carbon-based biomaterials have been widely used as coatings and fillers in implants as they have been well established for their thromboresistant properties [2]. However, one of the limitations in using coatings is the stability of the coating over time and in some cases, the occurrence of delamination under shear stress in blood flow. In addition to the complexity and processing cost, the regulatory approval process can in many cases be very taxing.

The effect of surface topography on platelet interactions has received tremendous attention and is a very active field of research [3]. Indeed, the concept of using nanoscale topography in carbon nanotubes (CNTs) has been demonstrated to improve the blood compatibility [4-7]. However, the influence of platelet response on

Chapter 1: Introduction

the orientation, spacing and other attributes of the CNTs have not been answered in those studies [4, 6]. Additionally, platelet interaction studies in polymeric biomaterials, involving the effect of surface chemistry [8-15] and surface topography [16-18] are still scarce, and studies on separating the effects of surface chemistry and topography in relation to platelet adhesion and hemocompatibility are still in a very preliminary state [19, 20]. This is understandable because topographical variations are usually accompanied by the presence of chemical heterogeneities on the surfaces. The challenge lies in the ability to control similar chemistries over the different topographic surfaces.

Recently, other researchers have also employed colloid-derived topographies [21], demixing of polymer blends [22], dual-scale structures with nano and micron dimensions [23], commercially pure Ti (cpTi) disks with different surface finishes [24], tapered polyurethane micro-fibers [25] as model substrates to investigate the impact of surface topography on platelet response. In another study, Milner et al. [26] showed that sub-micron polyurethane features with low aspect ratio influences the platelet adhesion when compared with pristine surfaces, but no attempts were made to optimize the topographic parameters to decrease the platelet adhesion. Thus, a comprehensive study on the effects of substrate topography on platelet adhesion for a single substrate has not been explored.

Layers of plasma protein can form rapidly on the surface of the implant after implantation and this surface subsequently governs the cellular interactions leading to thrombus formation [27]. Besides obtaining information on the amount of adsorbed

Chapter 1: Introduction

proteins on the surfaces, the adsorbed protein conformation is also important since it governs the interaction with the glycoprotein GPIIb/IIIa receptor in the platelet [28]. The exact mechanism of protein behaviour on the topographic surfaces with high aspect ratio is not well understood, in particular, for fibrinogen adsorption. Hence, a greater understanding on the structural conformational change upon adsorption on these textured features is important in order to elicit a low platelet response with biocompatibility improvement between the blood and biomaterials.

1.3 Scope of Study and Specific Aims

With this background in mind, the thesis aims to study the effects of chemical and particularly topographical factors in influencing the thrombogenicity of surfaces in contact with body fluids. In particular, this thesis provides a study on the effect of micro to nano surface modifications on the adsorption and conformational changes of fibrinogen and on the adsorption and activation of platelets. It was hypothesized that particular topographic features may induce specific physical attributes to a surface, such as changes in wettability, orientation effect, increase or decrease of effective surface contact area and increase or decrease of effective material stiffness, that may influence the adsorption of fibrinogen and platelets in the subsequent changes derived from the attachment to the surface. To address the above central hypothesis, three specific aims were pursued.

Specific aim 1: To study the effect of aspect ratio and orientation of surface features made of CNTs on thrombogenicity.

Chapter 1: Introduction

The working hypothesis for this aim was motivated by the promising results from the aforementioned studies on CNTs. Here, we aim to investigate the enhancement on blood compatibility of a polymer composite by incorporating CNTs with different topographical features, namely randomly and vertically aligned into a polymer matrix. The thrombogenicity of the nanostructured composite films will be compared to that of pristine graphite since the use of carbon-based biomaterials as coatings and fillers in implants is well established for its thromboresistant properties in blood-contacting medical applications [2]. By evaluating the influence of surface chemistry and topography on platelet adhesion, we aimed to determine the relative importance of each factor. The details are presented in *chapter 3 and 4*.

Specific aim 2: To investigate the effect on thrombogenicity of specific micro/nano topographic features fabricated on polymeric surfaces.

The working hypothesis for this aim was that the degree of platelet activation can be influenced by physical topographical alterations on the contacting surface. For this aim, a variety of geometrical parameters with micron and submicron dimensions on the substrates, i.e. size, aspect ratio, and density will be attempted. Through this evaluation, we aimed to investigate for two critical aspects in influencing the platelet response; a) to determine if the chemical effect, i.e. on a smooth graphite surface, can be overcome by the topographical effect, i.e. on a topographic polymeric surface, and b) to determine for the range of optimized dimensions in terms of aspect ratio and density on the topographic surface. The detailed investigation is presented in *chapter 5*.

Specific aim 3: To understand the effect on both the extent of fibrinogen adsorption, and the conformation of the adsorbed fibrinogen, on the topographic substratums.

The working hypothesis for this aim was that the conformation of the adsorbed fibrinogen on a topographical surface will differ from that of a pristine surface, which is an important determinant of platelet adhesion since the exposed specific platelet binding sites ultimately governs the surface thrombogenicity. In particular, the secondary structure of fibrinogen upon adsorption on the topographical structures will be analyzed and compared to that of conformation on a pristine surface. Through this investigation, we aim to: a) evaluate for the induced conformational changes to determine for the subsequent surface thrombogenicity and b) correlate the relationship between the induced fibrinogen conformational changes to the amount of adsorbed fibrinogen at different adsorption rates on the substrates. The detailed investigation is presented in *chapter 6*.

1.4 Significance of Research

The significance of this research is to first understand whether controlled topographical modification to surfaces is an effective means to improve the thromboresistance of biomaterials; second, to define the nature of such topographical features (orientation, dimensions, density) that have the maximum effect on thromboresistance and finally to correlate these effects with studies of fibrinogen adsorption and the induced conformational changes upon adsorption to specific topographical surfaces. These findings are significant because they highlight the

Chapter 1: Introduction

ability to significantly decrease platelet adhesion on the polymeric topographical surface compared to a carbon-based material (used in market for its thromboresistant properties) merely by altering the orientation, dimensions and density of the surface features without surface modification by chemical methods, thereby reducing the complexity of clinical trials since it provides an equivalent model (in terms of surface properties) for an effective comparison with that of a pristine surface.

Chapter 2

Literature Review

2.1 Evaluation on the blood compatibility biomaterials

Biomaterials can be divided into four major classes of materials: polymers, metals, ceramics and natural materials. They are used to interact, supplement or restore the function of living tissues in the human body [29]. Blood compatibility is viewed as a material that would not produce specific adverse reactions when placed in contact with blood; such as the adhesion and activation of blood elements, thrombus formation and emboli (detached thrombus) [30]. Indeed thrombogenicity is one aspect of ‘blood compatibility’, where platelet thrombus formation plays an important role in the initiation of many adverse cardiovascular events [31].

Although the complex mechanism of blood-materials interaction is reasonably well understood, still it offers surprises. For example, vascular grafts such as expanded polytetrafluoroethylene (ePTFE) and Dacron[®] are successful in large diameter (> 6 mm) vascular prostheses, but becomes a primary cause for failure when employed in small diameter prostheses leading to thrombosis implications [32]. Here,

Chapter 2: Literature Review

the thrombotic response due to geometric factors is highlighted. Additionally, the design of the device also affects the overall blood compatibility. While an anticoagulant long-term adjuvant therapy based on heparin and a vitamin K antagonist may overcome the thrombotic issues, its side effects are undesired [33].

The understanding of protein-surface interactions is an important factor for consideration in the design of biocompatible surfaces, where such interactions are largely dependent on the surface properties (topography and surface chemistry) [34]. Clearly, the design of blood-compatible materials presents a challenge to increase the success in all medical devices which comes into contact with blood. Unfortunately, even after 50 years of intensive research and development, there exists no clear consensus as to which materials are ‘blood compatible’. Moreover the compelling need for standardized methods to assess these blood compatible materials undeniably pose a greater challenge [35]. Nonetheless, the objectives to achieve the safe and long usage of the implant with minimal side-effects as well as post-implant surgery remain the foremost factor.

A rational blood compatibility evaluation that requires the consideration of a large number of factors have been emphasized by B. D. Ratner [36]. However, blood compatibility testing is not a simple proposition since *in vivo* models may be difficult to interpret especially when subjected to large experimental errors [37]. Indeed, in addition to a deeper understanding of the interactions occurring between the proteolytic cascades in the blood and the surface of a biomaterial. A substantial and focused effort is also required from the interdisciplinary team of hematology,

engineering and materials science in the development and testing of new blood-compatible materials.

2.1.1 Hemocompatibility of Biopolymers

Since the early 1950s, polymers have been used widely for a large range of applications, from the long-term implants to the short-term dressings and in all fields of medicine and surgery. Indeed, the application for blood compatible (hemocompatible) polymers has increased significantly in recent years and some of the applications for these polymers include catheters, heart valves, stents, dialyzers, pacemakers, blood pumps, orthopedic prostheses, vascular prostheses, cardiovascular implants, ophthalmic systems, arterial grafts, drug delivery systems, etc. [38-40].

Surface modification is one of the approaches for hemocompatible polymers and is achieved by passivation of the surface to achieve minimal interaction with the blood proteins and cells. Polymers in their soluble forms have a large range of applications and are mainly used for therapeutic aims in drug delivery systems, such as in coatings to improve the hemocompatibility of vascular grafts [41]. Hydrophilic polymers such as poly(ethylene glycol) (PEG) or polyethylene oxide (PEO) and phosphorylcholine based copolymers have demonstrated nonthrombogenic properties, due to the nonspecific protein adsorption and cell adhesion attributed to the steric hindrance effect [42]. On the other hand, hydrophobic coatings composed of silicon and fluorine polymeric materials are also popular due to their relatively good clinical performances, such as the use of GoreTex®, Teflon®, Impra® and Silastic® in cardiovascular and orthopedic implants and devices [43]. Though their vascular

Chapter 2: Literature Review

biocompatibility is adequate, they are less than that of polyurethanes. Additionally, surfaces with zwitterionic moieties such as phosphobetaines, sulfobetaines, carboxybetaines and poly(carboxybetaine acrylamide) have also showed promising results upon interactions with blood [44, 45].

Despite the wide use of biopolymers in the clinical environment, the biocompatibility of these materials is still far from ideal because a variety of foreign body reactions may be triggered when the medical device comes into direct contact with tissue. Blood compatibility on surfaces consists of a multi-step and interlinked process which include protein adsorption, platelet adhesion and activation process. Ideally, a blood compatible surface should have high protein resistance properties in order to reduce materials-associated thrombosis issues [46]. The undesirable effect of blood contact on polymer surfaces has been well discussed [47], being the release of polymeric compound as the dominant issue governing the activation of the blood coagulation system. Nevertheless, medical grade polymers (e.g. PLGA) have been employed as a resolution to meet this compatibility requirement due to their non-toxicity of released components and degradation products [48], hence is the polymer of choice adopted in this study.

Poly(lactic-co-glycolic-acid)

Poly(lactic-co-glycolic acid) (PLGA) is used in this study because it is an approved therapeutic device by the Food and Drug Administration (FDA) for its biodegradability and biocompatibility [49]. It is synthesized via the random ring-opening co-polymerization of two monomers (glycolic acid and lactic acid) as shown

Chapter 2: Literature Review

in Fig. 2.1. They are commonly used as a biodegradable polymer in many biomedical devices, such as implants, prosthetic, drug delivery systems, scaffolds and sutures because of its minimal systemic toxicity. The lactic acid and glycolic acid monomer, produced during the hydrolysis of PLGA, becomes by-products of various metabolic pathways in the body [38]. Besides, the controllable degradation time (weeks to a year) by alterations in the ratio of monomers and the processing conditions have made it a suitable biomaterial of choice in a wide range of medical applications.

Fig. 2.1: Chemical structure of poly(lactide-co-glycolide)(PLGA).

2.2 Proteins

2.2.1 Protein adsorption at interfaces

Protein interaction with solid surfaces is one of the major fields of interest in blood biocompatible materials. When artificial surfaces contact blood, plasma protein adsorption occurs rapidly at the biomaterial surface; this competitive adsorption behavior of proteins determines the pathway and the extent of intrinsic coagulation and adhesion of platelets that subsequently plays a crucial role in blood coagulation cascades [50]. The interactions between proteins and surfaces are complex, involving

Chapter 2: Literature Review

many types of noncovalent forces - electrostatic, hydrogen bonding, hydrophobic, or entropic — related to surfaces as well as surrounding water molecules [51]. The adsorption of plasma proteins is the first event to take place upon exposure of blood to a biomaterial surface and is the central event in the biofouling of surfaces. Therefore, the design of an inert surface to reduce protein adsorption is of importance on the performance of the device.

Protein adsorption is a thermodynamically driven process by an overall decrease in Gibbs energy [52], where protein interactions can occur through noncovalent and covalent adsorption [53]. Protein adsorption on a surface is a complex and dynamic process. Basically, all adsorbed proteins undergo conformational change and denaturation upon adsorption on surfaces and this is critical in the subsequent cellular events following blood contact with the biomaterial surface [54, 55]. The degree of conformation change is hard to predict as it depend on many variables, which include a) protein solution conditions, such as protein concentrations [56], contact time [57], adsorption rate [58], pH and ionic strength [59] and salt concentration [60], b) protein intrinsic properties, like charge or dipole moment [61], conformational stability [62] and intramolecular forces [63], and c) substrate surface properties, like hydrophobicity [64, 65], wettability [51], charge density [66], zeta potential [67], heterogeneity [68], chemistry [69] and topography [70]. The chemistry and topography effects on proteins and platelet interaction will be reviewed in details in *sections 2.5.1.1 and 2.5.1.2* respectively.

2.2.2 Role of fibrinogen in thrombus formation

Fibrinogen, being the third primary plasma protein in blood following serum albumin and immunoglobulin, has a concentration between 2 - 4.5 mg/ml is the key structural glycoprotein that has been studied extensively for its role in coagulation [71], and its ability to promote platelet adhesion and aggregation in enhancing the thrombogenic response [72]. Being part of the thrombotic fibrin network, it is one of the adhesive plasma proteins that are capable of initiating platelets and interacting with the activated form of GP IIb/IIIa located on platelet membranes and is the major ligand in mediating platelet thrombus growth on artificial surfaces [73]. Furthermore, fibrinogen can be indirectly activated by the Hageman factor (factor XII) when it comes into contact with the biomaterial surface [27]. Indeed, the highest degree of adsorbed fibrinogen has been observed to correspond to be the most thrombogenic material for an *in vivo* study on the thrombogenicity of catheters [74]. Additionally, the conformation of the surface-adsorbed fibrinogen has been determine to affect the behavior of platelet adhesion and activation resulting in the formation of thrombus [75-77].

2.2.3 Adsorption properties of fibrinogen

The composition of the adsorbed fibrinogen can change with time and is strongly dependent on surface properties, where it shows a maximum at a certain point followed by a decreasing surface concentration thereafter, known as the Vroman effect [78] and is observed at every surface except for highly hydrophilic ones [79]. The spreading kinetics of fibrinogen occurs in a two-phase process from a

Chapter 2: Literature Review

weakly bound (displaceable) to a tightly bound (non-displaceable) state, with the rapid initial adsorption followed by a slow exponential when more fibrinogen adsorbed as evidenced by changes in the fibrinogen's dimensions through atomic force microscopy (AFM) [80].

Fibrinogen displays high surface activity at solid-water interfaces [52] and adsorbed more than other proteins at high surface concentrations [81] and is less affected by the presence of other proteins [82]. Being a high molecular weight dimeric protein (340 KDa), they usually contain highly diverse structures and multi-domain organization to produce the close packing of adsorbed fibrinogen due to its strong lateral interactions and high surface activity [82].

Fibrinogen tend to relax or change its conformation in response upon interaction with polymer surfaces [81]. Indeed, studies have shown that the induced conformational change of the adsorbed fibrinogen on surfaces, and not just the adsorbed amount, is an important determinant of platelet adhesion and the subsequent thrombus formation [83-85]. Generally, hydrophobic surfaces tend to adsorb more fibrinogen and induce more conformation changes as compared to hydrophilic ones [86, 87]. However, this trend cannot be generalized as the opposite is observed on charged surfaces of polyethylene with plasma oxidation treatment [88], with increased plasma protein adsorption but reduced platelet adhesion at increased wettability. On the other hand, high fibrinogen adsorption were observed on glassy rigid polymers regardless of being a hydrophilic or hydrophobic polymer [89]. Nonetheless, the binding strength of fibrinogen was much lower on hydrophilic

polymers and on oxygen-containing hydrophobic polymers. Fibrinogen eluted by sodium dodecyl sulfate (SDS) on polyurethane showed a tighter binding (i.e. lesser elutability) and appears to correlate with decrease platelet binding activity [90]. Higher retention of fibrinogen with lowered platelet activity were observed on a pyrolytic carbon as compared to that of polyetherurethane urea [91], and was attributed to the rapid reorganization of the adsorbed fibrinogen to a less reactive state to interact with the platelets.

2.2.4 Structure of fibrinogen and conformation

The simplest fibrinogen molecule in the trinodular model consist of three spherical regions (called D-E-D domains) connected by two sets of coiled-coil regions [92]. A more complex model has been developed over the years [93], where fibrinogen is identified with three pairs of non-identical polypeptide chains ($A\alpha$, $B\beta$ and γ) and two pairs of oligosaccharides interconnected by disulfide bonds in the extended trinodular model of globular domains together with the coiled-coil regions. It has four distinct regions where the three hydrophobic domains comprise two outer D domains with two sets of carboxyl-termini chains and a central E domain with the amino termini of all six $A\alpha$, $B\beta$ and γ chains (Fig. 2.2). Generally, D and E domains are hydrophobic while the αC domain is hydrophilic [94], where the latter is formed by the folding of $A\alpha$ chain [95]. The coiled-coil connector regions are located between the E and each D domain and correspond to the α -helical structure of $A\alpha$, $B\beta$ and γ chains. The carboxyl-termini of the $B\beta$ and γ chains fold independently in their respective D domains. On the carboxyl-termini, the $A\alpha$ chains are longer than the $B\beta$

and γ chains where it depart from the D domains and fold back to form a single globular domain near to the central E domain. The plasmin cleavage sites are located along each coiled-coil region. Fibrinogen is a rod-shaped protein and has a molecular length of 47.5 nm and diameter of 6.5 nm and 5 nm in the D and E domains respectively. The diameter and length of the coiled-coil connector is 0.8 – 1.5 nm and 16 nm respectively.

Fig. 2.2: Schematic illustration of a fibrinogen molecule showing the different domains and tertiary structure of the protein.

2.2.5 Fibrinogen binding sites to platelet

Fibrinogen contains the arginine-glycine-aspartic acid (RGDs) peptide sequence which is known to bind and activate platelets [94]. The six platelet binding sites which have been identified on the fibrinogen are, three different pairs located at

Chapter 2: Literature Review

the D domains (γ 400-411), the α C domains (RGDS of A α 572-575), and the coiled-coil regions (RGDF of A α 95-98) [96]. Although the RGDs are common to other proteins, the dodecapeptide at the carboxyl-terminus of the γ chain is unique to fibrinogen and was shown to be the most important site in the mediation for platelet adhesion and aggregation [97]. A synergistic role is suggested that the dodecapeptide sequence to be necessary for fibrinogen RGD recognition in the activation process of the integrin GP IIb/IIIa [98]. Indeed, unstimulated platelet can bind to immobilized fibrinogen, but not with soluble fibrinogen via the carboxyl-termini of A α chains. However, the A α chain is not as effective when compared with the carboxyl-termini of B β and γ chains as they can response more sensitively to changes in which the γ chain possess a highly chain-specific region for platelets interaction [99, 100]. Upon fibrinogen adsorption onto surfaces, the D and E domains interact with the material surface with the release of fibrinopeptides [101]. The cleavage of the fibrinopeptides yields fibrin and the fibrin molecules in the E domain can interact with regions of negative charge in the D domain to enhance the fibrin interactions in the blood clotting process. The structure of fibrinogen becomes distorted upon adsorption on surfaces as it maximizes surface interactions due to its multi-binding ability. On the other hand, a particular domain on the fibrinogen could be favored to inhibit the platelet binding sites through surface alterations [94].

The binding of the D domain is stronger on a hydrophobic surface and this allows the available α C domains to interact with the platelets, whereas the amount of α -helical structure reduces upon contact with a hydrophilic surface [102]. However, other evidence shows that the A α chains is responsible for the surface activity in

Chapter 2: Literature Review

fibrinogen where the absence of the A α chains have displayed retarded polymerization, lower clottability with improved blood compatibility [103-105]. However, other studies have suggested that the α C chains (connected to each of the D domains) is the first to adhere to surfaces upon adsorption due to their high flexibility and mobility as they easily change their conformation as they adhere to surfaces by either their hydrophobic or hydrophilic regions [106, 107]. This 'stickiness' of the α C chains is attributed to the exposed 'caged' Trp residues [105], but fibrinogen adsorption via the α C domain alone is not strong and can be easily be displaced by other surface active proteins since the chain is neither very hydrophobic nor highly positive [108]. Conversely, fibrinogen behaves differently on other surfaces where the α C domain interacts with the surface and the D domain bind to platelets as observed on glass [94]. In fact, any deviations in the native secondary structure may lead to a loss of the fibrinogen physiological activity resulting in increased surface thrombogenicity [109]. Nonetheless, the binding of fibrinogen to platelet ability is also reduced on highly denaturized fibrinogen upon interaction with the surfaces [55].

2.2.6 Quantification of adsorbed proteins

The quantification techniques for studying the adsorbed amount and conformational change of protein have been well reviewed [110]. Information on the adsorbed amount as well as the orientation and conformational state of the protein is important because both approaches can complement each other to provide valuable insights about the adsorbed protein. The extent on which the adsorption and

Chapter 2: Literature Review

conformational changes of fibrinogen can affect the rate of platelet adhesion and activation onto biomaterials have been reported [111].

Methods to determine amount of adsorbed proteins

The amount of adsorbed proteins can be quantified in a variety of techniques and some of the most common direct measurements are performed using colorimetric methods with an enzyme linked immunosorbent assay (ELISA) [112], radioisotope labeling (RI) [113] or by measuring the radioactivity of proteins labeled with ^{125}I -radiolabeling [114]. The use of ELISA in the detection of proteins on surfaces is well established, where the conjugation of an enzyme to a specific antibody enable the detection of a surface immobilized protein via an enzyme catalysed oxidation by means of a colorimetric substrate solution, fluorescent or luminescent technique [115]. The proteins can be probed directly on the biomaterial surface or after the extraction from the surface; however the efficiency of protein extraction limits the sensitivity in the ELISA technique.

Although the radiolabeled protein adsorption measurements can determine the number of molecules and mass of protein adsorbed for all of the components in the proteins, it is not surface sensitive where it requires the exact surface area in the quantification on the amount of adsorbed protein. Moreover, it is a less popular technique because it requires specific apparatus that are relatively expensive and poses a health threat to the users.

Chapter 2: Literature Review

Proteins labeled with fluorophores, i.e. fluorescein can be localized on the target of interest and quantified to generate an image of their distribution on the sample surface by quantifying the change in the fluorescence spectrum of protein upon adsorption. However, they are not favored due to the erroneous data from their susceptibility to induce photochemical reactions and triggering of photophysical transitions [116]. Unlike the fluorescence technique, the use of surface plasmon resonance (SPR) has distinct advantages over the fluorescence methods with lesser complications because lower affinity interactions can be detected in the presence of a large excess of unbound protein [117]. SPR is used for its direct and nondestructive measurement in the protein adsorption kinetics by elimination of an optical tag use in the labeling of proteins [68].

Neutron reflectometry allows the simultaneous determination of adsorbed amount and composition of proteins adsorbed at a solid-liquid interface [118]. The frequency change of protein mass and the water trapped in the protein layer couple to the surface can be quantified by the quartz crystal microbalance (QCM) [119] and the electrode-separated piezoelectric sensor (ESPS) technique [120]. The refractive index measured by ellipsometry [121] provides the average adsorbed protein thickness and concentration, where the adsorbed state of protein can be predicted based on the two previously derived underlying principles. On the other hand, information on the total adsorbed amount and composition of the adsorbed protein layer can be acquired by the utilization of the total internal reflection fluorescence (TIRF) coupled with ellipsometry [122]. Alternatively, TIRF can be used individually in the study of

Chapter 2: Literature Review

various protein interactions with the surface to provide real-time information on competitive adsorption, kinetic studies and interfacial conformational change [123].

X-ray photoelectron spectroscopy (XPS) or known as electron spectroscopy for chemical analysis (ESCA) [124] is another surface sensitive technique that measures the atomic composition of the adsorbed protein layer, however it precludes the analysis of adsorbed proteins in their native configuration since the analysis is carried out in an ultra-high vacuum (UHV) environment ($\sim 10^{-9}$ torr). Therefore, the sample must always be dried prior analysis. Alternatively, a liquid nitrogen-cooled stage can be affixed to allow analysis of frozen hydrated samples. Near edge X-ray adsorption fine structure (NEXAFS) spectroscopy is a powerful tool due to its high sensitivity in detecting surface chemical functionalities and the adsorption behavior of fibrinogen on SWCNT have been demonstrated [125]. Recently, X-ray photoemission electron microscopy (XPEEM) [126] and electrochemical impedance spectroscopy (EIS) [127] have also been applied to study protein adsorption on surfaces. Additionally, an *ex-situ* wavelength dispersive spectroscopy technique (WDS) is developed recently to quantify the protein adsorption on metals or other conductive surfaces by direct measurement of the carbon and nitrogen content [128].

Surface- matrix-assisted laser desorption ionization (MALDI) mass spectrometry [129] is a rapid and experimentally convenient technique that allows the detection of adsorbed proteins in complex mixtures. The limitations in mass resolution and sensitivity are overcome as compared to the established biochemical

techniques like sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [130]. However, not all proteins, especially very large proteins are detectable at this early stage of development.

Characterization techniques for conformational and organization of adsorbed proteins

The conformational change in the fibrinogen does not definitely result in platelet binding because the specific exposed receptor is also necessary for a successful binding. The techniques employed to identify the specific sites on the protein are as described below.

The adsorbed fibrinogen conformation and orientation changes can be detected by the highly sensitive and specific polyclonal [76] (against multiple epitopes) and monoclonal [15] (against specific protein epitopes) antibodies to the conformation-sensitive epitope in the D-domain.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) provides extreme surface sensitivity and chemical specificity at the outermost surface [131], and can be employed in multivariate analysis to determine the surface composition, conformation and orientation of the adsorbed protein layers. The only limitation lies in the further denaturization of the protein layers due to the UHV condition.

The atomic force microscopy (AFM) offers excellent spatial resolution and therefore is able to provide information on the morphological changes of the adsorbed protein [80], but it has limited chemical sensitivity. Circular dichroism (CD)

Chapter 2: Literature Review

spectroscopy is sensitive to the secondary structure of proteins where it allows the ease of monitoring for the changes in the conformation of proteins in the aqueous environment [132].

Fourier transform infrared (FTIR) spectroscopy is based on the interaction of molecular vibrations induced by infrared radiation with a material; therefore detailed information on the protein structure can be obtained upon adsorption [54]. The kinetic study on conformational change in the protein is made possible by attenuated total reflection spectroscopy (ATR-FTIR) [133]. The information about the secondary structure of protein can be achieved relatively fast in the absence of an UHV environment and eliminating the need to establish a precise 3-D location of individual structural elements. Additionally, the ‘depth of penetration’ is also larger and can reflect more than just the outermost atomic layers, i.e up to 1 μm . Nonetheless, one of the issues it faces is the lack of direct quantification on the amount of adsorbed protein. The FTIR - reflection adsorption spectroscopy (FTIR-RAS) with ellipsometry [134] and the grazing angle - FTIR (GA-FTIR) with QCM [55] are some other techniques that can be employed to probe for the conformational change upon protein-surface interactions.

Recently, some other powerful vibrational spectroscopic techniques used to elucidate the fibrinogen structural changes upon interaction at interfaces include the Raman spectroscopy [135] and the sum frequency generation (SFG) vibrational spectroscopy [136]. Well documented in literature [137-139], SFG not only permits the identification of surface species with its excellent surface sensitivity, but also

Chapter 2: Literature Review

provide chemical structural information on the orientation of the chemical groups at interfaces or within thin films. Likewise, changes in the protein secondary structures can be obtained through the spectra to deduce valuable information on the conformational changes of adsorbed proteins in Raman spectroscopy [140-142]. Very recently, X-ray absorption near edge structure (XANES) spectroscopy is used to investigate for the structural details of protein changes occurring at the interface of carbon nanotubes [143]. Likewise, polarization modulation infrared reflection adsorption spectroscopy (PM-IRRAS) technique has been employed recently to determined the adsorption thermodynamics as well as the conformational changes of the proteins [109].

2.3 Platelets

2.3.1 Platelet biology

Platelets or thrombocytes are the cells fragments circulating in the blood and are involved in the cellular mechanisms of primary hemostasis leading to the formation of blood clots. They are nonnucleated blood cells produced in the bone marrow from megakaryocytes, where the latter arises by a process of differentiation from the haemopoietic stem cell which undergoes fragmentation of their cytoplasm to produce platelets [144, 145]. Platelets are small, disc-shaped cells with a mean diameter of 1 - 2 μm and circulate in relatively high concentrations between 150 – 400 $\times 10^6$ platelets/ml [144]. Platelets in their inactivated state are roughly discoid in shape with a smooth and rippled surface, where it contains cytoplasmic organelles,

cytoskeletal elements, invaginating open-canalicular membrane systems, and platelet-specific granules, called alpha and dense granules.

2.3.2 GP IIb/IIIa Receptor

GP IIb/IIIa ($\alpha_{IIb}\beta_3$) is one of the receptors on the platelet plasma membrane and is known to bind to four soluble adhesive proteins: fibrinogen, von Willebrand factor (vWF), fibronectin and vitronectin [146]. GP IIb/IIIa belongs to the integrin family of receptors which comprise of two distinct proteins: GPIIb (α_{IIb} according to integrin nomenclature) and GPIIIa (β_3) with its structure shown in [Fig. 2.3](#). Both proteins have a short intracellular C-terminal tail and GPIIb and IIIa form a complex on the membrane through non covalent bonding [147]. GP IIb/IIIa is a heterodimer which consist of a globular head with two receptors; these receptors consist of two subunits, alpha and beta. The alpha sub unit (136-kDa) consists of a heavy chain and a light chain; the light chain has a short cytoplasmic tail, a transmembrane region, and a small extracellular domain, whereas the heavy chain is entirely extracellular. The beta sub unit (92 kDa) consists of a single polypeptide of 762 aminoacids, with a short cytoplasmic tail, a single transmembrane region, and a large extracellular domain [148]. GP IIb/IIIa is essential for both platelet aggregation and platelet adhesion for binding to its soluble ligands (e.g. fibrinogen) which facilitate platelet-platelet interaction.

Fig. 2.3: Structure of platelet GP IIb/IIIa receptor. [149]

The recognition specificity of the GP IIb/IIIa receptor is defined by two peptide sequences, through which the agonists bind to it. One is the Arg-Gly-Asp (RGD) and another sequence is Lys-Gln-Ala-Gly-Asp-Val (KQAGDV). The RGD sequence is initially identified as the adhesive sequence in fibrinogen which is also present in fibrinogen, vWF and vitronectin. All these ligands contain at least one RGD sequence, whereas fibrinogen contains two RGD sequences per half molecule. The KQAGDV sequence is the other major sequence involved in the binding of fibrinogen to GP IIb/IIIa receptors and is located at the carboxyl terminus of the g-chain of fibrinogen [97]. Unlike RGD, this sequence is found only in fibrinogen and is probably the predominant site for the binding of fibrinogen to GP IIb/IIIa receptors.

2.3.3 Role of platelet in thrombus formation

Platelets play a central role in the process of thrombus formation (thrombosis) [150]. A series of platelet reactions are triggered when blood contact any

thrombogenic surface leading to the formation of a blood clot (thrombus). A thrombus is composed primarily of two types of blood cells, platelets and red blood cells (RBCs). Thrombogenic surfaces include artificial surfaces, an injured endothelium and subendothelium [151]. Rapid biochemical reactions and cellular change occurs upon contact on these thrombogenic surfaces, leading to the formation of a platelet-rich thrombus by the release of active compounds and changes in the platelet-vessel wall interactions in the following phases [147]: (i) platelet adhesion, (ii) platelet activation, (iii) platelet aggregation, (iv) platelet aggregation, and (v) procoagulant activity.

Platelet adhesion

The adsorption of plasma proteins on biomaterials is the initial event that follows immediately upon an implantation. Among the different adhesive plasma proteins, fibrinogen has shown evidence to play a major role in the thrombogenic process on artificial surfaces [73]. Platelets adhere to adsorbed proteins on the surfaces via the platelet membrane GPs [152]. Integrin- $\alpha_{IIb}\beta_3$ in the un-activated platelets in their resting state can selectively mediate platelet adhesion to fibrinogen [28]. Un-activated platelets each contain $\sim 50,000 - 70,000$ GP IIb/IIIa receptors per platelet which are uniformly distributed over the platelet surface and this number approximates the number of fibrinogen binding sites on the surface of normal platelets [153], where the expression of the newly transformed GP IIb/IIIa binds to fibrinogen and creates the ideal platform for the next platelet to adhere.

Platelet activation

Platelet activation occurs when the adhesive ligands binds to the related receptors on the platelet membrane and release a number of different coagulation factors and activating factors, where the platelets adhere to each other via these adhesion receptors [154]. Consequently, the platelets undergo a cytoskeletal activation that leads to a shape change from discoid to spherical with extrusion of the pseudopods after adhesion on artificial surfaces, resulting in the procoagulant activity of activated platelets to the subsequent generation of thrombin on the platelet surface [144]. The activated platelets extend their pseudopodia and the membrane between them as it is pushed out by the hyaloplasma until the platelets reach their fully spread state. In the final activation state, the platelets typically have an average diameter of 7 – 10 μm . The morphologies of activated platelets can be divided into 5 categories including round, dendritic, spread-dendritic, spread and fully spread [9].

Platelet aggregation

Platelet aggregation is the amplification step immediately after platelet activation leading to the accumulation of platelets into the hemostatic thrombus. A secondary irreversible phase of aggregation results when platelet secretion takes places with the release of platelet granule proteins and the recruitment of additional platelets [155], where the release of platelet granule proteins play an important role in the secondary or secretion-dependent phase of aggregation. The binding of fibrinogen is closely correlated with the platelet aggregation process, where the major platelet reactive sites of the γ -chain within the D domains on the fibrinogen molecule

“bridge” the adjacent activated platelets and subsequently initiate the platelet aggregation process [156]. Consequently, the binding of fibrinogen causes further changes in the conformation of the GPIIb/IIIa to mediate further platelet aggregation [157].

Procoagulant activity

The constituents of the platelet release reactions play a crucial role in the formation of a platelet thrombus. The triggering of the coagulation cascade can occur via the extrinsic and intrinsic pathways [151, 158] and both pathways can culminate in the same thrombin-mediated mechanisms because they join at a common pathway at the activation of factor X as shown in [Fig. 2.4](#). One of the two important mechanisms is that the presence of the chemicals released from the aggregated platelets stimulates the arachidonic acid cascade to promote more platelet aggregation and assist in the formation of a blood clot [159]. Next, the ultimate reaction in the coagulation cascade converts the prothrombin to thrombin by activated factor X [160], and the thrombin cleaves the fibrinogen to polymerize it into an insoluble fibrin clot to subsequently trap the red cells leading to thrombus formation [161].

Fig. 2.4: Overview of blood coagulation cascade showing both the intrinsic and extrinsic pathways [151].

2.3.4 Platelet characterization techniques

Quantification for adherent platelets

The amount of adherent platelets on the biomaterial surface can be quantified by microscopy, fluorescence technique and platelet adhesion assays [162, 163]. Platelet adhesion assays are usually used as an indication on the thrombogenicity of the biomaterial. The two relatively simple methods employed to evaluate the adhered platelets count are the cytosolic platelet lactate dehydrogenase (LDH) [164] and the platelet membrane acid phosphatase (ACP) [165]. Platelet adhesion is commonly

quantified by LDH assay, where this method is based on the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate from the activity of the cytoplasmic enzyme. This enzyme is released into the lysing solution upon damage of the plasma membrane and activity of the intracellular LDH allows the quantification on the number of platelets as a function of the amount of LDH released.

Quantification for activated platelet

Flow cytometric measurement on the changes in the platelet surface membrane antigens is gaining acceptance as a method for accessing platelet activation status both *in vitro* and in clinical studies [166-168]. Flow cytometry not only measures and analyzes the signals of cells and molecules that are required to be suspended in fluid. Here thousands of cells can be analyzed rapidly by providing the correlated data that links the different population profiles based on single cell analysis. Additionally, it also offers a rapid and quantitative analysis for a variety of cell types based on the cell size, molecular and antigenic composition [169].

Evaluation of platelets by the flow cytometry include quantification of immature (i.e. reticulated) platelets, detection of anti-platelets antibodies, and detection of activated platelets [170]. Flow cytometric tests which are developed to detect the circulating activated platelets include: binding of the fibrinogen receptor occupancy to platelets for studies in platelet-directed anticoagulation in thrombosis, detection for a specific activation-dependent modification in the platelet surface membrane, binding of monoclonal antibodies to activation-dependent markers on the platelet surface, detection of platelet microparticles for assessment of

Chapter 2: Literature Review

hypercoagulable states, platelet-associated immunoglobulin assays, reticulated platelet assay to detect “stress” platelets, detection of shape change and the detection of platelet-leukocyte aggregates [166-168, 170, 171].

Laboratory markers of platelets activation include activation-dependent changes in the GP IIb/IIIa complex, exposure of granule membrane proteins, translocation of granule-associated proteins to the platelet surface (P-selectin, GP 53, dense granule membrane protein), binding of secreted proteins to the platelet surface (thrombospondin), and development of a procoagulant surface [168, 170]. The most commonly known monoclonal antibodies used to assess platelet activation are Procaspase-activating Compound 1 (PAC-1) and P-selectin (CD 62P). Fluorescence-isothiocyanate (FITC) with PAC-1 conjugated binds to the activated form of GP IIb/IIIa receptor complex which is required for subsequent platelet aggregation that appears in the early stages of platelet activation. PAC-1 is an IgM antibody which recognizes and binds only to the activated form of GP IIb/IIIa that is critical for fibrinogen binding since PAC-1 and fibrinogen inhibit each other's binding in a competitive manner [172, 173]. Therefore, PAC-1 only binds to activated platelets and not to resting platelets, where PAC-1 has been useful in identifying the intracellular mediators responsible for fibrinogen receptor expression in activated platelets [174]. On the other hand, CD62P is directed against the granule membrane protein (GMP-140) or also known as PADGEM (platelet activation dependent granule membrane protein) [168]. Furthermore, a three-color analysis of platelet activation can be use to compare degranulation with CD62-PE, GP IIb/IIIa activation using PAC-1-FITC in human whole blood with CD61-PerCP acting as the pan-

Chapter 2: Literature Review

platelet marker. CD61-PerCP react with GP IIIa while CD62-PE express the fusion of the internal α -granule membrane with the external plasma membrane, which is a subsequent event after the platelets are activated [175].

Flow cytometric evaluation of P-selectin expression has been used to distinguish heparin-induced thrombocytopenia (HIT) from HIT with thrombosis, which may allow for early intervention for prevention of thrombotic complications [145]. P-selectin is normally expressed on the internal surface of alpha granules and is not detectable on the surface of resting platelets but upon degranulation, it is transported to the platelet plasma membrane [170]. P-selectin acts as the adhesion receptor by promoting cell-to-cell contact by recruiting both platelets and neutrophils to the sites of tissue injury and acute inflammation [144]. The effect of platelet activation on monoclonal antibody binding is shown in [Fig. 2.5](#), which depicts the binding of the monoclonal antibodies to the resting platelets and the relative change in the binding of the antibodies after the thrombin activation. S12 only binds to the surface of the activated platelets after the thrombin activation when P-selectin is translocated to the platelet plasma membrane.

Sample preparation is required prior to flow cytometry analysis, where platelets are washed, stained and fixed. The stained samples are washed to remove traces of any unbound antibodies and supernatant, followed by resuspending the platelets in a fresh medium in the final step for analysis. Staining with monoclonal antibodies not only allows definition of each individual cells, but also allows for cell population analysis [176]. There are 2 different staining methods; direct and indirect

Chapter 2: Literature Review

staining. Direct staining is preferred as manipulation of cells is minimized and easy to perform. Moreover, it also allows the combination of several markers for multiparameter analysis [167]. Indirect staining is performed with unconjugated antibodies against specific cellular determinants and usually consists of two or more antibodies. Additionally, it improves the sensitivity of detection when the number of fluorochromes per specific antigenic determinant is increased as several second fluorochrome antibodies may bind to a primary antibody.

Fig. 2.5: Schematic drawing showing the effect of platelet activation on monoclonal antibody binding [168].

Fixation is used to preserve the shape of cells and to stabilize the sub-cellular morphology and normally 0.5-2% of formaldehyde is used as the standard fixation of cells for immunofluorescence [168]. The optimal fixation is an important variable for each monoclonal antibody and must be defined because fixation prior to antibody labeling often lead to lower fluorescence intensity as compared to unfixed platelets prior to antibody labeling [173]. Additionally, the binding of some antibodies

decrease further after fixation in a time-dependent manner, hence samples should always be analyzed within 24 hours of antibody labeling [177].

Overall, flow cytometry is currently the preferred golden standard used in the study of platelet structure and function despite the complicated sample preparation and the costs associated with purchase and maintenance. Flow cytometry is not only a simple technique, but also a sensitive and accurate one to allow the simultaneous analysis of various cell types. A high degree of sensitivity of the platelet subpopulations can be detected with rapid and constant measurement even if the cells are suspended in a minuscule volume, making flow cytometric measurements particularly more attractive with these advantages [178].

2.4 Carbon-based materials in biomedical systems and devices

2.4.1 Carbon films

The passive nature of carbon in tissues has been known for several decades. The various forms of carbon that have been studied for possible use in biomedical applications include modified carbon, vitreous or glassy carbon, carbon nanotubes, carbon nitride (CN), pyrolytic carbon, low-temperature isotropic (LTI) and vacuum vapor deposited carbon coatings like diamond-like carbon (DLC) [91, 179-182].

Pyrolytic carbon, known for its resistant to thrombus formation are one of the few carbon-based materials that have been successfully used in the market for application in artificial clinical heart valves [183]. Though data from a clinical study involving 420 patients showed that pyrolytic carbon coated stents could not

Chapter 2: Literature Review

demonstrate a significant improvement in stent performance over high-grade stainless steel stents [184]. Likewise, a recent study indicate that blood biocompatibility is unlikely to improve even when pyrolytic carbon is alloyed with silicon [185]. A probable explanation for the contradictory observations could be due to differences in the fibrinogen contact times with the substrate since platelet adhesion is very sensitive to how tenaciously the fibrinogen is bound to the substrate [91].

The hydrogenated amorphous (a-C:H), tetrahedral amorphous carbon (ta-C) and the nonhydrogenated amorphous carbon (a-C) all belong to the same family of amorphous carbon film under the DLC classification [186]. Although LTI carbon is proven to be relatively blood compatible for its use as artificial heart valves, ta-C has shown even better anticoagulant property [187], and the reason is attributed to the effective work function rather than the surface energy. While the DLC stent has indeed reduced acute and subacute thrombosis, its benefit with respect to longer term patency did not show desirable results after a six-month follow-up [188]. Additionally, the high residual compressive stress and low adhesion strength limit their biomedical applications [189]. Despite such findings, the only appealing advantage of those DLC-coated stent is probably the considerable reduction of metal ion release over the bare metal stents. However, research efforts in the further optimization of DLC coatings remain active with developments in fluorine-doped DLC by various research groups [190, 191]. The tendency to induce thrombus formation was significantly lower on these fluorine-doped DLC and was attributed to the amount of fluorine doping that led to the much suppressed platelet adhesion and activation. Additionally, silicon doping of a-C:H films at relatively higher annealing temperatures of 300 °C

increase the I_D/I_G ratio, lowered the electrical resistivity, work function and surface energy resulting in improved hemocompatibility [192]. Indeed, the effect of surface roughness, microstructure, surface energy, electrical conductivity, work function, annealing, silicon doping, sp^2/sp^3 ratio and the level of graphitization play a role in the hemocompatibility of these carbon-based films [192-194]. Overall, the carbon surfaces are less thrombogenic and hemocompatible as evidenced by their existing usage in the commercially available coated heart valves and stents.

2.4.2 Carbon nanotubes as artificial prostheses

Lately, the impressive properties demonstrated by carbon nanotubes have spurred many research groups to investigate its promising applications in a variety of biomedical systems and blood-compatible devices for its non thrombogenic and surface topographical properties [6, 195]. The bio-applications of CNTs both at molecular and cellular levels [196] have been predicted and explored ever since the discovery of these one dimensional carbon allotropes. Indeed, CNTs have many interesting and unique properties potentially useful in a variety of biological and biomedical systems and devices [181, 183]. These nano topographical CNTs can be employed as coatings or fillers in composites for applications in blood-contacting environments due to their inert nature to cells and tissues because of their pure carbon composition and biocompatible chemical surface [5]. The interaction of biomolecules with CNTs has been studied [197] and this opens up new a potential for CNTs to be developed and applied in novel blood-compatible biomaterials and medical devices.

2.4.2.1 Background on CNTs

Multi-walled carbon nanotubes (MWCNTs) are introduced by Iijima in 1991 while investigating the soot of an arc-discharge experiment used to create C_{60} buckyballs from a Fullerene experiment [198]. About two years later, he made the observation of single-walled nanotubes (SWCNTs) [199]. The discovery of carbon nanotubes (CNTs) has the potential of revolutionizing the biomedical research as they can show superior performance because of their impressive structural, mechanical, and electronic properties due to its small size and mass, high strength, higher electrical and thermal conductivity, etc. Carbon nanotubes (CNTs) are unique, one-dimensional macromolecules, with near ideal whiskers containing folded graphene layers with well-ordered cylindrical hexagonal lattice structure of carbon atoms. CNTs originated from fullerenes and are referred to as tubular fullerenes or bucky tubes. They are the only form of carbon with extended bonding without dangling bonds. During growth and depending on the processing conditions, they could be assembled either as multiwalled co-axial tubules, which comprise an array of concentric cylinders (multiwalled nanotubes, MWCNTs) or as bundles (ropes) consisting of a single graphite sheet seamlessly wrapped into a cylindrical tube (single-walled nanotubes, SWCNTs) packed in two-dimensional tri-angular lattices [200] as shown in [Fig. 2.6](#). The SWCNTs and MWCNTs can be produced by arc-discharge [201], laser ablation [202], chemical vapor deposition (CVD) [203] or gas-phase catalytic process (HiPco) methods [204]. They have a high aspect ratio, with lengths ranging from several hundred nanometers to several micrometers and diameters of 0.4–2 nm for SWCNTs and 2–100 nm for MWCNTs [205].

Fig. 2.6: Idealized representations of different structures of defect-free and opened carbon nanotubes showing a) concentric MWCNTs and SWCNTs with different chiralities displaying the b) armchair structure, c) chiral structure and d) zigzag structure [206].

2.4.2.2 Adsorption of plasma proteins onto CNTs

Nonwoven SWCNT showed completely different plasma protein adsorption behavior [5], where the surface of nonwoven SWCNT strongly adsorbed fibrinogen, including exposure to very low-concentration fibrinogen solution. Additionally, the adsorption behaviors were reported to be different from those for individual CNTs and CNT bundles, which Shim et al. [207] reported that relatively small proteins such as streptavidin (60 kD) could adsorb more readily to the sidewall of individual SWCNTs. On the other hand, fibrinogen (340 kD) adsorption was not observed because of its relatively large size (~ 45 nm) compared with the diameters of individual SWCNTs (~ 2 nm). However in another study, fibrinogen was observed to be strongly adsorbed on the surface of SWCNT [125] when it was the only protein used. The competitive adsorption behavior of proteins in relation to their sizes in comparison to the SWCNTs could be a probable explanation for the above observed differences in the fibrinogen adsorption.

Bovine serum albumin (BSA) proteins attached to CNTs via diimide-activated amidation [208] show that the protein species were intimately associated with the nanotubes. Selective binding of serum proteins, plasma proteins and C1q to CNTs have been demonstrated [209], where fibrinogen and apolipoproteins (AI, AIV and CIII) are the proteins that bound to CNTs in its greatest quantities. The hydrophobic SWCNT has been demonstrated to affect the protein structure and function strongly by either enhancing the retention or the loss of its native-like protein properties [210]. Indeed, a significant degree of biological function on the fibrinogen is retained when adsorbed on CNTs, which suggested the possibility of usage in a coagulation reaction [211].

Nonwoven SWCNTs with immobilized fibrinogen showed low levels of platelet adhesion and activation as compared to nonwoven SWCNTs with immobilized albumin [5]. The differences in its protein adsorption behaviors arise from the three factors as discussed by J. Meng et al.; (a) a larger surface area was provided for larger proteins, as well as stronger interactions and a favored surface topography for protein binding as compared with macroscopic carbon materials and polymer materials, (b) studies have indicated that fibrinogen adsorbs preferentially onto hydrophobic surfaces where its binding strength increases with hydrophobicity and (c) many domains in fibrinogen molecules can bind readily to the material surfaces.

2.4.2.3 Carbon nanotube polymer composites for biomedical applications

The most widespread application has been the use of CNTs in polymer nanocomposites for their potential applications in a variety of biomedical systems and devices due to their unique structure and properties [212-218]. Polymer-based composites and blends are widely used in commercial products, where these polymers serve as the matrixes for inorganic, organic, or carbon fillers and have an enormous impact as engineering materials.

A multiwalled carbon nanotubes-polyurethane composite (MWCNT-PU) [6] displayed a significantly improved anticoagulant function where platelet activation and red blood cell disruptions were remarkably reduced as compared to a polyurethane. Likewise, a reduced thrombogenicity was demonstrated on microcatheter prepared from MWCNTs-nylon-based composite [7]. Additionally, nanostructured superhydrophobic films with MWCNTs coated with poly(carbonate urethane)s (PCUs) showed excellent anti-adhesion to platelets and have relatively low platelets activation. It demonstrated that thrombosis could be efficiently prevented on this nanostructured superhydrophobic composite and was proposed for applications in artificial organ implantation, manmade blood vessels and other blood-contacting medical devices [4]. Very recently, chitosan/carbon nanotubes (CS/CNTs) composites with high concentrations of CNTs (5.1 wt%) have shown excellent anti-adhesion properties with regard to platelets [219].

2.4.2.4 Techniques for dispersing CNTs

Dispersing CNTs individually and uniformly in the polymer matrix is the most fundamental and critical requirement in producing composites with reproducible and optimal properties. CNTs are highly polarizable, smooth-sided compounds with attractive interactions of 0.5V per nanometer of tube-to-tube contact [220]. The CNTs exist as bundled structures that are often referred to as ropes due to these high cohesive forces. The manipulation and characterization of large numbers of individual CNT is difficult because of the high molecular weights and strong intertubular forces (both van der Waals and electrostatic) [221]. This bundling or roping phenomenon contributes to the bulk materials and limits the solubility which causes poor dispersion in polymers [222]. The lack of solubility and the difficulty in manipulation in any solvents have imposed great limitations to the use of CNT.

One of the issues faced is that as-produced CNT are insoluble in all organic solvents and aqueous solutions, where precipitation immediately occurs when the sample is left to settle after the sonication process. The thermodynamic drive and agglomeration of the CNTs must be overcome in order to obtain well-dispersed CNTs. There have been many investigations into improving CNT solubility through optimizing the nanotube dispersion within the polymer matrix, where some successful and popular methods are: sonication, stabilization with organic surfactants [223], polymer-assisted melt blending [224], solution mixing [225] and via the chemical functionalization route involving covalent [226] and noncovalent functionalizations [227]. Chemical functionalization is employed to allow adhesion between the

Chapter 2: Literature Review

nanotubes and the polymer to enable an effective stress transfer at the polymer-filler interface. Additionally, CNTs can undergo chemical reactions to make them more soluble for their integration into organic, organic and biological systems [228].

Oxygenated functional groups are intrinsic defects prepared by inducing oxidative damage to the nanotube frame work through the use of strong acids such as nitric acid and sulfuric acid to remove the metal particles [229]. Acid oxidation treatment not only results in shortened nanotubes with the formation of carboxylic, carbonyl or hydroxyl groups on the end tips or sidewalls but also leads to smaller-diameter nanotube bundles. The presence of these functional groups is advantageous to ensure the chemical bond between polymer and filler and to enhances the solubility of CNTs in various solvents [230]. Carbonyl groups on nanotubes can bind to the polymer matrix through hydrogen bonds, and such functionalities serve as anchoring sites to open the route for further derivatization reactions. Indeed, these specific interactions are proven to be effective with a high degree of nanotube dispersion achieved in the nanotube-polymer composite through chemical modification [231].

The fluorination reaction is reported to be useful because further substitution could be accomplished [228]. Fluorination of CNTs significantly improves the chemical reactivity and solubility due to the interaction of the solvent and fluorine atoms on the surface of nanotubes while maintaining the mechanical properties of the CNTs [232]. Fluorinated nanotubes can be solubilized in various alcohol solvents by means of ultrasonication and are reported to have a moderate solubility ($\sim 1\text{ mg/ml}$) in alcoholic solvents [233]. The combination of acid treatment and subsequent

fluorination of SWCNTs was reported to increase the solubility in the solvent and facilitates an uniform dispersion in the epoxy resin [231].

Organic synthetic surfactants have been commonly used to improve CNT solubility in aqueous solutions to enable the easy access of the CNT with the chemical reagents [197]. The water solubility of functional groups (or adsorbed species), the extent of functionalization or modification, and the strength of interactions between the functional groups and the nanotubes are the most important factors for the aqueous solubility in nanotube dispersion.

For the ionic surfactants [234], sodium dodecylbenzene sulfonate (SDBS) gives the most well resolved spectral features with SDBS-assisted SWCNT suspensions stable over months without significant aggregation or bundling. For the nonionic systems, surfactants with higher molecular weight suspended more SWCNT material and have more pronounced spectral features. Besides, the ultrasonic treatment of the CNTs in aqueous surfactants such as sodium dodecyl sulfate (SDS) and Triton X-100 were reported to have a solubility of 0.1 mg mL^{-1} and 0.5 mg mL^{-1} respectively [197]. However, rapid rebundling [226] occurred when unfunctionalized micelle-coated CNTs are washed to remove the surfactant. Here, the removal of surfactant is necessary due to prevent the denaturization of biological molecules.

2.4.2.5 Toxicity and Biocompatibility Issues of CNTs

While many biomedical applications incorporating CNTs have been proposed, the toxicology of CNTs is not well-understood at the present time and hence the task

Chapter 2: Literature Review

necessary to establish the toxicological and pharmacological profile of such systems is required before any clinical studies can be undertaken further [235]. It is also imperative to have the occupational health and safety of CNT exposure to be thoroughly investigated before the use of CNT-based materials becomes widespread. Despite the clear need for a full scientific assessment of CNT toxicology, early research into the safety of CNT is scarce. This oversight is perhaps founded on the excellent performance of existing carbon based biomaterials, but the lack of the multidisciplinary expertise within CNT research groups required to perform such characterization. However, in the last five years, disturbing and often conflicting data have emerged concerning their safety [183]. Researchers suggested that CNTs possess health risks, where they are capable of producing inflammation especially in epithelioid granulomas (microscopic nodules), fibrosis, biochemical and toxicological changes in the lungs [195, 236]. Profound cytotoxicity of SWCNTs is observed in a test protocol [237], where the cytotoxicity of SWCNTs are higher as compared with fullerene (C_{60}). In another study, a cytotoxicity ranking study on the macrophages comparing SWCNTs and MWCNTs with C_{60} was reported to be in a mass basis order of $SWCNT > MWCNT > C_{60}$. Additionally, profound cytotoxicity was observed in SWCNTs even at a low concentration of $0.38 \mu\text{g}/\text{cm}^2$ [238]. Based on this premise, MWCNT would be employed throughout in all the experimental studies.

Particle toxicology study ranges from the adverse effects of tissue exposure (typically through the lungs, digestive tract or skin) to particulate matter. The emergences of nanotechnology and nanoparticulate pollution have raised questions as

Chapter 2: Literature Review

to the effect of these nanomaterials on human health. One of the major and recurring problems encountered by researchers in the investigation of CNT toxicity and biocompatibility is the tendency of CNT to aggregate in large bundles and ropes. The manipulation and characterization of large numbers of individual CNT is a difficult task due to its high molecular weights and strong intertubular forces (both van der Waals and electrostatic) which promote the formation of such bundles and ropes [221, 236]. It is very likely that a large portion of the discrepancies in toxicity and biocompatibility data are due to differences in CNT dispersion, which is a factor that ultimately dictates the presentation of CNT to the cells. It is discussed that different geometric structures will exhibit different cytotoxicity and bioactivity *in vitro*, although they may not be accurately reflected in the comparative toxicity *in vivo*. The high aspect ratio of CNT makes them unusual for toxicological investigation since they share similar shape characteristics with both nanoparticles and fibers [239]. More studies of the cytotoxicity of SWCNTs has been reported elsewhere [183, 236].

Toxicity studies including the biocompatibility of CNTs and its sensitivity to infections are potential areas of research work for the successful applications of CNTs in the biomedical field. Nanotubes in the nano-sized range will have their inherent toxic effects enhanced by virtue of a higher surface area/mass ratio. Their size as well as its surface area are important material characteristics from a toxicological perspective, therefore as size reduces, the potential to cause harm is expected to become greater [238-243]. There is still much work to be done in establishing the toxicity and biocompatibility of CNT, although no clinically relevant toxicity has yet to be reported [240] and there have been no investigations into the ability of CNT to

migrate or accumulate *in vivo*, despite their apparent potential as drug delivery vehicles. Preliminary hematological *in vitro* tests on New Zealand rabbit and human blood samples have indicated good biocompatibility with coatings of Fe@C nanoparticles produced using arc discharge methods similar to those used to produce fullerenes and CNTs [244].

Until now, it is still too early to draw meaningful conclusions about the inherent dangers of these engineered nanoparticles as the test methods for testing these nanoparticles are not straightforward enough. Detection methods definitely need to be developed for risk exposure assessment and the decision on the regulation of nanoparticles should be based on scientific evidence of toxicity where specific products or products lines and the likelihood of an exposure risk need to be considered [240]. Indeed, current studies showed that CNTs have yet to cross many technological hurdles in order to fulfill their potential as the most preferred material for biomedical applications and there remain too many challenges at the current research stage. All that need to be addressed before the full potential of CNTs for biomedical applications can be realized [183, 196, 236, 245].

2.5 Surface modifications

2.5.1 Surface modifications on polymeric surfaces

Surface modification on polymeric surface is a suitable method to create blood-compatible surfaces and various surface modification techniques which have been attempted by many researchers in order to control the proteins adsorption and

cell activity [25, 70, 246-248]. Thus, it is important to design blood-compatible surfaces in order to manipulate platelet-surface interactions to increase the thromboresistance of foreign surfaces towards blood. Specifically, the effects of surface chemistry and surface topography on fibrinogen and platelet response will be reviewed in the following *sections 2.5.1.1.1 and 2.5.1.4.1* respectively.

2.5.1.1 Chemically modified polymer surfaces

Chemical graft modification can be employed to immobilize either a protein or monomer to the surface to alter the surface chemistry, where the extensive techniques has been well reviewed by Kato et al. [42]. Additionally, chemical grafting is also used in the immobilization of heparin and PEO to surfaces to increase the blood compatibility though it faces a number of drawbacks such as the loss of protein mobility as well as the presentation of the protein in an unfamiliar conformation on the surface [249].

Self-assembled monolayers (SAMs) is a technique that allows a precise control in the density and conformation of a single or multiple specific functional groups on a surface, but are generally limited to gold-coated or silver coated surfaces [250].

Surface molecular imprinting containing functional groups, well reviewed by Wulff [251], are employed to introduce specific recognition sites into the polymers, in which the functional groups are arranged complementary to the structure of the

template. The low capacity of the molecularly imprinted polymers for the print molecule as well as its cost and availability are some of the drawbacks [252].

Plasma modification is a popular method used to alter the surface functionality of polymers without affecting their bulk properties, hence it is the technique of choice employed for a number of biomedical applications [253]. It does not only allow a high level of film chemistry controllability during the coating process, but also offers uniformly modified surfaces regardless of the complex shapes and geometry [254].

2.5.1.1.1 Fibrinogen and platelet interaction with varying surface chemistries

The effect of surface chemistry on fibrinogen adsorption has been extensively investigated and the results revealed that the amount of spontaneously adsorbed fibrinogen is a function of surface chemistry [84, 255-257]. Polymer surface with either super-hydrophilic or super-hydrophobic property may possess excellent blood compatibility [258]. Generally, improved blood compatibility were observed on surfaces with anionization and zwitterionization, with zwitterionomer being relatively less thrombotic than the anionomer, whilst the highest thrombogenic potential was observed on the cationized surfaces [259].

To minimize protein adsorption on the surface of biomaterial, rigorous research efforts have been placed on fabricating surfaces with functional groups that are able to “repel” proteins. Functionalities like carboxyl (-COOH), amino (-NH₂), hydroxyl (-OH) and methyl (-CH₃) groups are most commonly investigated with

Chapter 2: Literature Review

relation to biomaterial interactions. The presence of new functional groups on modified graft surfaces through argon plasma treatment revealed a higher inhibitory effect on fibrinogen deposition [260]. Similarly, surface functional groups can influence the surface-induced conformational changes of adsorbed fibrinogen on plasma functionalized surfaces [261]. A linear decrease of fibrinogen was demonstrated with an increase of -OH groups on a SAMs surface [84]. Additionally, an optimum concentration of -OH groups has been suggested to enhance the affinity of albumin binding over fibrinogen [256], while an abundant amount of adsorbed fibrinogen has been observed on -CH₃ terminated SAMs [262].

Conversely, fibrinogen adsorption on negatively charged surfaces, such as a carboxyl -COOH group, revealed a lesser amount than on the -NH₂ surface, since fibrinogen is unable to out-compete water to interact with the carboxyl surface whereas the -NH₂ surfaces can adsorb more fibrinogen by the formation of hydrogen bonds with fibrinogen to the surface [263]. Likewise, fibrinogen was observed to adsorb lesser on -COOH surfaces as compared to -CH₃, -NH₂ and -OH surfaces [255]. Indeed, another study showed similar result for fibrinogen adsorption to SAMs in the ascending order: -CH₃ >> -OH > NH₂ > -COOH [264]. Subsequently, platelet accumulation was observed on surfaces with -CH₃ functionality due to the high fibrinogen binding, leading to poor blood compatibility [265]. Correspondingly, reduced amount of platelet adhesion and activation was observed with decreased fibrinogen adsorption on SAMs surface with the -OH functionality [84]. Lowest platelet adsorption was observed on mixed SAMs surfaces with equal molar fractions of -NH₂ and -COOH functionalities, suggested the importance of surface neutrality

Chapter 2: Literature Review

related blood compatibility of biomaterials [266]. In fact, platelet adhesion has been demonstrated to reduce gradually to gradient surfaces with increasing surface density of functional groups [267].

An ionic PO_3H_2 surface with lower platelet reactivity than a $-\text{COOH}$ control was employed in a two series of binary mixed SAMs, and revealed the lowest level of platelet adhesion on $-\text{PO}_3\text{H}_2$ and $-\text{OH}$ mixed SAMs surfaces [268]. The results suggest that platelet compatibility of $-\text{PO}_3\text{H}_2$ is somewhere between the $-\text{OH}$ and $-\text{CH}_3$ groups. In addition, markedly enhanced antithrombogenicity has been observed on sulfonated PEO surfaces with low degree of platelet adhesion by the introduction of the negatively charged SO_3 groups [269].

In vitro assays show that a superhydrophobic surface of polydimethylsiloxane rubber (PDMS) and a superhydrophilic surface of hydroxyethylmethacrylate phosphatidylcholine (HEMA-PC-grafted surface) irradiated by a CO_2 -pulsed laser resulted in reduced platelet adhesion and subsequent activation [258]. Here, the phosphatidyl choline zwitterionic head group of the HEMA-PC-grafted materials create a strong bond hydration shield around the molecules and prevents the molecular recognition process from bonding to the blood proteins and cells. Conversely, the laser-treated PDMS surfaces with both roughness (porosity) and the charged chemistry nature of the surface (O_2 enriched) serves to alter the properties to nonplatelet activity as compared to the nature of the platelet activity of the pristine PDMS. Another similar study also showed the reduced platelet adhesion and activation onto a polyethylene terephthalate (PET) film when the surface was

irradiated with a CO₂ pulsed laser followed by acrylic acid (AAc) graft polymerization [270].

2.5.1.2 Topographical structures on polymeric surfaces

Surface patterning with features spanning over microns to nano scales have been gaining intense interests with the recent innovations to allow the production of these structured surfaces with greater geometrical complexity at much reduced operation time and cost. Approaches for creating intricate patterns on polymeric surfaces has been well reviewed [271]. Typically, our interest is focused on the effect of a) different carbon nanotube orientations and b) protruding structures with controlled geometrical parameters, directed to investigate their roles in affecting the fibrinogen and platelet behavior. This is reviewed in the following *sections 2.5.1.3 and 2.5.1.4* respectively.

2.5.1.3 Fabrication of Polymer Nanocomposite with CNTs

For the past decade till now, polymer nanocomposites have received significant attention from researchers. Polymer nanocomposites represent a radial alternative to conventional filled polymer and polymer blends in which discrete constituents incorporated in the polymer matrix are in the nanoscale range [272].

Dispersing the nanotubes in polymer matrices is one of the most common techniques in the fabrication for homogeneous nanotubes-polymer composites, where they can be fabricated by solution blending, melt blending, in situ polymerization and chemical functionalization [214, 223]. Though the agglomeration of CNTs can occur

Chapter 2: Literature Review

due to the slow solvent evaporation rate in solution blending, it can be reduced by employing the spin-casting technique [273], wet annealing method [274] and coagulation method [275].

Generally, melt blending is less effective at dispersing nanotubes in polymers since it is limited to lower nanotube loadings. However, high nanotube loadings can be achieved via a “coagulation spinning” method by coagulating the nanotubes into a mesh by wet spinning it together with aqueous poly(vinyl alcohol) solution followed by a slow draw process [276]. Apparently, in situ polymerization offers a better solution for homogenous dispersions because nanotubes are more likely to disperse easily in a precursor monomer than in the polymer. The routes to chemical functionalization of CNTs have been discussed in *section 2.4.2.4*.

Layer-by-layer (LBL) assembly method is another technique used in the fabrication for polymer nanocomposite. It is based on a sequential dipping between alternating solutions of anionic and cationic polyelectrolytes on charged surfaces (Fig. 2.7). The driving force comes from the electrostatic interaction between the opposite charges and van der Waals interaction [277]. The substrate surface must be electrically charged, either by oxygen plasma or chemical modification, in order for the surface to bind with the first polyelectrolyte layer. Starting with a negatively charged substrate, the first layer of polycations is deposited onto the substrate electrostatically which overcompensates such that the sign of the surface charge reverses upon dipping. Similarly, the subsequent dips follow with alternating polyelectrolytes, resulting in a stepwise growth of polymer films [278].

Chapter 2: Literature Review

Poly(diallyldimethylammonium chloride) (PDDA) and poly(ethyleneimine) (PEI) are often used as polycations and poly(sodium-p-styrenesulfonate) (PSS) as polyanions in LBL assembly. PDDA/PSS and PEI/PSS systems are the two most commonly used combinations [279]. Prepared nanotube-polymer composite based on this technique has been reported with nanotube loading as high as 50 wt % [280]. Similarly, nanocomposites with layers of coated CNTs prepared by the LBL technique have been demonstrated [281-285].

Fig. 2.7: Schematic illustrating the sequential dipping of alternating polyelectrolytes with different charges in the electrostatic layer-by-layer self-assembly.

The alignment of CNTs in the polymer matrix can be fabricated by an ex situ alignment of CNTs as demonstrated by the well-aligned polyaniline (PANI)/MWCNT composite films [286]. Alternatively, alignment of CNTs can be achieved by mechanically stretching the nanotube-polymer composite above its glass transition temperature, followed by cooling down to room temperature when the desired stretching ratio was attained [287]. Composite containing vertically aligned

MWCNTs coated with poly(carbonate urethane)s (PCUs) has been demonstrated [4]. However, the issue observed in that study was not performed on a free standing film, where the composite film was prepared by dipping the MWCNTs substrate into the PCU. Further studies have been well reviewed by other research groups [214, 223].

The effect of proteins adsorption and platelet studies on CNT composites has been previously reviewed in *sections 2.4.2.2 and 2.4.2.3* respectively.

2.5.1.4 Fabrication of topographic surfaces in polymers with physical attributes

The review in this chapter is focused at controllable high aspect ratio protruding features ranging from micron to submicron meter scales. Some of the simplest and conceptual forms of polymers micron scale processing include injection moulding, hot embossing, thermoforming, stamping or printing [288]. Techniques which are capable to produce periodic array of nano features of high-resolution consist of electron beam lithography [289], ion beam lithography [290], nanoimprint lithography (NIL) [291], deep ultraviolet lithography [292], extreme ultraviolet lithography and X-ray lithography [293], where a master template is use to transfer the features onto the polymer following the deep reactive ion etch (DRIE) process [294]. In addition, colloids can also be employed as a resist material with DRIE on the silica [295] to create a master template. Subsequently, techniques that are employed in the transfer of features to allow for the fabrication of high aspect ratio features in polymers include: temperature-based processing via hot embossing [296] or nano-imprinting [297], thermal activated polymerization via soft lithography [298]

Chapter 2: Literature Review

or capillary force lithography via either temperature-induced capillarity or solvent-induced capillarity [299] and solvent-based casting [300].

Sun et al. has demonstrated nanocasting in PDMS via a replication process using a lotus leaf as the template [300]. Similarly, PDMS structures with micron features was fabricated via a replication technique on a master prepared by macromachining [301]. Nanostructured polystyrene (PS) surfaces was constructed on a nanoporous anodic aluminum oxide (AAO) template via the nanoimprint lithography [302]. Capillary lithography has been employed in the fabrication of nanostructured PEG surfaces [303]. Likewise, a capillary lithography approach on an alumina membrane was used in the fabrication for aligned PS nanotubes [304].

Alternatively, topographic features may be created on the surface via surface capillary waves as a consequence of inherent polymer relaxation processes [305] and through phase separation of incompatible mixtures [306], whom both effects exploited the soft-matter ordering phenomena to construct the periodic features. It occurs as a result of surface instabilities at temperatures above the glass transition at high field gradients; hence eliminating the use of any mask or mould. Additionally, topographical structures can be fabricated by temperature-induced instabilities via lithography induced self-assembly (LISA) [307] and lithographically induced self-construction (LISC) [308]. Besides, periodic patterning at submicron scale can be achieved in thin films of block copolymers with microphase separation [309]. Surface structures created by a plasma treatment via the anisotropic etching of the surface

layers can cause considerable changes in the surface structure and have been demonstrated on various polymeric surfaces [310, 311].

2.5.1.4.1 Fibrinogen and platelet interaction on topographic surfaces

Different proteins and cell types have been reported to react differently to surfaces with varied topographic features which include a diversity of shapes, scales and symmetry of the feature [312-314]. The influence of surface topography on protein adsorption has received keen interest only recently [18, 314, 315], yet it was found that the cell's behaviour could be influenced by its surroundings as early as in 1964 [316]. To date, the structural information on surface-bound protein conformations and geometries are yet to be elucidated and is comprehensible due to the diverse knowledge required across multidiscipline, involving material science, life science and nanometer science. Nevertheless, some important progresses have been made by various research groups.

It has been proposed that differing protein-surface interactions could force the protein molecules to adopt specific conformations on the topographic surface, specifically on the same length scale as the protein molecules to hinder the protein function for specific interaction with the cells [317]. Surface topography on the order of nanometers (up to a several hundreds) has been shown to influence the conformation of bound proteins as demonstrated by P Roach et al. [315] on the conformation of fibrinogen and albumin using the surface curvature effect of varying sized colloidal particles. Likewise, a decrease in fibrinogen uptake was observed on colloidal structured silica with radius 21 nm when compared with a range of radius at

Chapter 2: Literature Review

7, 14 and 21 nm [318]. As proposed by various groups [319, 320], the interaction areas between the protein and nano particles increased as the particle size increased, thereby inducing more secondary structure changes in the protein. Indeed, the curvature may have caused diverse effects on the conformational and organization of various adsorbed proteins.

Diverse surface topographies, including the shapes, roughness, dimensional aspect and symmetry of the feature can result in a variety of fibrinogen responses. Surface feature in the nano-scale roughness has been suggested to be the mediating factor for the differences in the behavior of protein adsorption rather than at the micron scale [321, 322]. Controversial views have been reported regarding adsorbed fibrinogen induced by surface roughness, where one study reported no statistically significant differences on titanium surfaces with nanometer scale surface roughness (R_{rms} - 2 to 21 nm) [313]. On the contrary, another study clearly demonstrated the influence of surface roughness (R_{rms} - 2.0 to 32.9 nm) with an enhanced fibrinogen adsorption on a tantalum surface [323]. Similarly, increased fibrinogen adsorption have been demonstrated by Nygren et al. [324] with increased platelet adhesion on rough TiO_2 surfaces ($R_{\text{rms}} \sim 1.9 \mu\text{m}$). Fibrinogen adsorption process can be influenced by a specific feature sizes, e.g. an enhanced fibrinogen adsorption was observed on a whisker-like nano-roughness platinum surface [325]. Likewise, high level of adsorbed fibrinogen was observed on a patterned PDMS surface than on a flat surface [18]. The differing conclusions in the aforementioned studies were attributed to the varied nanotopography between the diverse tested surfaces and the differing methods to determine the protein adsorption. Thus, further studies should be performed on the

Chapter 2: Literature Review

same substrates with varied topographies in order to validate the effect of surface topography on the amount of adsorbed fibrinogen.

Cellular interactions on topographic surfaces with physical attributes have received tremendous attention [3, 326] and numerous studies on the effect of surface topography to cellular responses have been published [70, 327-329]; particularly in areas of orientation [329], proliferation rate [330] and gene expression [331], cell function regulation [248], cell adhesion [332], morphology [333, 334], tissue engineering [335] and cytoskeletal arrangement [336]. On the contrary, the effect of surface topography on platelet response is less explored. While promising results in relation to platelet response have been demonstrated on a variety of model substrates, such as on colloidal topographies [295], polymer demixed films [22], dual-scale structures with nano and micron dimensions on a PDMS surface [23], topographically modified polyetherurethane surfaces [337], machined and polished TiO₂ surfaces [321] and a textured polyether(urethane urea) film fabricated via a soft lithography two-stage replication molding technique [26].

The aforementioned studies describe platelet interactions with a variety of nanotopographies and a range of materials. However, none of the reports provided a systematic study from which a general behavior of platelets on topographic surfaces can be derived. Therefore, in this thesis we analyze the behavior of platelets in terms of adhesion and activation when they come into contact with a range of surfaces with topographic features with well defined geometrical parameters. The effect of material chemistry will be also considered by comparing the results from two materials, i.e

Chapter 2: Literature Review

carbon and PLGA. From the studies, it will be determined if controlled topographical manipulation is a feasible approach to reduce the thrombogenicity of surfaces for the materials employed in medical implants and other body fluid contacting applications.

Chapter 3

Platelet Adhesion Studies on Nanostructured Polymer Carbon Nanotube Composite

3.1 Introduction

Thrombosis is a complicated interaction between platelets and plasma proteins that occurs when blood contacts a foreign material. Blood-contacting medical devices such as stents and artificial heart valves usually fail due to thrombotic occlusion over time, caused primarily by platelet adherence to the material surface. Furthermore, thrombosis and thromboembolism complicate, to some extent, the use of all blood-contacting cardiovascular devices, since no artificial surface is completely inert towards blood. In addition, the current control of thrombus formation by systemic anticoagulant therapy is clearly not ideal due to the number of side effects associated such as thrombocytopenia, neutropenia and hemorrhage. Due to thrombogenicity,

platelets are undeniably activated on the surface of foreign matter and patients with artificial blood-contacting implants constantly require anti-coagulation therapy. The minimization of thrombosis is without doubt an important criterion for biomaterials for uses either as a temporary support or as permanent implants in a human body. However, till date, the search for blood-compatible surfaces remains on-going for the realization of a surface with minimum platelet-surface interactions.

The relatively passive nature of carbon in tissues is well known and documented, and many forms of carbon are used as coatings and fillers in biomedical implant applications which include the pyrolytic carbon, diamond-like carbon (DLC), carbon nitride (CN), carbon fibers and glassy carbon as discussed in *section 2.4.1*

Recently, the impressive properties demonstrated by carbon nanotubes (CNTs) are viewed as a class of nano-materials for promising applications in a variety of biological and biomedical systems and devices. These materials possess high tensile strength with Young's modulus about 5 times and tensile strength more than 100 times those of steel, and are even stronger than diamond due to their shorter carbon-carbon bond lengths as compared to diamond [338]. Being ultra-light weight with their excellent thermal and chemical stability, they can be used as reinforcement nano-fillers in polymeric materials for improvement in the mechanical properties as well as to create a highly anisotropic nanocomposite [339-341]. Based on this premise, carbon nanotubes (CNTs) were evaluated [195-197] for their potential applications in a variety of biomedical systems and devices due to their unique structure and properties. The most widespread application has been the use of CNTs

in polymer nanocomposites [212-215] as attributed to their inert nature and biocompatible chemical surfaces to cells and tissues as discussed in *section 2.4.2.3*. Motivated by these promising results, we aim to investigate for a blood-compatible biomaterial by incorporating CNTs onto a polymer film for its reduced thrombogenicity effect.

In this work, we study the thromboresistance of poly (lactic-*co*-glycolic-acid) – multiwalled carbon nanotube (PLGA-MWCNT) composite as a building material to fabricate artificial blood prostheses. Nanocomposite-based biomaterial will be prepared by the electrostatic Layer-by-Layer (LbL) deposition technique as discussed in *section 2.5.1.3*. Within the numerous proteins present in plasma, fibrinogen is one of the most adhesive plasma proteins that are responsible for thrombus formation on surfaces. Therefore, it will be immobilized on the PLGA-MWCNT composite before incubation in non-stimulated platelet-rich plasma (PRP) for platelet studies. Interactions between the plasma proteins, particularly fibrinogen and platelet-rich plasma (PRP) are investigated on the prepared PLGA-MWCNT composites and compared with the pristine surface.

3.2 Materials and Methods

3.2.1 Materials

Carbon nanotubes were purchased from Shenzhen Nanotech Port Co., Ltd, China; 70 % nitric acid (HNO_3); 95 wt% sulphuric acid (H_2SO_4); hydrogen chloride (HCl); sodium hydroxide (NaOH); poly(diallyldimethylammonium chloride) (PDMAA);

poly(sodium styrenesulfonate) PSS; 80/20 poly(lactic-*co*-glycolic acid) 5.01 (PLGA) was purchased from PURAC biochem and dichloromethane (DCM).

Human fibrinogen (Hfg) was purchased from Sigma-Aldrich (~ 35-65 % protein, $\geq 95\%$ of protein clottable). Platelet-rich plasma (PRP) was supplied from US Biological, USA. The PRP is stored at - 20 °C when not in use and are stable up to 12 months. Phosphate buffer saline (PBS) consist of sodium chloride (NaCl); monobasic potassium phosphate (KH_2PO_4); dibasic sodium phosphate (Na_2HPO_4) and potassium chloride (KCl).

3.2.2 Functionalization of MWCNTs

Carboxylic acid groups were introduced into the multi-walled carbon nanotubes (MWCNTs) by acid treatments [231]; whereby MWCNTs were added into a solution of H_2SO_4 and 70 % HNO_3 in a ratio of 3:1. The mixture was sonicated for 2 hrs, followed by adding 40 ml of HCl for 2 hrs to allow for the conversion to carboxylic acid groups on the surface. The mixture was then filtered using a vacuum pump after diluting the mixture with deionized (DI) water. Subsequently, it was washed with NaOH solution and DI water to achieve pH 7. Finally, the functionalized MWCNTs were baked at 70 °C for 24 hrs.

3.2.3 Preparation of PLGA-MWCNT composite by LbL technique

The PLGA film was mixed with DCM in a ratio of 1 g: 15 ml, casted into an aluminium petri-dish and vacuum baked at 80 °C for 5 days. A piece of PLGA film was first hydrolyzed with 6 M NaOH aqueous solution ranging from 5 - 15 mins at 40

°C in water bath. After rinsing with DI water, the film was immersed in 1.5 wt% PDDA solution (with 0.5 M NaCl) for 5 - 30 mins at room temperature followed by rinsing with DI water. The PLGA film was then dipped into a range of 0.08 - 0.3 wt% PSS (with 0.5 M NaCl) for 5 - 30 mins and rinsed with DI water. The PDDA/PSS adsorption treatment was repeated for 2 cycles with a fresh solution each time before a final treatment in PDDA solution as illustrated in Fig. 3.1. The film was dipped into a homogenized dispersed sonicated solution of functionalized MWCNTs in DI water ranging from 1 - 30 mins before drying at temperatures varying from 30 – 50 °C.

3.2.4 Plasma protein adsorption for platelet studies

PBS solution of pH 7.4 was prepared by dissolving 8.0 g of NaCl, 0.2 g of monobasic kalium phosphate, 2.9 g of dibasic sodium phosphate and 0.2 g of KCl into 1 L of DI water. Hfg was immobilized onto the PLGA-MWCNT composite and pristine PLGA film (for control) before incubation in non-stimulated platelet-rich-plasma (PRP) for platelet studies. The PLGA-MWCNT composite was cleaned in PBS before immersion in Hfg in concentration of 4 mg/ml and placed in a water bath 37 °C for 1 hour. Subsequently, it is washed in PBS to remove any loosely bound Hfg before incubation in PRP for 2 hrs at 37 °C. The samples were rinsed in PBS to remove the non-adhered platelets and fixed with 2.5 % glutaraldehyde for at least 2 hrs at 4 °C. After which, the samples were rinsed in PBS and dehydrated through a series of graded alcohols (0%, 25%, 50%, 75%, 100%) before they were freeze dried at - 80 °C for Field Emission Scanning Electron Microscope (FESEM) analysis. Surface characteristic of the PLGA-MWCNT composite were determined by the

contact angle (CA) measurement. The functionalized MWCNTs were characterized by Attenuated Total Reflectance – Fourier Transform Infrared (ATR-FTIR), Energy Dispersive X-ray (EDX) microanalysis and Transmission Electron Microscopy (TEM). TEM was performed on the functionalized MWCNTs on its structural integrity and investigation for the presence of any unremoved catalysts after the acid treatment.

Fig 3.1: Schematic diagram on the fabrication process for the PLGA-MWCNT composite by employing the LbL technique.

3.3 Results and Discussion

3.3.1 Analysis of functionalized groups on MWCNTs

Optimized acid treatment times are absolutely required in order to avoid breakage of the nanotubes and to prevent the sidewalls of the nanotubes from acquiring defects. The structural integrity of the MWCNT remained intact as shown in Fig. 3.2 with no signs of any catalysts presence. Dark spots appeared to be in-line with the walls of the MWCNTs and could be induced by the aggressive acid treatment where defects (kinks) were being introduced as lattice distortion.

Fig. 3.2: TEM micrograph on a segment of MWCNT showing (a) the presence of dark spots from carboxylic acid treatment and (b) magnified view of the dark spots on the walls of the MWCNTs indicated the possibility of defects induced as lattice distortion from the aggressive acid treatment.

ATR-FTIR spectra of functionalized MWCNTs showed the attachment of carboxylic acid groups in Fig. 3.3. Carboxylic acid groups (carbonyl C=O stretch) is

indicated at 1738cm^{-1} (presence of COOH is within $1700 - 1790\text{cm}^{-1}$ range). The existence of the C-O stretch is indicated at 1217cm^{-1} . Previous study has shown that oxidized MWCNTs have better solubility and can form stable dispersions when they are terminated with oxygenated functionalities which includes the carboxylic, carbonyl and hydroxyl groups [231]. These functionalized MWCNTs also act to provide bonding sites to the polymer matrix due to crosslinking since the ends of the MWCNTs are expected to carry more than one carboxylic acid group.

Fig. 3.3: ATR-FTIR spectra of a) MWCNTs (control) and b) functionalized MWCNTs with the attached carboxylic acid groups at 1217cm^{-1} and 1738cm^{-1} .

We observed that the functionalized MWCNTs were easily dispersed within minutes under sonication which remained stable overnight till usage; hence this demonstrated an effective mean to improve the solubility and dispersion of the MWCNTs through the chemical modification technique. The heat generated by

sonication over a prolonged period heat up the suspension and the solvent is evaporated away, causing damage and re-aggregation of the MWCNTs. Therefore, sonication in an ice-bath is necessary in order to prevent this from occurring. It is noteworthy that intensive ultrasonication is not encouraged as it might cause severe damage to the CNT walls and decrease the dispersion stability when the solution stability reached a saturation limit [233].

3.3.2 Adsorption of MWCNTs onto PLGA film by LbL technique

Efficient adsorption of MWCNTs was observed on the PLGA film as shown in Fig. 3.4. This sample was dipped in PSS and PDDA for 15 mins, followed by 30 mins of MWCNTs adsorption and dried at 50 °C for 1 hr. We observed that using PSS with 0.08 wt% resulted in maximum adsorption among all the samples when the concentration of PSS was varied between 0.08 - 0.3 wt% with other conditions remaining the same. With lower charge density, more molecules can be adsorbed to overcompensate and invert the surface charges for adsorption in the next layer, which resulted in an increase in the adsorption amount of polyelectrolytes. As discussed by Kolarik *et. al* [342], the principle behind LbL assembly arises from the electrostatic interactions which can be affected by the charge density of the polyelectrolytes, where the adsorbed amount can be increased by decreasing the charge density. This observation correlates well in that a reduction in weight concentration leads to a reduced charge density that subsequently resulted in an enhanced adsorption of MWCNTs. The maximum adsorption at a lower concentration suggests the presence of a critical charge density, in which an optimum minimum charge density for the

polyanions could lead to adsorption enhancement with increase in thickness layers but this would result in weak interlayer bonding due to low charge density that is available for electrostatic interactions.

Fig. 3.4: PLGA-MWCNT composite with an enhanced MWCNTs coverage with 0.08 wt% of PSS treatment.

Generally, the adsorption of polyelectrolytes takes about 10 to 20 mins [343-345], but Chen J Y et al. states that the adsorption of the polyelectrolytes on the substrate is relatively fast and reaches its saturation point within 2 mins [346]. In our study, the dipping times of substrate in polyelectrolyte and the MWCNTs solutions were varied between 1 to 30 mins. A longer dipping time resulted in a better surface coverage as shown in Fig. 3.5a with 15 mins and 30 mins dip in the polyelectrolytes and MWCNTs solutions respectively. In comparison with Fig. 3.5b, where the sample was dipped in both polyelectrolytes and MWCNTs solutions for 1 min, non homogeneous MWCNTs coverage can be observed on the surface especially around the dust particles. This can be explained by a two step process of polyion adsorption [347]. During the first 1 to 2 min of the process, the polymer chains are adsorbed on

sites with a higher concentration of local charges such as scratches, holes and edges, which resulted in a non homogeneous surface. But with a longer deposition time, the macromolecules will equilibrate and relax to form a flattened and a homogeneous monolayer. Since the second step requires a longer period, this explained why longer dipping times effect in higher adsorption thereby resulting in a homogenous coverage of MWCNTs on the PLGA film.

Fig. 3.5: (a) PLGA-MWCNT composite showing a homogeneous MWCNTs adsorption with 15 mins and 30 mins dip in polyelectrolyte and MWCNTs solutions respectively and (b) a non homogeneous MWCNTs coverage due to the presence of local charges with a short dipping time of 1 min.

3.3.2.1 Stability of PLGA film adsorbed with polyelectrolytes

Polymer degradation was observed in Fig. 3.6 on the surface of the PLGA-MWCNT composite film at regions without any MWCNTs attached. Polymer degradation could have occurred during the immersion in PSS as white visible lines could be seen on the sample surfaces after the first dip regardless of the dip time

which varied from 5 - 30 mins. The PSS solution turned from transparent to slightly white in color after every dip. In order not to affect subsequent dipping process, the PSS and PDDA solutions were changed after every dip. The polymer degradation was displayed as pit-holes with an uneven surface as shown in [Fig. 3.6](#). The non-CNT covered regions could arise from NaCl (or Na⁺ and Cl⁻ ions) competing with the polyelectrolyte for adsorption, resulting in the dissolution of the multilayer formation and being washed away subsequently. The caustic effect of the NaOH pretreatment as a result of its alkalinity could also have resulted in the pit-holes formation. NaOH is a surface treatment to introduce negative charges on the substrate so that a good binding of the first polyelectrolyte can be achieved to ensure satisfactory binding in the subsequent layers. In theory, adsorption of polyelectrolytes and MWCNTs on the uncharged substrate is not anticipated since surface charge density is relatively small for most substrates, and the amount of polyelectrolyte adsorbed is dependent on the type of substrate used and its surface treatment [279]. In our study for substrates without the pretreatment, the surface was observed to be macroscopically patchy with an inhomogeneous coverage of MWCNTs. Hence, a minimal surface charge on the substrate is essential to ensure a successful LbL assembly with a homogenous coverage of MWCNTs.

Fig. 3.6: Regions with no MWCNTs adsorbed on the PLGA-MWCNT composite showed signs of polymer degradation with the presence of pit-holes and uneven surface.

The addition of salt to the polyelectrolytes is used to contribute to an increment in the adsorption amount to allow for adjustment in the thickness of the adsorbed layers and to speed up the adsorption process [279, 348]. Here, a higher degree of coiling occur in the polyelectrolytes chain with increasing salt concentration and result in a thicker formation of adsorbed polyelectrolytes in the presence of salt. When no salt was added, either very little or no MWCNTs were adsorbed on the PLGA film. To counter this problem, a longer dipping time was used to enhance the adsorption of the MWCNTs to the PLGA film to enable a more homogenous adsorption of MWCNTs on the PLGA film.

Drying procedure is another parameter that influences the adsorption process. We realized that the duration of drying is crucial for the amount of adsorbed MWCNTs on the PLGA film. Maintaining the wetness of adsorbed layers during

deposition always results in a linear adsorption of thinner layers as demonstrated by the increase in absorbance measurements due the complete decoiling of polymers with the loosely adsorbed layers [349]. In contrast, drying of the film between each adsorption step is reported to either contribute to the success of film growth or interference with the film growth under extreme conditions [345, 349]. In our study, the substrate surface is observed with little or no adsorbed MWCNTs when it was dried at 40 °C for more than 3 hrs. It is inferred that contamination such as impurities and dust particles may compensate the electric charge of the top polyelectrolyte layer to some extent with extended periods of drying, and consequently reduced the adsorption efficiency. In addition, improper sample handling, storage and transportation of the sample before placing into the oven could have also introduced the contaminants onto the film. On the other hand, MWCNTs adsorption was observed on samples dried between 1 - 2 hrs. Here, re-organization of surface charges takes place and caused the enhancement of the hydrophilic part of the PDDA on the surface through drying which makes it favorable for MWCNTs adsorption. As discussed by Chen et al. [345] that re-adsorption only takes place after the film is washed in water and dried. And a drying step is crucial for the re-adsorption of the next layer of polyelectrolyte as the surface charges reorganize themselves for a more compact layer that is less susceptible to hydration due to an entropy-increasing process. This study indicates that an optimum drying time is vital for a successful adsorption of MWCNTs onto the PLGA film.

3.3.3 Platelet studies on PLGA-MWCNT composite

The surface properties of a biomaterial can affect its behavior towards other materials or molecules, where the attachment behavior of platelets can be effectively controlled through the modification of the surface properties (surface charges, surface hydrophilicity or hydrophobicity, surface roughness, wettability and surface tension) of the substrate [15, 317, 350].

In our analysis, we observed that platelet adhesion as well as activation (as deduced from platelet morphology) is greatly reduced, on the PLGA-MWCNT composite (Fig. 3.7a) when compared to the pristine PLGA control sample (Fig. 3.7d). This observation correlates well to contact angle measurement made on the PLGA-MWCNT composite with an angle of 64.94° as compared to the pristine (93.43°). As a result, reduced platelet adhesion was observed on surfaces with increased hydrophilicity having $CA < 90^\circ$, which agrees well with studies performed by other authors [15, 350, 351]. Additionally, the reduced platelet adhesion is attributed to the electrostatic repulsion between the MWCNTs and the Hfg/platelets. Fibrinogen is known to be a relatively large protein having a molecular weight (MW) of 340 K Daltons with an overall net negative charge [94], while human blood platelets at physiological pH in saline or plasma exhibit a net negative charge [352]. Moreover, the hydrophilic functional groups attached into the surface of MWCNTs by the strong acids (e.g. H_2SO_4 , HNO_3 and HCl) from the acid treatment resulted in negatively charged surface groups. It has been shown that surfaces with negatively charged groups are antithrombogenic, while positively charged surfaces are thrombogenic

[353]. Thus, the decrease in the platelet adhesion on the PLGA-MWCNT composite is influenced by the surface chemistry (highly hydrophilic after functionalization with negative surface charges) as well as the surface morphology of the MWCNTs, whereby repulsion prevented the direct contact between the Hfg and subsequent bridging with the platelets on the PLGA-MWCNT composite surface.

Likewise, as observed on the PLGA-MWCNT composite in [Fig 3.7b and 3.7c](#), the amount of platelet adhesion on regions with no adsorbed MWCNTs is greater as compared to the regions with adsorbed MWCNTs. Clearly, there is a distinct preferential adhesion of platelets with modest platelet activation on the PLGA surface of the PLGA-MWCNT composite as evident in [Fig. 3.7c](#), and could be attributed to the surface properties of the MWCNTs. In particular, nonwoven single-walled nanotube (SWCNT) has been demonstrated to show extremely low levels of platelet adhesion and activation [5]. Likewise, reduced thrombogenicity was demonstrated on microcatheter prepared from MWCNTs-nylon-based composite [7]. Additionally, nanostructured superhydrophobic films with MWCNTs coated with poly(carbonate urethane)s (PCUs) showed excellent anti-adhesion to platelets and have relatively low platelets activation [4]. Reduced platelet adhesion was observed on the PLGA-MWCNT composite ([Fig. 3.7c](#)) where the platelets preferred to adhere on the PLGA surface instead of a region with adsorbed MWCNTs. We ascribed this observation due entirely to unfavourable interaction between platelets and to the dense surface topography of the MWCNTs adsorbed on the PLGA film, since gas pockets in or on the surfaces act to increase the likelihood of thrombus formation [354].

In comparison, both platelet adhesion and activation are observed on the control sample, where the activated platelet displayed a dendritic morphology from their initial discoid shapes in the resting state (Fig.3.7d). We postulate that on hydrophobic surfaces such as pristine PLGA, the adsorption of Hfg results from the unfolding of the Hfg that exposes the interior hydrophobic domains. The adsorbed Hfg then interacts with the appropriate receptors on the platelet surface to enhance platelet adhesion. Subsequently, if the conformation of the adsorbed Hfg is favourable, then the platelet becomes activated via activation of the GPIIb/IIIa receptors that eventually leads to spreading. The conformational change induced by the surface-adsorbed Hfg has been determined to affect the behavior of platelet adhesion and activation on substrates [355, 356]. In principal, glycoprotein GP IIb/IIIa is essential for both platelet aggregation and platelet adhesion for binding to the Hfg to facilitate platelet-platelet interaction [152]. In contrast, this spreading is not observed for the MWCNT-PLGA composite surface, such that a conformational rearrangement of the adsorbed Hfg adopts a structure unfavourable for succeeding platelet activity, and hence the platelets do not assume an extended morphology.

Fig. 3.7: (a) Reduced platelet adhesion showing no signs of activation on the PLGA-MWCNT composite, (b) increase platelet adhesion on the PLGA-MWCNT composite at regions with no adsorbed MWCNTs, (c) distinct platelet adhesion preferential on regions without any MWCNTs adsorbed and (d) platelet adhesion and activation observed on the pristine PLGA.

3.4 Summary

A homogeneous dispersion of MWCNTs throughout the polymer matrix has been demonstrated by the improved the nanotubes dispersion in polar organic solvent (DCM) through functionalization of the MWCNTs by the acid treatment. Suitable surface functionalizations of the MWCNTs are important to increase the MWCNT-polymer interfacial adhesion and chemical bonding as demonstrated by the carboxylic acid functional groups grafted into MWCNTs that offers an in situ chemical integration of the MWCNTs into the polymer matrix.

A simple technique to fabricate a thin and reproducible multilayer formation of MWCNTs by the LbL assembly technique has been demonstrated under the influence of different factors. These are summarized as below:

- Surface charging of the substrate is required for a homogeneous surface coverage and an overcompensation of the original surface charge of polyelectrolytes is important for a successful adsorption.
- A low weight concentration led to a low charge density of the polyelectrolytes and resulted in an enhanced adsorption of MWCNTs onto the PLGA film when more molecules are adsorbed to overcompensate and invert the surface charge charges for adsorption in the next layer.
- The addition of salt can improve both the adsorption rate and adsorption amount, but affects the mechanical properties when the film undergoes subsequent degradation. Therefore, a longer dipping time is suggested in a

salt free solution to enhance the adsorption of the MWCNTs to the PLGA film.

- Extended drying can improve the smoothness of the adsorbed films and result in the formation of more compact layers. However, a dry surface tends to have a higher chance of contamination by the dust particles than wet surface hence careful handling is necessary when drying is used. An optimized drying time between 1-2 hrs was demonstrated for an efficient adsorption.

The optimal parameters to achieve an efficient adsorption of MWCNTs onto the PLGA film are: PDDA and PSS at 1.5 wt% and 0.08 wt% respectively, with PDDA/PSS dipping duration of 15 mins, followed by 30 mins of MWCNTs adsorption and oven dried at 50 °C for 1 hr.

We have demonstrated a significant reduction level of platelet adhesion with the absence of platelet activation on PLGA-MWCNT composite fabricated by employing the LbL technique. Though the formation of pit-holes were observed due to the NaOH pretreatment in the LbL assembly, reduced platelet adhesion on the PLGA-MWCNT composite is clearly attributed to the surface chemistry as well as to the surface morphology of the MWCNTs. The observations suggested that platelet activation could be efficiently suppressed on this nanostructured composite with its excellent anti-adhesion and subsequently low platelet activation. Such composites may be used as a suitable anti-thrombotic material in artificial blood prostheses. However, a greater understanding of the influence of surface topography and of

Chapter 3: Platelet Adhesion Studies on Nanostructured Polymer Carbon Nanotube Composite

surface chemistry is required in order to design the optimum surface. This will be explored in the next chapter, where the elucidation on the role of topography and chemistry on Hfg and platelet adhesion on foreign surfaces chemistry will be presented.

Chapter 4

Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

4.1 Introduction

Numerous approaches have been employed in fabricating blood-compatible devices by either surface modification (by chemical and physical methods) as discussed in *section 2.5.1.1* or topographical modifications in *section 2.5.1.2*.

In the previous chapter, we demonstrated a significant reduction of platelet adhesion and absence of platelet activation on a poly(lactic-co-glycolic-acid)-carbon nanotube (PLGA-CNT) composite fabricated by the electrostatic Layer-by-Layer (LbL) technique. Enhanced platelet adhesion was observed on the surface of pristine PLGA as compared to surfaces with adsorbed CNTs. The reduction in platelet adhesion on the PLGA-CNT composite was attributed to the effect of the surface chemistry and topography of the CNTs, however no attempt was made to establish the relative importance of each factor. In view of these previous results, in this work,

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

we investigate the influence of surface chemistry and topography attempting to establish the contribution from each effect.

While many studies have demonstrated the promising results of reduced platelet adhesion and activation on a variety of carbon nanotubes based polymer composites [4, 6], the effect of a different surface topography presented by the various orientations of carbon nanotubes has not been explored. Indeed, platelet interaction studies in polymeric biomaterials, involving the effect of surface chemistry [6, 8-12] and that of surface topography [4, 16, 17] are scarce, and studies on both the effects of surface chemistry and topography in relation to platelet adhesion and hemocompatibility are still in a very preliminary state [19]. This is understandable because topographical variations are usually accompanied by the presence of chemical heterogeneities on the surfaces, which complicates the evaluation of blood interaction on biomaterial surfaces. In light of this, in this study, fibrinogen adsorption and platelet interactions were studied in poly (lactic-co-glycolic-acid) multi-walled carbon nanotube (PLGA-MWCNT) composites with randomly dispersed and vertically aligned MWCNT orientations and compared to that of pristine films to study whether the different surface topography provided by the MWCNTs can modulate the platelet interaction behaviour. The material used as control was the highly ordered pyrolytic graphite (HOPG) as it is well recognized as being thromboresistant in blood-contacting implants [357] and coatings [185].

4.2 Materials and Methods

4.2.1 Materials

PLGA, Hfg, PRP, PBS, MWCNTs were used as described in *chapter 3 of section 3.2.1* and vertically aligned MWCNTs from Nanoloab, Inc. Newton, MA. The chemicals used were 70% nitric acid (HNO_3), 95 wt% sulphuric acid (H_2SO_4), hydrogen chloride (HCl), sodium hydroxide (NaOH), dichloromethane (DCM) and Tween 20. Monoclonal antibody binding (horse radish peroxidase (HRP) conjugated with human fibrinogen goat antibody) and 3,3',5,5'- tetramethylbenzidine (TMB) were purchased from US Biological, USA. Highly Ordered Pyrolytic Graphite (HOPG) was supplied from SPI Supplies and Structure Probe. The HOPG and pristine PLGA films were used as controls in this study.

4.2.2 Fabrication of randomly dispersed PLGA-MWCNT composite

The carboxylic acid groups were introduced into the MWCNTs as described previously in *chapter 3 of section 3.2.2*. The functionalized MWCNTs (~0.059 g) were sonicated in 18 ml of dichloromethane (DCM) in an ice bath to achieve a homogeneous dispersion before adding PLGA pellets to the sonicated mixture. 1 g of PLGA pellets were dissolved in 18 ml of DCM to obtain a solution with low viscosity as the subsequent addition of MWCNTs increases the viscosity substantially. The solutions were mixed and cast into aluminium pans and left to dry at room temperature for a minimum of 3 hrs before placing into the vacuum oven at 55 °C for 4 days for the solvent evaporation. Oxygen plasma treatment as performed at 250

mTorr, 40 W, 12 sccm of O₂ to expose a partial layer of the embedded MWCNTs in the composites.

4.2.3 Fabrication of vertically aligned PLGA-MWCNT composite

A polymer solution of PLGA pellets to DCM (1 g / 20 ml) was infiltrated into vertically aligned arrays of MWCNTs grown on a silicon substrate by chemical vapor deposition (CVD). Prior to PLGA infiltration, the vertically aligned arrays of MWCNTs were exposed to O₂ plasma treatment at 250 mTorr, 40 W, 12 sccm of O₂ for 2 mins to enhance the PLGA infiltration in between the interstitial sites of the MWCNTs. The samples were placed at room temperature for 2 hours to allow the PLGA solution to infiltrate into the MWCNTs arrays before placing them into the vacuum oven at 55°C for 4 days. The samples were allowed to cool at room temperature before peeling the PLGA film from the silicon substrate.

4.2.4 Fibrinogen adsorption quantification by ELISA

The amount of Hfg adsorbed onto the substrate surfaces was determined by enzyme-linked immunosorbent assay (ELISA) array [358]. The adsorbed Hfg was quantified after binding to its monoclonal antibody by colorimetry. A calibration curve was established before quantification. The procedure is as follows: samples of 3 x 3 mm were first equilibrated in PBS for 30 mins in micro centrifuge tubes. Subsequently, the samples were placed in 96-well microtiter plate with 0.05 mg/ml of Hfg for 1 hr at 37 °C. The samples were rinsed 5 times with washing buffer (0.1% v/v Tween 20 in PBS) before transferring the samples into new wells on the microtiter

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

plate. The samples were then incubated in HRP conjugated goat antihuman fibrinogen for 1 hr at 37 °C at 1:100 000 dilutions. The samples were rinsed subsequently for 5 times before transferring into new wells. Finally, a 100 µl colorimetric agent 3,3',5,5'-tetramethylbenzidine was added into each well and left to incubate in the dark for 20 mins where the solution undergoes oxidation and turns blue upon reaction with HRP. Subsequently, stop solution with 100 µl of 0.5 M H₂SO₄ were added to the wells to stop the enzymatic reaction. The colour of the solution changes to yellow due to the conversion to diimine and correlate proportionally to the amount of bound antibody adsorbed onto the substrates. The solutions were quickly aspirated using a multiple pipette tool and transferred to new wells, where the absorbance values of the solutions were measured on a microtiter plate reader at 450 nm within 5 mins. Adsorption experiments were done in triplicate (n = 3) and repeated separately for 3 times.

4.2.5 Platelet adhesion studies

The procedure followed is same as described in *section 3.2.4* in *chapter 3*.

4.2.6 Characterization of PLGA-MWCNT composites

Functionalized MWCNTs and samples were characterized by Attenuated Total Reflectance – Fourier Transform Infrared (ATR-FTIR) (Spectrum GX) for the detection of surface chemical functional groups. Raman spectroscopy was used to determine the tangential band regions in the vertically aligned MWCNTs and vertically aligned PLGA-MWCNT composite. Atomic Force Microscopy (AFM)

(Nanoscope III, Digital Instruments, Inc.) and Field Emission Scanning Electron Microscope (FESEM) (JEOL 6340F) were used to image the surface topography of the PLGA-MWCNT composite and quantify for the platelet adhesion and spreading. AFM images were recorded at room temperature by non-contact method using a silicon nitride probe. The surface characteristic wettability was determined by contact angle measurements (FTÁ 200). Transmission Electron Microscopy (TEM) (JEOL 2010) was employed to examine the randomly dispersed PLGA-MWCNT composite, where microtome sections of the films were deposited on a copper grid holder and coated with a thin layer of carbon.

4.2.7 Statistical analysis

All experiments were run in triplicate. The PRP and plasma was from the same donor and kept at -20°C while stored. Data determined from contact angle, ELISA and platelet counting are presented as mean values with standard deviations. Statistical significance differences between two substrates were analyzed using single factor analysis of variance (ANOVA) for ($p < 0.05$); H_o is rejected when ($F > F_{crit}$).

4.3 Results and Discussion

4.3.1 Functionalized MWCNTs in randomly dispersed PLGA-MWCNT composite

A homogeneous and uniform dispersion of nanotubes in the polymer matrix is highly desirable in order to maximize the interaction between the nanotubes and the host polymer. The difficulty for uniform dispersal of carbon nanotubes lies in the

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

attractive van der Waal's forces between the surfaces of nanotubes and the polymer long chains. To improve the dispersability, PLGA pellets were added progressively to the dispersed MWCNTs in DCM after sonicating in an ice-bath to reduce the evaporation of the solvent, and to minimize the damage and re-aggregation of the MWCNTs. Prior to dispersion, the MWCNTs were acid treated to improve dispersion in the solvent as well as in the polymer matrix. Films of the MWCNTs composites were obtained by casting the mixture into aluminium pans and oven drying, as shown in Fig. 4.1a.

The ATR-FTIR spectra for all substrates are shown in their absolute intensities with background corrected. ATR-FTIR spectra of the control substrates are shown in Fig. 4.2a and 4.2b. The presence of carboxylic acid groups [359] on the fabricated nanocomposites was verified by ATR-FTIR spectroscopy in Fig. 4.2c and 4.2d, wherein the characteristic bands at 899 cm^{-1} , 1092 cm^{-1} and 1740 cm^{-1} can be observed. The existence of -COOH is indicated by the broad stretching bands between 3120 and 2890 cm^{-1} with the presence of 2970 cm^{-1} sharp peak due to CH stretching vibrations.

a)

Exposed Tube diameter: $\sim 24 - 43\text{ nm}$ (h)
Exposed Tube length: $\sim 2 - 8\text{ }\mu\text{m}$ (w)
Aspect ratio*: $\sim 0.003 - 0.02$

*Aspect ratio = height, h/width, w

b)

Tube diameter: $\sim 100 - 150\text{ nm}$ (w)
Protruding tube length: $\sim 2 - 5\text{ }\mu\text{m}$ (h)
Aspect ratio*: $\sim 13 - 50$

Fig. 4.1: Illustration on the orientations and dimensions of the multi-walled carbon nanotubes in the PLGA-MWCNT composite for a) randomly dispersed and b) vertically aligned.

Most of the MWCNTs in the composite were exposed to the surface after the O₂ plasma treatment and as verified by the ATR-FTIR (Fig. 4.2d) where a distinctive peak of -COOH group at 1092 cm⁻¹ is observed. The presence of this peak is also observed on the acid-treated MWCNTs, HOPG and the vertically aligned PLGA-MWCNT, confirming that the existence of the carboxylic groups is attributed to the acid treatment, whereas the carboxylic groups observed on the HOPG surface are possibly due to atmospheric oxidation as observed by other researchers [360]. The analytical data are summarized in Table 4.1. Both the acid treated MWCNTs and HOPG showed the same ATR-FTIR spectra, indicating that the HOPG could be used as a control material to determine comparatively the influence of topographical features on platelet adhesion. Figure 4.3 shows a TEM image of the individually dispersed MWCNTs throughout the randomly dispersed PLGA-MWCNT composite obtained by this process proving that the acid treatment of the MWCNTs was effective in the enhancement of the MWCNTs dispersion by reducing its tendency to agglomerate in bundles.

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

Fig. 4.2: ATR-FTIR spectra on (a) acid treated MWCNTs and HOPG, (b) PLGA, (c) vertically aligned PLGA-MWCNT composite and (d) randomly dispersed PLGA-MWCNT composites, plasma treated PLGA and aged PLGA. The peaks at 1092 and 899 cm^{-1} indicate the presence of the $-\text{COOH}$ groups.

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

Table 4.1: Wavenumber and assignment of ATR-FTIR spectra for PLGA, HOPG, randomly dispersed and vertically aligned PLGA-MWCNT composites.

Wavenumber (cm ⁻¹)	Assignment
<i>Individual peak characteristic in PLGA</i>	
3016-2916	C-H stretch in methyl groups
1746	C=O stretch
1424	C-H deformation in O-CH ₂ structure present in glycolic acid structure in PLGA
1380	Stretching and deformation of methyl groups
1268	O-H deformation vibrations
1180 and 1128	C-O stretch
1081	Alcohol C-O stretching vibrations in saturated primary alcohols
1043	C-CH ₃ stretch vibrations of methyl groups
956	Alkene C-H bend
867	Aldehydes C-H deformation vibrations
755-743	Interaction of O-H deformation and C-O stretching vibrations
<i>Common peaks in all samples</i>	
1470-1429	C-H stretch in methyl groups
~1365	Alkane C-H deformation vibrations
<i>Common peaks in all samples except PLGA</i>	
~3120-2890	-OH in carboxylic acids
~2970	C-H stretching vibrations
~1740	C=O stretching vibrations in ketone or carboxylic acid groups
~1217	Alkane C-C skeletal vibrations
~1092	Carboxylic acids –COOH (monomer)
~899	Carboxylic acids –COOH (dimer)

Fig. 4.3: TEM image of the randomly dispersed PLGA-MWCNT composite showing (a) a well dispersed MWCNTs throughout the PLGA and (b) a magnified view of a single MWCNT well blended into the PLGA matrix from inset (a).

4.3.2 PLGA-MWCNT composites with oxygen plasma treatment

The randomly dispersed PLGA-MWCNT composites films were treated with 1 min of O₂ plasma in order to expose the MWCNTs' topography for platelet adhesion studies. The diameter and length of the MWCNTs used in this study ranged from 20-50 nm and 5-15 μ m respectively. After 1 min of O₂ plasma treatment (250 mTorr, 40 W and 12 sccm of O₂) on the composite, the average diameter (Ra) of the MWCNTs protruding from the surface was $\sim$ 39 nm and the length (L) was $\sim$ 6.5 μ m as measured from the AFM images. Figure 4.4a shows the AFM image of the composite surface prior to oxygen plasma treatment and Fig. 4.4b, the nanostructured topography of the randomly dispersed PLGA-MWCNT composite after the treatment. The 3D AFM images and quantitative roughness data on the randomly dispersed

PLGA-MWCNT composite and the controls (pristine PLGA and HOPG) were shown in A4.1. Both the controls demonstrated similar quantitative roughness data, indicating that both could be used as a control surface to determine comparatively the influence of topographical features on platelet adhesion. The O₂ plasma treatment not only partially etched the surface of the composite, but also modified the surface chemistry of the PLGA-MWCNT composite. The randomly dispersed PLGA-MWCNT composite exhibited reduced contact angle readings (Fig. 4.5) due to the introduction of polar groups (carboxylic, hydroxyl, carbonyl) by the O₂ plasma treatment [203]. The formation of –COOH groups on the surface of PLGA by plasma treatment was verified by ATR-FTIR spectroscopy (Fig. 4.1d) by the 899 cm⁻¹ peak. However, ATR-FTIR measurements on the aged plasma treated PLGA after 20 days did not show the presence of carboxylic groups but displayed the same spectra to that of pristine PLGA films with the disappearance of the –COOH peak (Fig. 4.6). In contrast, the randomly dispersed PLGA-MWCNT composites showed the presence of carboxylic groups even 20 days after the O₂ plasma treatment, indicating that the origin of the –COOH can be attributed primarily to the acid treatment on the MWCNTs and not to the O₂ plasma effect on PLGA. The adhesion studies were subsequently carried out on aged substrates.

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

Fig. 4.4: AFM images (amplitude) showing the well-dispersed functionalized MWCNTs within the PLGA matrix with little agglomerations. (a) Before O₂ plasma treatment with an average Ra ~13nm and (b) when a partial layer of PLGA surface was etched after 1 min of O₂ plasma treatment with an average Ra ~39nm.

Fig. 4.5: Contact angle variation on randomly dispersed PLGA- MWCNT composite with different O₂ plasma treatment times. An increase in hydrophilicity with treatment times can be observed. Contact angle readings (n=3) were taken 24 hrs after O₂ plasma treatment.

Fig. 4.6: ATR-FTIR spectra of the aged plasma treated PLGA films showing the disappearance of the -COOH peak ($\sim 899 \text{ cm}^{-1}$) after a period of 20 days.

To fabricate vertically aligned PLGA-MWCNT composites, uniformly distributed vertically grown MWCNTs were used with length varying between 2 – 5 μm and tip diameter between 100 – 150 nm (Fig. 4.7a). The resulting film of an array of vertically aligned MWCNTs embedded in a PLGA matrix is shown in Fig. 4.1b. The vertically grown MWCNTs were subjected to O_2 plasma treatment prior to PLGA infiltration to improve the wettability and aid the infiltration of the PLGA solution into the MWCNTs (Fig. 4.7b) as compared to the substrates without treatment (Fig. 4.7c). The O_2 plasma treatment removes contaminants from the MWCNTs and at the same time increases the wettability of the MWCNTs by introduction of carboxylic groups [203], thus enhancing the infiltration of the PLGA into the array of MWCNTs as shown in the cross-sectional view in Fig. 4.7d. However, controlling the exposure time is important since prolonged O_2 plasma

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

exposure (> 3 mins) results in a strong adhesion of the PLGA to the silicon substrate and makes its removal from the underlying silicon substrate difficult.

Fig. 4.7: FESEM images showing a) the original vertically grown MWCNTs, vertically aligned PLGA- MWCNT composites b) with 2 mins of O₂ plasma treatment revealing an enhanced PLGA infiltration, c) without O₂ plasma treatment prior to PLGA infiltration and d) the cross-sectional view of the nanotubes within the PLGA matrix (arrows refer to the protruding MWCNTs).

Raman spectroscopy on vertically aligned PLGA-MWCNT composite in Fig. 4.8b shows the D peak (1351cm^{-1}), G peak (1577cm^{-1}) and the 2nd order of D peak (2704cm^{-1}) typical of MWCNT (Fig. 4.8a). The D peak intensity is lower than the G peak which implies a low ratio of defect since the D mode is induced by defects [359, 361]. The I_D/I_G intensity ratio is comparable, at 0.988 and 0.987 before and after infiltration respectively. These results confirm that the structure of the MWCNTs is well preserved after the plasma treatment and PLGA infiltration process.

Fig. 4.8: Raman spectroscopy of the vertically aligned MWCNTs a) before PLGA infiltration treatment and b) after PLGA infiltration (PLGA-MWCNT composite) showing the presence of D ($\sim 1351\text{cm}^{-1}$) and G ($\sim 1577\text{cm}^{-1}$) peaks.

4.3.3 Analysis and quantification for adsorbed fibrinogen on substrates

Fibrinogen was used like in previous tests, which is initially adsorbed on the substrates as fibrinogen mediates the adhesion and activation of platelets to artificial surfaces. The amount of fibrinogen adsorbed on the different substrates was

determined by ELISA. A calibration curve for Hfg bound to microtiter plate was first established. A linear correlation ($r = 0.951$) between the coating concentrations (0.001 – 0.05 mg/ml) of Hfg on the microtiter plate and the absorbance values measured by ELISA were obtained (Fig. 4.9). The calibration curve for the absolute amount of adsorbed Hfg was:

$$\text{Adsorbed Hfg (mg/mm}^2\text{)} = 4.31 \times 10^{-3} \text{ OD} - 1.489 \times 10^{-4}$$

where OD indicates the optical absorbance of the reaction solution as obtained by the microplate reader.

Fig. 4.9: Calibration curve showing the amounts of adsorbed Hfg on the microtiter plate against different concentrations of Hfg measured by ELISA. Data are expressed as the means $\pm$ SD of three independent measurements.

As shown in Fig. 4.10a, the contact angle readings of the substrates in the ascending order were as follows: randomly dispersed pristine PLGA-MWCNT < randomly dispersed etched PLGA-MWCNT < HOPG < PLGA < vertically aligned PLGA-MWCNT. On the other hand, the amount of Hfg adsorption onto the substrates (Fig. 4.10b) in the ascending order was: vertically aligned PLGA-MWCNT < randomly dispersed etched PLGA-MWCNT < randomly dispersed pristine PLGA-MWCNT < PLGA < HOPG. Although the loading in the PLGA-MWCNT composites (randomly dispersed vs. vertically aligned) is not exactly the same, the differences in fibrinogen adsorption and platelet adhesion are different being the vertically aligned PLGA-MWCNT composite is much lower.

The statistical significance on adsorption between substrates tabulated from Fig. 4.10 is shown in Table 4.2. Typically, hydrophobic surfaces tend to favour protein adsorption while highly hydrophilic surfaces impede adsorption [86, 87]. In our result, we observed that although the PLGA film and HOPG film (Fig. 4.10a) displayed similar contact angle readings ($\sim 81^\circ$), the amount of Hfg adsorption was different on these substrates (Fig. 4.10b). The HOPG is observed to adsorb the highest Hfg amount when compared to the other substrates. In contrast, a lesser amount of Hfg was adsorbed on the surface of the PLGA film. The result indicates that the adsorption of Hfg is not only determined by the surface energy of the substrates, but most importantly by the surface chemistry of the substrates since both the PLGA and HOPG have similar contact angle readings but displayed different amount of Hfg adsorption. A study by Feng et al. [362] is consistent with our results,

in that strong adsorption of proteins was found on Low-Temperature Isotropic Carbon (LTIC), which is similar to the HOPG used in our study.

Fig. 4.10: Graphs showing a) contact angle measurements in which readings were taken 20 days after O₂ plasma treatment on the randomly dispersed PLGA- MWCNT composite, b) amount of adsorbed Hfg determined by ELISA and c) amount of adherent platelets. Data were run in triplicate (n=3) and expressed as mean $\pm$ standard deviation of 3 independent experiments.

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

Substrate	HOPG			Vertically aligned			Randomly dispersed pristine			Randomly dispersed etched		
	a	b	c	a	b	c	a	b	c	a	b	c
Pristine PLGA	≠	*	*	≠	*	*	*	≠	≠	*	*	*
HOPG				≠	*	≠	*	*	*	*	*	≠
Vertically aligned							*	*	*	*	≠	≠
Randomly dispersed pristine										*	*	*

Table 4.2: Tabulated data from Fig. 8 for the comparison between substrates for their statistical significant differences in a) contact angle measurement, b) amount of adsorbed Hfg determined by ELISA and c) amount of adherent platelets. The statistical significance ($p < 0.05$) is indicated by asterisks and ≠ denotes non-significance. Data were run in triplicate ($n=3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments.

We observed a reduction of Hfg adsorption on the surfaces for both of the PLGA-MWCNT composites. Comparing the randomly dispersed etched PLGA-MWCNT composite between its pristine composite, the randomly dispersed etched PLGA-MWCNT composite had the least Hfg adsorption, whereas maximum Hfg adsorption was observed on the pristine composite without plasma etching treatment. Carboxylic groups on the pristine composite of randomly dispersed PLGA-MWCNT were almost non-existent because the MWCNTs are not exposed on the surface but covered by PLGA. This was confirmed by the much suppressed -COOH peak ($\sim 1092\text{cm}^{-1}$) for the pristine PLGA-MWCNT composite in the ATR-FTIR spectra shown in Fig. 4.2d. In addition, the difference in Hfg adsorption between these two substrates could be influenced by the exposed topographic features of the MWCNTs on the surface.

Carboxylic acid group has been reported to prevent or reduce protein adsorption as well as prevent platelet adhesion and subsequent spreading [10, 363]. Charge repulsion may contribute to reducing Hfg adsorption on the randomly dispersed etched PLGA-MWCNT composite film due to the presence of the carboxylic acid groups with negative charges. As reported by J. H. Lee et al. [353] serum proteins tend to adsorb more readily on positively charged surfaces than on negatively charged ones. Fibrinogen has a net negative charge in PBS (pH 7.4) that may contribute to a repulsive force between the Hfg molecules and the substrate surface of the etched PLGA-MWCNT composite film. Therefore, we attribute the reduction of Hfg adsorption to the presence of -COOH groups on the etched and vertically aligned PLGA-MWCNT composites, in addition to the significant influence of the topography effect presented by the MWCNTs as compared to the HOPG surface.

4.3.4 Platelet adhesion analysis on substrates and response to nanostructured surfaces

To quantify the number of platelets adhered on the substrates studied in this work, FESEM images were taken on the five types of substrates and the total number of platelets observed (from average of 6 images) was divided by the surface area ($n=3$) and expressed in mm^2 . The amount of platelet adhesion found on the substrates (Fig. 4.10c) in the ascending order was: vertically aligned PLGA-MWCNT < randomly dispersed etched PLGA-MWCNT < HOPG < PLGA $\cong$ randomly dispersed pristine PLGA-MWCNT.

Comparison between the vertically aligned PLGA-MWCNT composite and randomly dispersed etched PLGA-MWCNT) show similar level of platelets adsorption although the former displayed the least adsorbed Hfg and platelet adhesion. In comparison, the randomly dispersed pristine PLGA-MWCNT composite (Fig. 4.11a and b) displayed the highest platelet adhesion tendency and had a large amount of Hfg adsorbed when compared with the etched PLGA-MWCNT composite. In contrast, low amount of platelets was observed on the HOPG surface (Fig. 4.11c and d) and does not correlate with the large amount of Hfg adsorbed. On the HOPG, due to the negatively charged carboxylic acid groups, the re-organization of Hfg upon attachment does not appear to favour the subsequent binding of the platelet glycoprotein GPIIb/IIIa epitopes. Fibrinogen conformation is a requisite for platelet adhesion to surfaces as the binding of the GP IIb/IIIa receptor called LIBS (ligand-inducing binding site) on the membrane of the platelets forms a specific bond with the RIBS (receptor-inducing binding site) on the fibrinogen, whereby the latter becomes available when it reveals its RIBS sequence for specific interaction with the former when undergoing conformational changes [364]. Similar observations have been reported by other researchers, wherein the conversion of adsorbed fibrinogen to fibrin was comparatively ineffective on the negatively charged surface of self-assembled monolayers (SAMs) containing the carboxyl-terminated group [255]. In another study, SAMs of of alkanethiols with 100% -COOH displayed the lowest platelet activation among the rest of surfaces with mixed functional groups although results were not statistically significant [250]. On the other hand, PLGA adsorbs large amount of fibrinogen, and a large number of platelets also attach to this surface (Fig 4.12a) as

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

compared to HOPG. The inference therefore is that the adsorbed Hfg undergoes an adequate conformational rearrangement on these surfaces, thus allowing it to bind to the platelet GPIIb/IIIa binding receptors effectively. This conformational change is required for subsequent platelet adhesion.

Fig. 4.11: High platelet adhesion amount with discoid platelet morphology were observed with connecting membrane links that fuse platelets from one to another to form elongated (string like) aggregates on a) randomly dispersed pristine PLGA-MWCNT composite, b) magnified view from inset (a), and c) HOPG, d) magnified view from inset (c).

The PLGA-MWCNT composites showed a marked decrease in Hfg adsorption and platelet adhesion in comparison to control samples without any topographical features. The vertically aligned PLGA-MWCNT composite had lesser adhered platelets as compared to the randomly dispersed etched PLGA-MWCNT composite (Fig. 4.12b). Platelet adhesion was evidently decreased when compared with the pristine PLGA surface and that of HOPG surface. Although the HOPG showed considerably reduced platelet adhesion, the adhesion on the nanotopographic surface of the MWCNT composites was noticeably less. Even though the chemistry of MWCNT and HOPG are similar, the surface containing MWCNTs exhibited lower fibrinogen adsorption as well as platelet adhesion, and this certainly indicates the influence of surface topography. A similar study by J. Meng et al. [6] displayed significantly improved anticoagulant function where platelet activation and red blood cell disruptions were remarkably reduced on a multiwalled carbon nanotubes-polyurethane composite (MWCNT-PU), however the topographical effect was not characterized due to the highly agglomerated nanotubes in their study. In another study by T. Sun et al. [4], poly(carbonate urethane) (PCU) – MWCNTs composite films with protruding nanostructured topography showed superhydrophobic properties that largely improved the blood compatibility. In a very recent study, surface morphology in conjunction with electrical negatively charged surface has been demonstrated to induce lower thrombogenicity on carbon based a-C:H thin films [365]. While other researches have attributed the reduced platelet adhesion to the bonding structure (increasing I_D/I_G ratio) as well as to the surface topography on the magnetron-sputtered amorphous carbon (a-C) films [366]. Indeed, as reported by

Chapter 4: Platelet Interactions on poly(lactic-co-glycolic-acid) - Multi-walled Nanotube Composites with Different Nanotube Orientations

Roach et al., the conformation of Hfg upon binding to a surface is dependent on both the chemical and physical nature of the surfaces describing that Hfg is denatured to a greater extent when adsorbed onto particles with higher surface curvature [315]. Hence, we postulate that the conformational change that Hfg undergoes on the different topographic surfaces is responsible for all the observed differences in the subsequent platelet adhesion.

Fig. 4.12: FESEM images showing the morphology of adhered platelets on a) pristine PLGA film with large amount of platelet adhesion forming elongated (string like) aggregates and b) randomly dispersed etched PLGA-MWCNT composite with modest platelet adhesion and low spreading.

Therefore, the least platelet adhesion was observed on the vertically aligned PLGA-MWCNT composite (Fig. 4.13a) and can be attributed to both the reduced amount of Hfg and the restricted conformation adopted in the small contact area offered by the protruding MWCNTs sharp tips (~100 - 150 nm in diameter). In

addition, the nanotopography of the MWCNTs' tips reduces the platelet adhesion by minimizing the number of contact points a platelet can anchor onto the surface (Fig. 4.13b). Correspondingly, the randomly dispersed etched PLGA-MWCNT composite follows on the platelet adhesion affinity owing to the smaller surface topographical feature since the MWCNTs are embedded in the diameter-wise orientation within the PLGA matrix as compared to the vertically aligned.

Fig. 4.13: Vertically aligned PLGA-MWCNT composite displayed a) the least platelet adhesion amount with low spreading as compared with other substrates and b) magnified view from inset (a).

4.4 Summary

We have shown that the tendency of platelets to adhere onto artificial surfaces varies with the material properties and surface topography. The results in this study substantiate the fact that specific materials with the appropriate chemical and nanotopographical modifications can elicit the desired platelet response. For three of the four surfaces studied, the extent of Hfg adsorption correlates with platelet adhesion. The exception is HOPG, for which high Hfg adsorption is observed with

low platelet adhesion. The reason for this may be that on HOPG, the adsorbed Hfg does not adopt the appropriate conformation for the platelet receptors to bind. From the observations presented here, we can infer the following:

1. The contact angle data shows no correlation to the Hfg adsorption. This is primarily because the contact angle only measures the overall interfacial tension, and is insensitive to chemical details.
2. Chemical effects play a role in determining fibrinogen adsorption onto these surfaces. However, high fibrinogen adsorption does not always induce platelet adhesion, as the conformation of the adsorbed Hfg is also critical.
3. Platelet adhesion appears to correlate strongly to the presence of -COOH groups on the surface and to a lesser extent to the MWCNT topographic features on the substrates, with vertical alignment of MWCNTs favouring lower platelet adhesion and is clear that topography also plays an important role, in addition to chemistry.

Encouraged by the low adhesion of fibrinogen and platelets obtained on the vertically aligned PLGA-MWCNT surfaces, further investigations will be carried out to substantiate the effect of micrometer to nanometer and high aspect ratio surface topographical features on the adhesion and activation of platelets onto these surfaces. We will use principally PLGA in the investigation as it is a widely used biomaterial to determine for the optimized parameters in reducing platelet interactions on surfaces, which is presented in the next chapter.

Chapter 5

Platelet Interactions on Surfaces with Topographically Controlled Features

5.1 Introduction

Most recently, cellular interactions on topographic surfaces with physical attributes have received tremendous attention where numerous studies on the effect of surface topography in several aspects of cell biology have been published as discussed in *section 2.5.1.4.1*. In contrast, the effect of substrate topography on platelet adhesion has not received as much attention, because most efforts have been focused on chemical modifications as discussed in *section 2.5.1.1.1*. In the previous chapter, platelet adhesion on poly (lactic-co-glycolic-acid) multi-walled carbon

nanotube PLGA-MWCNT composites with different surface topographies has shown a significant reduction level of platelet adhesion on a vertically aligned PLGA-MWCNT composite and was ascribed to the sharp topographic features presented by the MWCNT. Recently, other researchers have also employed other techniques to investigate for the impact of surface topography on platelet response which have been previously discussed in *section 2.5.1.4.1*.

Following our previous results in *chapter 3 and 4*, here we aim to fabricate a variety of topographic features on PLGA films to investigate the role of geometrical parameters, (i.e. size, aspect ratio and density), in influencing platelet adhesion to ultimately determine an optimum topography with much reduced thrombogenicity. We also envision that the study of platelet adhesion on specifically defined geometrical features with homogeneous chemical composition may help to better understand the complex platelet behavior on surfaces.

The topography of interest in this study is high aspect ratio protruding features in the submicron to nanometer range. Methods of fabricating micron to nanometer scale patterns in polymers include hot embossing [296] and nanoimprint lithography [291] respectively, where a master template is used to transfer the features onto the polymer by mechanical deformation at precise conditions of heat and pressure. Technologies such as polymer casting [300] and capillary force lithography [299] allow for the fabrication of high aspect ratio features in polymers and are therefore the techniques adopted in this work. Additionally, we investigate the platelet response on topographical surfaces with similar features on different materials; namely the

vertically aligned multi-walled nanotubes (MWCNTs), where HOPG was used as the material for control. The adsorption of fibrinogen and subsequent platelet adhesion from platelet-rich plasma (PRP) on the topographic surfaces were quantified and analyzed. The morphology of platelets after exposure to the topographically modified substrates was also studied to determine the degree of activation inflicted in relation to the specific topographic features tested.

5.2 Materials and Methods

5.2.1 Materials

5.2.1.1 Substrates

PLGA, HOPG and vertically aligned multi-walled nanotubes (MWCNTs) were used as described in *chapter 4 of section 4.2.1*.

5.2.1.2 Buffers, biochemicals and antibodies

PBS, washing buffer and stop solution were used as described in *chapter 4 of section 4.2.4*. Lactate dehydrogenase (LDH) cytotoxicity assay kit from Cayman Chemical Co (Cat # 1008882), lysis buffer containing 2% v/v Triton X-100 in PBS and stop solution of 1 M HCl in PBS were used for LDH assay. Calcium free tyrode buffer consist of 4.0 g NaCl, 0.5 g NaHCO₃, 0.1 g KCl, 0.03 g Na₂HPO₄, 0.5 g D-glucose, 0.1 g MgCl₂ in 500 mL de-ionized water and 2% formaldehyde was adjusted to pH 7.4 with NaOH or HCl; were all prepared for flow cytometric analysis.

CaCl_2 , H_3PO_4 , 3,3,3-trifluoropropyl-trimethoxysilane, glutaraldehyde, acetone and ethanol were all purchased from Sigma-aldrich. Micro centrifuge tubes and 96-wells polystyrene microtiter plate were used in the incubation study and enzymatic assays respectively.

Hfg, PRP, HRP conjugated goat antihuman fibrinogen and TMB were used as described in *chapter 4 of section 4.2.1*. Fluorescently labeled IgM monoclonal antibody, procaspase activating compound-1 fluorescein isothiocyanate (PAC-1 FITC) was purchased from BD Biosciences, San Jose, CA for flow cytometric analysis.

5.2.2 Fabrication of nano porous anodized alumina (NPAA) template

To fabricate an ordered NPAA template, an indentation process as described in [367] was followed. Briefly, the surface of an aluminum foil (99.999%, Alfa Asear) was indented using pressure at 4000 psi for 2 mins with a substrate where silica colloids of particle diameter ~ 270 nm had been assembled. The foil was subsequently cleaned in an ultrasonic water bath to release the attachment of silica colloidal spheres on the surface. The Al film was then anodized at 3 °C in 0.3 M H_3PO_4 aqueous solution, with a voltage of 112 V for a time ranging from 1 – 20 mins depending on the desired depth of the pores.

5.2.3 Fabrication of master template in silicon

The Si master templates were fabricated either via deep UV photolithography or electron beam lithography, followed by inductively coupled plasma – deep reactive ion-etching (ICP-DRIE) BOSCH process. A variety of templates were fabricated with

features ranging from nanometer to micrometer size to produce the structures with dimensions as summarized in [Table 5.1](#). A schematic diagram illustrating the fabrication steps on both the NPAA and silicon template is shown in A5.1. Additionally, a schematic diagram which defines the topographical features of the substrate is shown in A5.2.

5.2.4 Fabrication of topographical structured features in PLGA film

The prepared templates were cleaned in acetone for the removal of contaminants traces and rinsed in de-ionized water before being oven dried. Subsequently, to facilitate the removal of casted PLGA film after drying, the templates were treated with 3,3,3-trifluoropropyl-trimethoxysilane (anti-adhesion agent) for ~ 1 hr in the vapor-phase. The casting mixture was prepared by dissolving PLGA pellets in dichloromethane (DCM) at the ratio of 1:28 w/v. This solution was then poured onto the coated templates and vacuum baked at 70 °C for 4 days before releasing the topographic features from the templates.

5.2.5 Fibrinogen adsorption determined by ELISA

The technique has been previously described in *chapter 4 of section 4.2.4*.

5.2.6 Platelet adhesion determined by LDH assay

A calibration curve was initially plotted by measuring the OD values of solutions of known platelet concentration (determined by hemocytometer). To quantify the amount of platelets adhered on the test surfaces, the substrates (3 x 3 mm)

were first equilibrated with PBS for 30 minutes at 37 °C. Then, 200 µl of Hfg (4 mg/ml) were added into the microtiter plate wells and incubated for 1 hr at 37 °C. Subsequently, the wells were rinsed 3 times with PBS to remove any unbound Hfg, followed by adding 200 µl of PRP into wells and incubation at 37 °C for 2 hrs. After incubation, the wells were again rinsed with PBS for 3 times to remove any non-adhered platelets and the substrates were transferred into new wells. Then, 25 µl of lysis buffer were added and incubated at 37 °C for 1 hr. After that, 50 µl of LDH reaction solution were added and incubated for 20 minutes at room temperature. Afterwards, 25 µl of stop solution were added into the well and aspirated before transferring to new wells, where the OD readings were taken at 490 nm using a microtiter plate reader. Experiments were done in triplicate ($n = 3$) and repeated separately 3 times.

5.2.7 Platelet activation determined by flow cytometry

The PRP suspension extracted from the platelet-bound Hfg substrates after PRP incubation in *section 5.2.6* was used for the flow cytometry studies. The washing solution (calcium free tyrode buffer) was added to the extracted PRP and then centrifuged at 2000 rpm for 15 mins. The supernatant was removed after centrifugation and the activated platelets were stained by direct immunofluorescence using PAC-1 FITC, in which the ratio of diluted PAC-1 FITC stain to PRP was 4:1. The stained samples were incubated at room temperature for 20 mins in the dark. After that, an equal volume of 2% formaldehyde was added to fix the platelets. The fixed samples were stored at 4 °C in the dark and were analyzed within 18 hrs.

Platelet activation was quantified using BD FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA) equipped with 488 nm laser excitation wavelength. A total of 10,000 platelets were gated per sample from the dot plot which was set in the forward side scatter (FSC) and side scatter light (SSC) channels to obtain the mean intensities of fluorescence for platelet-bound Hfg. The fluorescence intensity was collected through a 530/30 (FL1) bandpass filter. Logarithmic amplification was used due to the small platelet size. The data obtained from flow cytometry were re-analyzed with WinMDI 2.9 software package. (<http://en.bio-soft.net/other/WinMDI.html>)

5.2.8 Observation of adhered platelet on substrates by FESEM

The substrates prepared in *section 5.2.6* were fixed for FESEM observation to investigate for the changes in the platelet morphology. The technique has been previously described in *chapter 3 of section 3.2.4*.

5.2.9 Statistical analysis

All experiments were run in triplicate and repeated separately for at least three times. Data obtained from the different assays are presented as mean values with standard deviations. Statistical significance of differences between two substrates was analyzed using single factor analysis of variance (ANOVA).

5.3 Results and Discussion

5.3.1 Topographical dimensions of substrates

Several cell types have been reported to react differently to surfaces with varied topographic features which include a diversity of shapes, scales and symmetry aspects [70, 248]. Thus, a precise topographic control appears to be a powerful approach in the evaluation for specific platelet responses to a surface. The difference in platelet response to the varying topographical substrates was assessed where surface chemistry variation was negligible. For this purpose a fabrication process based on solvent casting was employed throughout to produce the topographically modified PLGA films. Solvent casting reproduces precise topographical features from a master template, is inexpensive and allows for reuse of the template. The pillars of PLGA were obtained from casting on several NPAA templates or silicon molds. The NPAA templates had a relative constant interspacing and diameter, and the height was controlled by varying the duration of anodization time. A height of $\sim 1 \mu\text{m}$ was achieved with ~ 20 mins of anodization time. The dimensions of the topographical features fabricated were analyzed using Smile View 2 and are tabulated in [Table 5.1](#), along with the corresponding aspect ratio (height/width) of each substrate.

Table 5.1: Summarized table showing an overview on the topographical dimensions and aspect ratio for all substrates.

Substrate	Interspacing (I) nm	Width (W) nm	Height (H) nm	Aspect Ratio
P(1,1,2)*	120 ± 27	90 ± 28	200 ± 68	~ 2
P(1,1,8)	130 ± 29	90 ± 26	800 ± 131	~ 8
P(1,5,8)	140 ± 27	500 ± 5	840 ± 161	~ 2
P(1,2.5,8)	140 ± 32	250 ± 6	840 ± 159	~ 4
P(8,10,0.4)	840 ± 12	1000 ± 60	40 ± 4	~ 0.04
P(14,6,0.4)	1400 ± 15	600 ± 55	40 ± 6	~ 0.07
P(9,30,150)	900 ± 35	3000 ± 12	15 ± 3.7 µm	~ 5
C(~,1.5,190)	≠	150 ± 47	19 ± 5.8 µm	~ 130

Substrate notation: P refers to the textured PLGA film and C refers to vertically aligned carbon nanotubes on a silicon substrate;

(1,1,2) → refers to the dimensional aspect (interspacing, width, height) of the pillars and is expressed in (x100 nm) unit.

Numbers in bold refer to the area of investigation between substrates; i.e. P(1,1,2) refers to height.

≠ Not measurable due to the high density of carbon nanotubes.

5.3.2 Adsorbed fibrinogen and platelet activity

Fibrinogen being one of the most important proteins in the blood mediates the adhesion and activation of platelets to artificial surfaces through the GPIIb/IIIa receptor on platelet cells [368]. Hence, in this work, it was pre-adsorbed on the substrates prior to contacting the surfaces to the platelets. The amount of adsorbed Hfg on the substrates was determined by ELISA and the calibration curve for Hfg was established. A linear correlation ($r = 0.951$) between the coating concentrations (0.001 – 0.05 mg/ml) of Hfg and the absorbance measured by ELISA was first obtained. The calibration curve for the absolute amount of adsorbed Hfg is expressed as:

$$\text{Adsorbed Hfg (mg/mm}^2\text{)} = 4.31 \times 10^{-3} \text{ OD} - 1.489 \times 10^{-4}$$

where OD indicates the optical density or absorbance of the reaction solution as measured with the microtiter plate reader. The amount of adsorbed Hfg on the substrates tested is shown graphically in [Fig. 5.1a](#).

The improvement in hemocompatibility on a biomaterial surface aims at reducing protein adsorption with the eventual goal of discouraging platelet adhesion. A preliminary evaluation of the thrombogenicity of surfaces can be carried out *in vitro* by quantifying the number of adherence platelets to a surface during the period of incubation. In this work, quantification was performed using the LDH assay. Initially, a calibration curve for the LDH assay was constructed and the linear correlation ($r = 0.9882$) between the read optical density and the absolute amount of suspended adherent platelets is expressed as:

$$\text{Number of adherent platelets/mm}^2 = 370.37 \text{ OD} + 21.26$$

The optical density values are primarily attributed to the suspended platelets as interaction of pre-adsorbed Hfg with the LDH reaction solution showed an insignificant absorbance value ($p > 0.05$). The amount of adherent platelets measured on the substrates tested is displayed in [Fig. 5.1b](#).

Platelet activation takes place when platelets interact or attach to foreign surfaces and as a result undergo a series of complex reactions to promote thrombin formation and platelet aggregation [147]. The PAC-1 antibody was used to investigate for the degree of platelet activation on substrates. This antibody binds to the epitope of the glycoprotein GP IIb/IIIa complex in the activated platelets bound

with the fibrinogen receptor [168]. Studies have shown that platelets interacting with surfaces will develop the same internal transformations as platelets activated in suspension [369]. In this study, adhered platelets were first stained with PAC -1 prior to fixation because PAC-1 does not bind to formaldehyde - fixed platelets [173]. Following the incubation of platelets with the substrates, the degree of platelet activation was estimated by flow cytometric analysis of the platelet suspension. The mean intensities of the activated platelets were analysed using the WinMDI program, and its quantification of PAC-1 expression for platelet activation on all substrates is shown in [Fig. 5.1c](#). The activated platelet population was gated and defined by the flow cytometric region (R1) according to their size and morphological characteristics ([Fig. 5.2](#)). A higher platelet activation was observed on the pristine PLGA surface as compared to that of P(1,2.5,8) with a lower mean intensity.

Fig. 5.1: Quantification graphs showing the a) amount of fibrinogen adsorbed on substrates determined by ELISA, b) number of adherent platelets determined by LDH assay and c) PAC-1 expression on platelet activation derived from flow cytometric analysis. Data were run in triplicate ($n = 3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments.

Fig. 5.2: Forward versus side light scatter dot plots with mean intensities for stained platelet-bound fibrinogen obtained through gating displaying a) platelets only (positive control), b) higher platelet activation on the pristine PLGA as compared to c) P(1,2,5,8) with a lower mean intensity. All events were gated for platelet specific surface marker using PAC-1 FITC.

Platelet adhesion on surfaces is known to be influenced by the presence of fibrinogen binding to the platelet GP IIb/IIIa receptor [146]; however the adhesion of the platelets is also dependent upon the fibrinogen conformation on the substrate [55]. In our study, different amount of Hfg adsorption was observed on the test substrates with varied surface topographies, and the result is relatively well-correlated to the subsequent amount of platelet adhesion on the substrates. A correlation plot on the amount of platelet adhesion to the adsorbed Hfg shows a strong correlation ($r = 0.88876$) as shown in Fig. 5.3a. The resultant $r^2 = 0.789$ indicates that the adherence of the platelets is mostly dependent on fibrinogen adsorption ($\sim 79\%$) but that other factors ($\sim 21\%$) such as variation in surface chemistry (e.g., HOPG vs. PLGA) also play a part. However, if we use data for PLGA samples only, a stronger correlation is seen ($r^2 = 0.93$) (data not shown), indicating that once surface chemistry is fixed; fibrinogen adsorption differences dictate the subsequent platelet adhesion amount on those topographical PLGA surfaces.

A strong correlation was also observed for the number of adherent platelets determined by the LDH assay against the PAC-1 expression for activated platelets determined by flow cytometric analysis ($r = 0.84492$) in Fig. 5.3b. The related r^2 value of 0.714 indicates that $\sim 71\%$ of the variance in platelet activation can be attributed to the amount of adherent platelets and that $\sim 29\%$ of variability in the platelet activation quantity is unexplainable at this point. Similarly, a correlation study of the same correlation between the number of adherent platelets against the PAC-1 expression for activated platelets on all the PLGA substrates (i.e. textured and pristine PLGA) displayed a slightly stronger correlation ($r^2 = 0.72$) (data not shown).

Low amount of adsorbed Hfg was observed on substrates with submicron sized features, suggesting that such surface features may be tailored to regulate Hfg adsorption on these surfaces. Conflicting views have been reported in the literature regarding the relationship between fibrinogen adsorption and the subsequent platelet adhesion; one study reported no significant correlation between the level of platelet adhesion and the absolute amount of adsorbed fibrinogen [370]. Likewise, it has been suggested that direct cell sensing and response to topography effects may have a significantly independent role (as distinct from the indirect mediation via adsorbed proteins on the surfaces) [371]. On the contrary, another study claimed that platelet adhesion is a strong function of the adsorbed fibrinogen on topographically modified polyetherurethane surfaces ($\sim 100 - 750$ nm) [337]. Additionally, adsorption studies of fibrinogen by Roach et al. [315] on a series of colloids with diameters of $\sim 15 - 165$ nm assembled on a surface demonstrated that fibrinogen is denatured to a greater extent on the smallest nanoparticles owing to their highly curved surfaces, indicating that the native conformation is lost which subsequently resulted in the reduction of binding to the platelet receptors. As demonstrated recently, Hfg adsorption process can be influenced by a specific morphology, e.g. whiskerlike surface protrusions as well as the sizes of the nano-roughness surface features [372]. Although a strong correlation between the amount of adsorbed fibrinogen and adhered platelets is found in this work ([Fig. 5.3a](#)), more research is required to determine this relationship further because not only the amount of adsorbed Hfg is important, but also its conformation on the surface [77]. In other words, fibrinogen adsorption may be necessary but is not the only factor required for platelet adhesion. Consequently, a

more precise probing of Hfg conformational changes on these featured surfaces will be needed to determine this relationship in respect to the thrombogenicity of the surface topography.

Fig.5.3: Scatter diagrams tabulated from Fig. 5.1 showing a strong correlation on the a) amount of adsorbed fibrinogen by ELISA with the number of adherent platelets by the LDH assay ($r = 0.88876$) and the b) number of adherent platelets against the PAC-1 expression for activated platelets determined by flow cytometric analysis ($r = 0.84492$).

5.3.3 Platelet adhesion and activation on topographically modified substrates

An intriguing phenomenon in the platelet response on the structured polymeric surfaces was observed when the topographical dimensions (interspacing, width & height) were varied. The degree of platelet response and Hfg adsorption amount were clearly different on the textured PLGA films with varying topographical dimensions whereby markedly reduced levels of adsorbed Hfg and platelet response were observed on all the textured PLGA films when compared to that of pristine surfaces. Correspondingly, statistically significant differences were observed in Hfg adsorption and platelet response when comparing the textured substrates to that of pristine surfaces in terms of topographic features such as size, aspect ratio and density

in all three experimental studies, indicating the importance of these parameters in influencing the hemocompatibility of a surface.

5.3.3.1 Interspacing effect

Wilkinson et al. [373] have found that pillars with an interspacing of < 300 nm demonstrated low adhesion of epitenon cells on a polycaprolactone surface, and a large number of adherent cells on the pristine surface. In another study, pillars with an interspacing in the range of 75 - 200 nm have been observed to be a determining factor in favoring for reduced epitenon cell adhesion [374].

In our study, the effect of interspacing between structural features was first assessed by comparing the platelet response on substrates, namely P(14,6,0.4) and P(8,10,0.4) with constant height and varied interspacing. As summarized in [Table 5.2](#), no statistical difference was observed between the two substrates for Hfg adsorption and platelet adhesion; but platelet activation was significantly different. The result is confirmed by the electron images demonstrating similar platelet adhesion amounts on P(14,6,0.4) in [Fig. 5.4a](#) and P(8,10,0.4) in [Fig. 5.4b](#). Qualitatively, the explanation for the enhanced platelet activation is as follows: when the inter-pillar spacing is small ($< 1 \mu\text{m}$), the attachment of platelets is weak in the sense that the contact area of platelets with the surface is lesser, and hence the conditions are not favorable to establish a stable contact with the surface and to acquire the right cellular morphological phenotype, which leads to activation. Low level of platelet activation was therefore observed when the inter-pillar spacing is reduced as the platelet morphology becomes restricted by its inability to contact the entire surface area. This

effect is clearly discernible in the marked reduction of activation of platelets on the topography of substrate P(8,10,0.4) in Fig. 5.4d as compared to P(14,6,0.4) in Fig. 5.4c. In fact, a recent study by H. Chen et al. [18] have demonstrated a huge quantity of confined platelets in the valleys of dot-like protrusions on a poly(dimethylsiloxane) (PDMS) surface but to a lesser extent on the crest, suggesting the importance of having an appropriate dimensional control for the topographical structures on surfaces; however the authors did not report any results on platelet activation.

To study the effect of a greater range of interspacing on platelet adhesion, an additional investigation was conducted on substrates at a constant aspect ratio. Here, the results demonstrate the importance of interspacing control on platelet adhesion as demonstrated in Fig. 5.4e and f, clearly revealing a reduced level of platelet adhesion on the region with $\sim 0.15 \mu\text{m}$ interspacing and a high level of adherent platelets on the region with $\sim 1 \mu\text{m}$ interspacing. When the interspacing becomes less than about 200 nm, adhesion is clearly reduced, and it is reasonable to attribute this to decreased area of contact (Fig. 5.4g). Hence, based on the results from Fig. 5.4g, an interspacing smaller than $\sim 200 \text{ nm}$ appears to be effective in the minimization for the platelet adhesion on these topographic surfaces.

This result also explains the insignificant platelet adhesion difference observed on P(14,6,0.4) and P(8,10,0.4), because these substrates have interspaces of $1.4 \mu\text{m}$ and 840 nm and are not dense enough to impede adhesion to the increased surface area.

To sum up the effect of interspacing:

- When the spacing is greater than about 200 nm, platelet adherence is not dependent on spacing; however platelet activation increases with interspacing in this range.
- When the interspacing is below 200 nm, adhesion and activation are both lowered with decreased interspacing.

Our study indicates that the dimension of the inter-pillar spacing should be much smaller than the platelet size in order to reduce the probability of platelet entrapment between the pillars and the formation of stable contact with the surface which leads to platelet activation.

Table 5.2: Comparisons between the statistical significance performed by ANOVA and tabulated from Fig. 5.1 for a) the amount of adsorbed fibrinogen, b) the number of adherent platelets and c) platelet activation intensity. Data were run in triplicate ($n=3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments.

Substrate	P(1,1,8)			P(1,5,8)			P(1,2.5,8)			P(8,10,0.4)			P(14,6,0.4)			P(9,30,150)			Pristine PLGA			HOPG			C(-,1.5,190)		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
P(1,1,2)	☼	☼	*	*	*	≠	☼	*	☼	☼	*	*	☼	☼	≠	☼	☼	≠	☼	☼	☼	☼	☼	≠	≠	*	≠
P(1,1,8)				☼	☼	*	☼	*	*	☼	☼	≠	☼	☼	≠	☼	☼	*	☼	☼	*	☼	☼	*	☼	*	≠
P(1,5,8)							☼	☼	☼	≠	≠	*	*	≠	≠	*	*	≠	☼	☼	☼	☼	*	*	≠	☼	≠
P(1,2.5,8)										☼	*	☼	☼	☼	☼	☼	☼	☼	☼	☼	☼	☼	☼	☼	☼	≠	☼
P(8,10,0.4)													≠	≠	*	*	*	*	☼	☼	☼	*	*	*	*	*	≠
P(14,6,0.4)																≠	≠	≠	*	☼	☼	☼	≠	≠	*	☼	≠
P(9,30,150)																			≠	*	☼	☼	≠	≠	*	☼	*
Pristine PLGA																						*	*	≠	☼	☼	*
HOPG																									*	☼	*

≠ denotes non-significance, * denotes significance for ($p < 0.05$), ☼ denotes significance for ($p < 0.001$).

Chapter 5: Platelet Interactions on Surfaces with Topographically Controlled Features

Fig. 5.4: Platelet response on the substrates a) $P(14,6,0.4)$ and b) $P(8,10,0.4)$ showing similar amounts of adhered platelets, magnified view showing the morphologies of platelets adopting the c) spread-dendritic morphology where a high level of platelet activation is observed with increased interspacing on $P(14,6,0.4)$ as compared to d) a reduced interspacing on $P(8,10,0.4)$, adopting a dendritic morphology e) a supplement study comparing platelet adhesion on a topographical surface with interspaces of $\sim 0.15 \mu\text{m}$ versus a pristine surface showing a much higher adhesion level on the pristine surface, f) a supplement study on the effect of interspacing on platelet adhesion across the topographic PLGA substrate (constant aspect ratio with varied interspacing) displayed a higher amount of platelet adhesion with interspaces $\sim 1 \mu\text{m}$ (right) as compared to $\sim 0.15 \mu\text{m}$ (left) and g) tabulated data from (f) obtained by visually counting platelets over an average of 6 images, ($n = 3$).

5.3.3.2 Aspect ratio effect

Figure 5.5 shows the tabulated results of platelet adhesion per area versus the aspect ratio of the substrates tested. It can be seen that platelet adhesion was decreased on substrates with increasing aspect ratio. Our results are consistent with previous studies performed by other research groups using different types of cells [334, 375]. It appears that platelets are able to interact only with the tips of the pillars when interspacing becomes sufficiently small (~ 100 nm). Pillars with high aspect ratio such as in the substrate P(1,1,8) (Fig. 5.6a) are likely to get deformed and bent in a liquid media even under slight agitation during incubation; this flexibility results in any unstable adherent platelets being dislodged from the pillar's surface. This effect is illustrated in Schematic 5.1a.

Conversely, pillars with low aspect ratio are stiffer and will remain upright (Schematic 5.1b). Indeed, Dalby et al. have reported no significant difference in platelet adhesion between a planar surface and a 95 nm high surface topography [16], which we believe that such an aspect ratio is not sufficient to influence the platelet attachment. While we do not have direct evidence of this phenomenon at this point, the differences in platelet adherence as observed on P(1,1,8) in Fig. 5.6c when compared to P(1,1,2) in Fig. 5.7a are obvious with tabulated data showing high significance ($p < 0.001$). The clustering of the pillars observed in Fig. 5.6a is due to capillary forces during drying, we presume that these pillars exist individually separated in liquid phase during the PRP incubation. It is also worth noting that the impaired platelet response on P(1,1,8) could be also due to the low level of adsorbed

Hfg measured or inadequate conformational change adopted on these surfaces. Both effects, i.e. topography and fibrinogen structural changes may act jointly to decrease the adhesion of platelets. In this study, an aspect ratio value of 3 - 5 appears to be sufficient to reduce a significant amount of adherent platelets on the surfaces.

Fig. 5.5: Plot of platelet adhesion per area versus aspect ratio of structures on the substrates tested. Substrates with increasing aspect ratio demonstrated reduced level of adherent platelets. An aspect ratio of 3 - 5 (arrow) appears sufficient to achieve a low level of adherent platelets. Dotted lines refer to the controls. Data are tabulated from Fig. 5.1b.

Fig.5.6: a) P(1,1,8) showing the clustering of pillars after drying, b) an unactivated platelet with a round shape morphology and c) platelet adhesion on P(1,1,8) displaying the lowest amount of adherent platelets (circled) as attributed by their high aspect ratio.

Schematic 5.1: Illustration on the effect of aspect ratio on platelet adhesion. a) The bending movements of the high aspect ratio flexible pillars prevent the platelets from making a successful contact on the topographic surfaces compared to b) the low aspect ratio pillars where they remain stiff and upright in liquid media even under agitation, thereby permitting platelet attachment at a greater ease. Dimensions are not drawn to scale.

Fig. 5.7: Platelet adhesion on P(1,1,2) showing a) moderate amount of adherent platelets and b) magnified inset from (a) displaying an activated platelet with spiky pseudopods.

5.3.3.3 Width effect

Cell response has been reported to be significant when the width of topographic features was reduced to the width of the cells or lesser [329]. Hence, a range of structures from 100 nm to 3 μm in width were studied for platelet response in this study. The amount of adsorbed Hfg and adherent platelets with its subsequent activation were observed to be significantly lower ($p < 0.001$) on the substrate with 250 nm pillar width (Fig. 5.8a and b) as compared to that of 500 nm topography (Fig. 5.9a and b). When we compare P(1,2.5,8) with P(1,5,8), we note that the fibrinogen adsorption differences are statistically significant, and hence are responsible for the observed increased adsorption on the P(1,5,8) surface. In other words, on the wider pillars, fibrinogen adsorption is increased, perhaps due to greater area of contact and hence the platelet adherence is also increased. Likewise, the same statistical significance was also observed on 100 nm width pillars, P(1,1,8) when compared to that of 500 nm wide pillars in P(1,5,8), with reduced Hfg and platelet response observed on P(1,1,8).

Similarly, the width is observed to play a role in affecting platelet response on P(9,30,150), where a micron sized width of 3 μm is observed to induce a high level of platelet adhesion (Fig. 5.5) than on all the other submicron textured PLGA films. These results indicate that in general, the reduction in platelet adhesion is more pronounced at submicron scale dimensions when compared to micron sizes in P(9,30,150). Additionally, even though both substrates P(1,1,2) and P(1,5,8) have approximately similar aspect ratio of ~ 2 in Fig. 5.5, it can be seen that the level of

adherent platelets was noticeably lesser on P(1,1,2). These results indicate that the absolute dimensions of the pillars are also important in the reduction for platelet activity in addition to the aspect ratio. This finding is in consonance with the work of Fan et al. [376], where neurons cultured on silicon dioxide (SiO_2) surfaces with different topographic scales resulted in differential cell adhesion. Similar observation was also shown for platelet studies with decreased adhesion on smaller width pillars [26]. Furthermore, high epithelial cell spreading has been observed on larger width pillars [377]. Therefore, it can be envisaged that pillars with an enlarged width act to increase the surface contact area for enhanced fibrinogen adherence and consequently, enhanced platelet adherence/interactions by allowing adhesion to occur with greater ease.

Fig. 5.8: Platelet adhesion on P(1,2.5,8) demonstrating the a) preferential adhesion on the planar region in significant quantity as compared to the region with nanotopographical features and b) platelets with a dendritic morphology.

Fig. 5.9: Platelet adhesion on P(1,5,8) displaying a) the slight increase of adhered platelets (circled) observed on the 500 nm (width) pillars, b) magnified view showing a platelet with a spread-dendritic morphology on the tips of the pillars.

5.3.3.4 Micron scale dimensions

The results clearly demonstrated a higher amount of Hfg adsorption and its subsequent platelet response (Fig. 5.10a) on the micron scale, P(9,30,150) as compared to that of PLGA surfaces at submicron scale dimensions. This finding is comparable with several studies, where a greater amount of protein [378] and platelet adhesion [322] was observed on surfaces with larger feature size.

For a comparative study, the pillar's width (3 µm) on P(9,30,150) is relatively of the same order of magnitude as the size of a resting platelet (1 - 2 µm). No significant influence on Hfg adsorption amounts was observed between the pristine PLGA and micron-sized substratum, P(9,30,150), signifying that Hfg adsorption amounts on micron sized topography is comparatively similar to that on the pristine PLGA surface. However, results displayed statistical significance between these two

substratums for the subsequent platelet adherence and activation. Despite the large width (3 μm) on the P(9,30,150) as compared to the Hfg molecules (47 x 4.5 x 4.5 nm), dimension at this scale seems to have a strong effect on the quality (i.e, the conformation) of Hfg adsorption. The periphery of the pillars was observed to be covered with fibrinogen (Fig. 5.10b), where fibrin fibers are formed and stretched from one pillar to the next. Similar observation was reported by Guan et al. where such fibers were found to form on micron-textured pillar width of $\sim 7 \mu\text{m}$ through a dewetting mechanism [379], a dimension similar to the $\sim 3 \mu\text{m}$ investigated in our study. The result implies that the conformation of Hfg on the 3 μm pillars could be different from that on a pristine PLGA to account for the differences in platelet adhesion even though the amounts of adsorbed Hfg was similar on these substrates.

Fig. 5.10: a) Platelet aggregation and adhesion observed on micron-sized PLGA pillars, P(9,30,150) and b) an overlaid of fibrinogen on the periphery of pillars stretching from pillar-to-pillar.

5.3.3.5 Chemical versus topographical effect

The highest level of Hfg adsorption and platelet response was observed markedly on the pristine surfaces without any surface topography, e.g. PLGA and HOPG. This is clearly due to the fact that fibrinogen or platelet adsorption on such surfaces is conformationally optimum, and not constrained by the presence of physical features. In order to further investigate the impact of topographically controlled features on platelet response, the planar carbon surface of HOPG was evaluated versus its topographic equivalent, on the vertically aligned multi-walled nanotubes (MWCNTs), C($\sim$,1.5,190). The surface chemistry of these substrates is similar and has been characterized in *chapter 4*.

In this current study, the results showed statistically significant differences between HOPG and C($\sim$,1.5,190) in all three aspects (i.e. adsorbed Hfg, adherent and activated platelet), inferring the influential role of surface topography. However, the chemistry of the surface is also important [380], as seen in the differences in the behavior of C($\sim$,1.5,190) and the topographically-modified PLGA films. The chemistry of the surface needs to be taken into account since the initial adhesion of proteins would be quite different due to the protein-surface binding affinity [315]. The platelet adhesion and activation on the pristine PLGA (Fig. 5.11a and b) is clearly the highest in all the tests performed on pristine PLGA, followed by pristine HOPG (Fig. 5.11c). This exemplifies the effect of surface chemistry on adhesion between PLGA and HOPG, where statistically significant difference ($p < 0.05$) was observed in the Hfg adsorption and adherent platelets. However, it is noted that there

was no statistical differences in the platelet activation as seen in [Table 5.2](#). This result indicates that the chemistry of surfaces influences differentially during the initial adsorption and binding stages but not in the later stage of platelet activation.

Comparing to the pristine surfaces, platelet response was evidently lower on the topographically modified PLGA and carbon substrates, in this case the vertically aligned CNTs. However, the adhesion level on C($\sim$,1.5,190) ([Fig. 5.11d](#)) is comparable to that of high aspect ratio topographic PLGA films ([Fig. 5.1](#)). Hence this indicates that the chemical influence on adhesion can be overcome by suitable topographical modifications of a surface. However, we do not eliminate the possibility that both effects topography and chemistry may act together to determine the platelet response on artificial surfaces.

We conclude from our results that in addition to a surface chemistry, designing surfaces with specific topographical features may result in a much reduced thrombogenicity. Nevertheless, more studies on different materials with varying surface chemistries and similar topographies are required to refine these arguments further.

Fig. 5.11: a) Platelet adhesion on pristine PLGA observed with the highest adherent platelet amount where this morphology increase the potential for multivalent adhesive interactions by enabling maximal surface contact area with the substratum surface and is evidenced in, b) showing the platelet spreading at its advanced stage, c) HOPG surface showing a moderately high level of adherent platelets and d) vertically aligned CNT C($\sim$,1.5,190) displaying much reduced platelet adhesion level when compared to (c), implying the influential role of surface topography in the regulation for platelet activity.

5.3.4 Morphology of activated platelets

Platelet activation leads to a change in its morphology and is characterized by the cytoskeletal rearrangements, ruffling of the surface membrane with the formation of multiple pseudopods [158]. The morphologies of activated platelets are classified into 5 categories from the lowest to the highest level of activation including: round, dendritic, spread-dendritic, spread and fully spread [9].

The least amount of platelet activation observed on the substrate with a high aspect ratio P(1,1,8) has a round platelet morphology (Fig. 5.6b), followed by P(1,2.5,8) with a dendritic morphology as shown in Fig. 5.8b compared to that of a larger pillar width, P(1,5,8) with a spread-dendritic morphology (Fig. 5.9b). The result shows that platelet activation is increased as the pillar's width size becomes larger since the potential for multivalent adhesive interactions on the platelet is increased with an enlarged surface contact area.

Conversely, a substrate with a low aspect ratio, P(1,1,2) displayed high level of activation as platelets lose their discoid shape and became extended with spiky pseudopods adopting a spread morphology (Fig. 5.7b). Likewise, a similar activation level was also observed on the micron-sized pillars, as the platelets accumulate on top of the adhered fibrinogen layer (Fig. 5.10a).

The activation level on C(~,1.5,190) in Fig. 5.11d and P(14,6,0.4) in Fig. 5.4c are comparable, both adopting a spread-dendritic morphology compared to that of a smaller interspacing substrate, P(8,10,0.4) with a dendritic morphology (Fig. 5.4d).

The result reveals that platelet activation increases with increased interspacing between the topographical features, attributed particularly to the ability of the platelet to interact with the surface as the effective contact surface area increases.

The amount of platelet activation on the pristine PLGA is clearly the greatest where the platelets are fully spread as the cytoplasm expands through the surface causing the platelets to spread into a thin film with the continual adherence of platelets (Fig. 5.11b). In comparison, the platelet activation level on HOPG (Fig. 5.11c) is relatively lower and adopts the spread morphology due to the surface chemistry effect.

The results presented underline the importance of an appropriate dimensional control of structured surfaces in directing a desired platelet response. Topographical features with reduced interspacing - P(8,10,0.4), reduced width - P(1,2.5,8) and increased aspect ratio - P(1,1,8) were found to result in a significant reduction of adsorbed Hfg and platelet response. In reducing the risk of clot formation, not only is it important to reduce the amount of fibrinogen adsorbed to a surface, but also to influence the adopted conformation on the surface as it determines the subsequent platelet adhesion/activation. The geometrical features of the topography play an important role on this, as it has been shown that a pristine PLGA surface becomes minimally thrombogenic upon specific topographical modifications. In addition, carbon based surfaces in the form of vertically aligned CNT become minimally thrombogenic when compared to a thromboresistant material such as HOPG.

5.4 Summary

In this study, we have shown that platelet adhesion and activation can be influenced by surface topographic features at the submicron meter level with the significant reduction of platelet response compared to that of controls. In contrast, dimensions in the micron scale did not reduce the platelet response over controls, suggesting that the effective features to reduce platelet response are in the size range of platelets or below.

The specific dimensions of the topographical features have seen to significantly influence the platelet adherence and activation, in the following manner:

- (a) Increasing the height of the structures results in lower platelet attachment when the width and interspacing are decreased.
- (b) Increasing the width increases the likelihood of platelet adherence, due to the increase in surface contact area and presumably via the conformation of the adsorbed Hfg.
- (c) Increased interspacing to dimension > 200 nm results in the entrapment of platelets and its subsequent activation due to increased surface area of contact and entrapment.
- (d) Increased aspect ratio in submicron structures reduces the number of adherent platelets due to the inability of the platelets to form stable contacts with the reduced surface area for attachment.

Based on the above, for a reduced level of platelet activity, we suggest the optimal range of topographical dimensions of the pillars as follows: height = 300 - 800 nm, width = 100 - 200 nm, interspacing = < 200 nm.

In view on the promising result from the substrates with submicron features, further investigation will be carried to understand the conformational changes that Hfg undergoes upon adsorption onto the submicron topographic surfaces to determine the potential influence on platelet adhesion. The study on conformational changes of Hfg upon adsorption is presented in the next chapter.

Chapter 6

Surface Induced Conformational Changes of Adsorbed Fibrinogen on Surfaces

6.1 Introduction

The adsorption of plasma proteins is the first event to take place upon exposure of blood to a biomaterial surface and is the central event in the biofouling of surfaces. Adsorbed proteins undergo conformational change or denaturation upon protein-surface interactions and this resultant structural change ultimately determines whether a foreign material will be accepted by the host system. Thus, the understanding of the protein structural changes upon adsorption is of great importance to the development of biomedical materials with improved biocompatibility [50].

Fibrinogen ($M_w \sim 340$ KDa), being the third primary plasma protein in blood following serum albumin and immunoglobulin, has a concentration between 2 - 4.5 mg/ml in blood. It is the key structural glycoprotein that has been studied extensively for its role in coagulation [71], and its ability to promote platelet adhesion and aggregation in enhancing the thrombogenic response [72]. Fibrinogen undergoes surface conformational changes upon adsorption to surfaces [356]. Indeed, studies have shown that the induced conformational change of the adsorbed fibrinogen on surfaces is an important determinant for platelet activity on surfaces [83-85]. The conformation of the surface-adsorbed fibrinogen has been determined to affect the behavior of platelet adhesion and activation, by providing the specific platelet binding sites which results in the formation of thrombus [75-77]. In fact, it has been demonstrated that not only is the amount of fibrinogen adsorbed important in the determination for thrombogenicity, but also the conformation of the adsorbed fibrinogen [90]. The structure of fibrinogen and its binding sites to the platelet has been reviewed in *section 2.2.4 and 2.2.5*, respectively.

The experimental techniques employed to study the conformational and organization of adsorbed proteins has been discussed in *section 2.2.6* under “characterization techniques for conformational and organization of adsorbed proteins”. The analysis of the fibrinogen vibrational spectrum is characterized by the Amide I band ($1700\text{-}1600\text{ cm}^{-1}$), which is used widely to provide information on the changes in the secondary structure of the adsorbed fibrinogen on surfaces since the amino acid sequence of the RGDs absorb along the Amide I band, where a change in

the intensities of the component bands (related to secondary structure) is indicative of a conformational change [381].

Recently, the biocompatibility of surfaces has been improved with surface topographical features [25, 317]. The effects of platelet response on topographical surfaces, particularly with surface features in the sub-micron range have been demonstrated to be significantly influential in our work as well, as described in the previous chapter. Additionally, many studies have revealed a low level of platelet response on these topographical surfaces [22, 23, 26]. However, studies on the influence of topographic feature regarding the conformational change of the fibrinogen are scarce especially on surface features with high aspect ratios. We hypothesize that a high aspect ratio surface feature in the submicron scale could be mediating the differences in the behavior of protein adsorption by inducing a less favorable conformational change as compared to a pristine surface. Hence, in this study the adsorbed conformational change of the fibrinogen on the topographic features will be elucidated. Additionally, a time dependent study will be quantified to correlate the relationship between the induced fibrinogen conformational changes to the amount of adsorbed fibrinogen at different adsorption rates by the enzyme linked immunosorbent assay (ELISA).

6.2 Materials and Methods

6.2.1 Materials

Hfg, monoclonal antibody binding HRP conjugated with human fibrinogen goat antibody, PBS and TMB were used as previously described in *chapter 4 of section 4.2.1*.

6.2.2 Conformational assessment of fibrinogen by Attenuated Total Reflection – Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The conformational change of fibrinogen was assessed by infrared spectroscopy using a variable angle reflection attachment in the ATR accessory (HARRICK/Seagull™) in a FTIR spectrometer (PerkinElmer/Spectrum 2000). A polarizer, using only p-polarized light for enhanced spectra, was reflected on the substrate. Two substrates were investigated, namely P(1,2.5,8) and PLGA to compare the behaviour of Hfg adsorption on the high aspect ratio surfaces to that of a pristine surface, respectively. The incident angle used in all experiments was 30°. Hfg (~ 4 mg/ml) was used within 10 mins of their preparation time and was adsorbed onto the substrates at various time intervals (5, 30 and 60 mins), rinsed in deionized water and nitrogen dried before immediate examination. The spectra were recorded at 2 cm⁻¹ resolution with an average of 128 acquisitions. The substrate spectra was collected as background spectra and subtracted from the spectra of adsorbed Hfg on the tested surfaces, hence the final spectra shown has the background spectra eliminated. The spectra for Amide I band were fitted with a linear baseline with a non-linear fitting

scheme using Gaussian band profiles and the area of each band component was expressed as a percentage of the total area of Amide I band. The range of wavenumber position were obtained from literature [54, 77, 136, 355] and were used as a constraint during curve fitting. The underlying component bands' wavenumber position, width and intensity were allowed to vary from their initial estimates till a best fit was achieved. Initially, the resulting wavenumber position of each component band was fixed, and only the width and intensity were allowed to vary during the non-linear least squares fitting. Subsequently, all the wavenumber positions were allowed to vary. The positions of the component bands maintain their relevance found in the literature without any erroneous shift during the non-linear least squares fitting, which indicated a final satisfactory fit with the experimental data. Subsequently, direct comparison of the fitted peaks were used to monitor the conformational changes in the secondary structure of the adsorbed Hfg since each different secondary structures are assigned to specific wavenumber positions based on their dissimilar intermolecular interactions and strength of hydrogen bonding.

6.2.3 Fibrinogen adsorption quantification by ELISA

The technique has been previously described in *chapter 4 of section 4.2.4*. The amount of adsorbed fibrinogen on various substrates, namely P(1,2,5,8), PLGA, C(~,1.5,190) and HOPG were carried out over a range of time intervals (1,5, 15, 30, 45 and 60 mins) to investigate for the kinetics of Hfg adsorption.

6.2.4 Statistical analysis

All experiments were run in triplicate and data obtained are presented as mean values with standard deviations. Statistical significance of differences was analyzed using single factor analysis of variance (ANOVA) for ($p < 0.05$).

6.3 Results and Discussion

6.3.1 Conformational assessment of fibrinogen by ATR-FTIR

The degree of surface thrombogenicity can be assessed by the extent of induced conformational change of the surface-adsorbed Hfg that subsequently supports the attachment of platelets [382]. Indeed, the adsorption of Hfg onto an implant surface can increase the level of platelet attachment, though not a sufficient condition; the other determining factor is the exposure of the Hfg functional domains to be accessible by the platelets upon interaction with a surface. Thus, it is of importance not only to determine the adsorption kinetics of Hfg, but also the induced changes in the secondary structure of the Hfg upon adsorption onto surfaces.

The IR spectral regions of interest of all proteins are characterized by the Amide I and Amide II bands. The Amide I band ($1700-1600\text{cm}^{-1}$) arises principally from the C=O stretching vibrations of the peptide group with some contributions from C-N and CCN deformation, while the Amide II band ($1600-1500\text{cm}^{-1}$) represents the N-H bending with contributions from the C-N stretching vibrations [383]. Because the Amide II band is less sensitive to any conformational changes, hence only the Amide I band will be our focus to extract the information on the

secondary structures of the surface induced change of the adsorbed fibrinogen, due to its strong and distinct signal. Band assignments of the various secondary structure components on the Amide I band are determined according to the literature [54, 77, 136, 355]; component bands between ($1661\text{--}1650\text{ cm}^{-1}$) have been assigned to α -helix, ($1683\text{--}1673\text{ cm}^{-1}$) to β -turns, ($1695\text{--}1690\text{ cm}^{-1}$) to β -sheets, ($1640\text{--}1618\text{ cm}^{-1}$) to extended chains, ($1648\text{--}1641\text{ cm}^{-1}$) to random chains and ($1615\text{--}1610\text{ cm}^{-1}$) are assigned to side chain moieties. Studies have shown that major secondary structures on the native fibrinogen are attributed to the α -helix, β -sheet and β -turn structures [355]. A time dependent conformational study of the surface-adsorbed fibrinogen is performed by integrating the area under each curve to give a relative corresponding approximation of the secondary structure content induced on the native Hfg (Fig. 6.1) and the substrates (Fig. 6.2), with the Amide I band resolved into six symmetric Gaussian curves and was chosen according to literature [384]. The sum of the fitted band components yield a spectrum (solid line) that is in good agreement with the experimental (dotted line) band contour as shown in Fig. 6.1b and Fig. 6.2. The root mean square (RMS) was evaluated as a measure of the precision of the curve fitting and the goodness-of-fit is determined by a small RMS value, e.g. a value closer to 0 indicates that the fitted curve is closer to experimental data. To improve the quality of fitted curve, fitting was performed on the Amide II band (data not shown in plot for simplicity).

Fig. 6.1: Native Hfg dissolved in PBS displaying a) FTIR spectrum with Amide bands I and II, b) fitted FTIR spectra of the Amide I band into its resolved component bands of (i) β -sheets, (ii) β -turns, (iii) α -helix, (iv) random chains, (v) extended chains and (vi) side chain moieties.

Fig. 6.2: Fitted FTIR spectra of the Amide I band into its resolved component bands of (i) β -sheets, (ii) β -turns, (iii) α -helix, (iv) random chains, (v) extended chains and (vi) side chain moieties on a-c) pristine PLGA and d-f) P(1,2,5,8), respectively at adsorption times of a) & d) 5 mins, b) & e) 30 mins, c) & f) 60 mins.

The changes on the secondary structure content in the Hfg respective to the native Hfg are expressed as a percentage of the total area of Amide I band in Fig. 6.3, obtained from the data in Fig. 6.1 and Fig. 6.2. The results demonstrate that Hfg starts to lose its secondary structure once adsorbed, with the highest induced change observed in the random chains content on the PLGA when compared to the native Hfg after 60 mins of adsorption. The random chains is identified by the C-terminal region of the B β and γ chains, since the A α chain has restrictions on randomness imposed by the presence of the disulfide bonds (S-S bonds) as well as by the constraint with its C-terminus docked at the E-domains [385]. A substantial increase in the random chains (triangles) was observed on the pristine PLGA (~25%) in Fig. 6.3a as compared to P(1,2,5,8) in Fig. 6.3b with ~ 15% after 60 mins when compared with the native structure of ~ 5%. It was accompanied by a large decrease in the α -helix content (circles), with the pristine PLGA demonstrating a structure content of ~ 7% and P(1,2,5,8) with ~ 14% at the same adsorption time of 60 mins when compared with the native structure of ~ 23%. The adsorption of Hfg onto hydrophilic and hydrophobic surfaces has shown to increase and decrease the α -helix content respectively [355], and is in agreement to the obtained results showing a decreasing trend since the PLGA used in this study displayed hydrophobic properties (CA ~ 93.43°), as discussed in chapter 3. Indeed, denatured protein often leads to a random coiled structure and this occurs because of destabilization of the α -helix structure leads to more of the random coiled structure [52]. In fact, early studies has shown that ~ 70% of the fibrinogen's total α -helix content is found in the coiled coils which connect the protein's outer D domains to the central E domain [386], while the D and

E domains contain a α -helix content of $\sim 50\%$ and $\sim 35\%$ respectively [387]. From the result, it indicates that the pristine PLGA induce an extensive Hfg conformational change in that much of the α -helices are converted to random chains as compared to P(1,2,5,8). This implies that the A α chain is readily available to interact with the platelets at the E domain on the pristine PLGA as compared to P(1,2,5,8). As aforementioned, the random chains are located mostly in the C-terminal region of the B β and γ chains [385]. Therefore, this implies that the B β and γ chains of the adsorbed Hfg on the pristine PLGA become exposed and are readily available to interact with the platelet receptor as compared to P(1,2,5,8). In particular, direct evidence have showed that both B β and γ chains are responsible for the interaction of the Hfg with the platelet receptor [99, 100]. The above results are in consensus with the previous findings in *chapter 5*, where the platelet response on P(1,2,5,8) was markedly lower compared to the pristine PLGA.

Fig. 6.3: Comparison plot showing the changes induced in the secondary structure of Hfg with adsorption times for a) pristine PLGA film and b) P(1,2,5,8).

To better compare the results in Fig. 6.3, the obtained secondary structure contents on the substrates are presented in relation to the adsorption time intervals together with the corresponding Hfg native structure in Fig. 6.4. The result demonstrates significant induced conformational change in the α -helix content during the first few minutes of adsorption on the pristine PLGA compared to that of P(1,2.5,8) as shown in Fig. 6.4e. The α -helix content significantly decreases from $\sim 23\%$ in the native state to $\sim 18\%$ and $\sim 15\%$ after 5 mins of adsorption on the P(1,2.5,8) and pristine PLGA, respectively. This result is in agreement with the aforementioned observation on α -helix, with the higher conformational change observed on the pristine PLGA surface as demonstrated by the decreasing trend in the α -helix structural content. Similar result has been reported where the loss of helical structure was observed on nanometer silica spheres under the influence of the surface curvature effect [315].

Likewise, the extended chains content decreases from $\sim 33\%$ in the native state to $\sim 30\%$ and $\sim 24\%$ after adsorption on the P(1,2.5,8) and pristine for 60 mins, respectively. Indeed, previous investigations have indicated that a conformational change is characterized by the lower extended chains, β -sheets and side chains content, and is accompanied by the higher β -turns and random chains content with respect to the native structure of Hfg. [109, 355]. This is in agreement with the obtained results (Fig. 6.4) showing a significant level of Hfg conformational change on the surface of pristine PLGA to that of the textured substrate, P(1,2.5,8) towards the end of 60 mins period of Hfg adsorption. Significant difference of adsorbed Hfg was observed on pristine PLGA when compared with the native structure after 5 mins

of Hfg adsorption on β -sheet (Fig. 6.4a), extended chains (Fig. 6.4c) and random chains (Fig. 6.4d), but insignificant for P(1,2.5,8). In contrast, though P(1,2.5,8) displayed significance when compared against the native Hfg, a significant change was also noted on the pristine PLGA in the side chains (Fig. 6.4b) and α -helix (Fig. 6.4e) when compared with P(1,2.5,8). Based on these observations, the substrate P(1,2.5,8) evidently shows an overall lower induced conformational change which indicates that the adsorbed Hfg adopts a conformational structure closer to that of native Hfg. The results obtained further substantiate our hypothesis that the lesser conformation change in the Hfg is mediated by the differences in the adsorption behavior of Hfg on the textured surface of P(1,2.5,8) and is inferred to be induced by the high aspect ratio surface submicron features given that the surface chemistry of the two substrates remain constant.

Fig. 6.4: Comparison of the induced changes in the secondary structure content of the adsorbed Hfg showing the band components of a) β -sheet, b) side chain moieties, c) extended chains, d) random chains, e) α -helix and f) β -turns on the pristine PLGA and P(1,2.5,8) substrates with increasing adsorption times to the corresponding content in the native Hfg. * denotes significance for ($p < 0.05$).

The extent of conformational change in Hfg resulting from the adsorption onto an implant surface governs the surface-induced platelet interactions, and ultimately the surface thrombogenicity [75]. Specifically, the closer the secondary structure of the adsorbed Hfg to that of native structure, the lower the resultant surface thrombogenicity. In particular, the extent of surface thrombogenicity can be assessed by comparing the ratios of α -helix to β -turn and β -sheet to β -turn structures in the native Hfg to that of adsorbed Hfg on substrates [54, 388]. The ratios of these

two structures for the substrates are shown graphically in Fig. 6.5 and compared to the native Hfg. The results revealed that Hfg undergoes significant changes in the secondary structure following adsorption on the pristine PLGA surface as compared to P(1,2.5,8) after 60 mins Hfg adsorption, as demonstrated by the lower α -helix to β -turn and β -sheet to β -turn ratios. In comparison, a higher α -helix to β -turn and β -sheet to β -turn ratios was observed on P(1,2.5,8) which evidence that its secondary structure is closer to the native state. From the result, the textured surface of P(1,2.5,8) is expected to result in a lower attachment of platelets as shown in the previous chapter and consequently lower surface thrombogenicity.

Fig. 6.5: Ratios comparison of a) α -helix to β -turn structures and b) β -sheet to β -turn structures on the pristine PLGA and P(1,2.5,8), with respect to the native Hfg. * denotes significance for ($p < 0.05$).

6.3.2 Adsorption kinetics of Hfg

Using ELISA, the dynamics of the Hfg adsorption process was investigated onto the various substrates at different time intervals (1, 5, 15, 30, 45 and 60 mins). Well documented and shown by other research groups [389-391], a plateau region is generally observed approximately after 1 hr of adsorption time. From the adsorption measurements ([Fig. 6.6](#)), the amount of adsorbed Hfg on the substrates in the ascending order was: P(1,2.5,8) < C(~,1.5,190) < PLGA < HOPG. The obtained result is consistent with the previous results as obtained in *chapter 5*, demonstrating the least and most Hfg adsorption on P(1,2.5,8) and HOPG, respectively. The Hfg shows a rapid initial adsorption in [Fig. 6.6](#) and after which, it gradually slows down reaching a plateau after ~ 30 mins. The kinetic profile of the Hfg adsorption process is explained by a two regime process and has been well explained [392, 393].

Post-adsorptive molecular rearrangements of Hfg is suggested to take place at increasing adsorption rate with increasing adsorption times [394]. Therefore, the rate of Hfg adsorption amount can provide useful information related to the rate of induced conformation change in Hfg at increasing adsorption times. A correlation plot on the amount of adsorbed Hfg to the induced structural changes of the adsorbed Hfg is shown in [Fig. 6.7](#). The correlation study is performed by fixing each respective parameter at time intervals of 5, 30 and 60 mins, whereby only the three major band components, namely β -sheets, β -turns and α -helix are investigated. A higher conformational change as well as adsorption amount was observed on the PLGA as compared to P(1,2.5,8). The adsorbed Hfg amounts correlate well with the secondary

structure components, where a moderately strong negative linear correlation ($r = -0.76276$) was observed on P(1,2,5,8) for the α -helix band component, while the rest displayed either very strong positive/negative linear correlations. The result clearly indicates that a greater Hfg conformational change occurred when the Hfg adsorption amount increases with longer adsorption times. Specifically, it means that Hfg adopts a different conformation structure with different adsorption rates, given that the structural rearrangement of the adsorbed Hfg is likely to occur with increasing adsorption times. The results demonstrated that both the adsorbed Hfg amounts and the adsorption-induced conformational change can potentially affect the adhesion behavior of platelets in influencing the subsequent surface thrombogenicity.

Fig. 6.6: Adsorption kinetics of Hfg onto the various substrates at various adsorption times quantified by ELISA. Data were run in triplicate ($n = 3$) and expressed as mean $\pm$ standard deviation of 3 repeated independent experiments.

Fig. 6.7: Correlation plot on the amount of adsorbed Hfg to the induced changes in the secondary structure content of the adsorbed Hfg on the 3 major band components; a) β -sheet, b) β -turn and c) α -helix at various time intervals of 5, 30 and 60 mins, with their respective corresponding r values. Data are tabulated from Fig. 6.6 (amount of adsorbed Hfg) and Fig. 6.2 (secondary structure content).

6.4 Summary

In this study, ATR-FTIR is employed for the study in the conformational change of Hfg on the surface of the structured PLGA substrate P(1,2.5,8) which has pillar structures of 250 nm dimensions and aspect ratio of $\sim 3:1$ times, and a pristine PLGA over time. The ATR-FTIR results indicate that the adsorbed Hfg undergoes a greater structural change on the pristine PLGA surface, as compared to that of P(1,2.5,8). Moreover, the higher α -helix to β -turn and β -sheet to β -turn ratios observed on P(1,2.5,8) substrate further confirms that the secondary structure of the adsorbed Hfg is closer to that of native structure, and consequently is expected to have lower surface thrombogenicity. Trends in surface-adsorbed amounts correlate well with the extent of conformational change of the adsorbed Hfg on both substrates. Together these data are consistent with the understanding that a non-topographic surface, i.e. PLGA tend to be adsorb Hfg more strongly and induce substantial conformational change, while the surface topography effect on the topographic surface of P(1,2.5,8) is noticeably lesser as a function of time and, hence is expected to result in a surface with lower platelet attachment. The findings are in consistency with the previous observations in *chapter 5*. Here we attribute these observations to the high aspect ratio surface feature on the textured substrate of P(1,2.5,8).

This study highlights the ability to control the Hfg conformational change merely by tailoring the polymer surfaces with appropriate topographical features to maximally retain the native state of adsorbed Hfg. With this understanding, the design of these surface features can be directed to lower the thrombogenicity of biomaterials.

Chapter 7

Conclusions and Perspectives

Minimizing thrombus formation on blood-contacting devices in the context of medical device implantation is crucial in the development for blood-compatible materials. The work described in this thesis research contributed to the field of biomaterials by elucidating the effects of fibrinogen adsorption and conformation as well as the subsequent platelet responses on surfaces with nano and submicron topographic fabricated structures. These findings are significant because they bring new insights to the complex interactions between the fibrinogen and platelets on structured surfaces, with implications in achieving thrombo-resistant surfaces. The approach adopted in this thesis has been to study systematically the effect of topography, separating it from the chemical effects in influencing platelet adhesion.

Chapter 7: Conclusions and Perspectives

Carbon nanotube composites with nano-scale features, investigated in *chapter 3*, were used as a model surface to gain further insight into the platelet-surface interaction. The result of this study highlights the significant influence of surface chemistry as well as the surface morphology effects of the MWCNTs with the much reduced level of platelet adhesion on surfaces with adsorbed MWCNTs.

An extended study with particular interest on the influence of surface morphology and chemistry effect on platelet response was investigated in *chapter 4*, designed to determine the relative importance of each factor. The result revealed markedly lowered level of platelet adhesion to –COOH surfaces, and confirms that topography also plays an important role, in addition to chemistry, with the vertical alignment of MWCNTs favouring the least platelet adhesion.

The aforementioned studies laid the basis for the determination of the range of dimensions of topographical features required to reduce platelet response, presented in *chapter 5*. The approach taken to make a polymeric surface low adhesive for platelets is described based on appropriate design of feature dimensions. It has been shown that specific topographic features can affect the platelet response distinctively. Topographical features, particularly in the submicron and nanoscale range showed significant reduction in platelet response as compare to the pristine surfaces. Of great significance is the finding that merely by altering the surface topography, the degree of platelet adhesion can be reduced to much lower levels to that observed on the “minimally thrombogenic” surfaces such as HOPG.

Chapter 7: Conclusions and Perspectives

Specifically, the results reveal:

- (a) On unstructured surfaces, surface chemistry plays a dominant role on adhesion, as shown by the reduced platelet adherence on HOPG as compared to PLGA.
- (b) When surfaces are structured, these features are able to overcome the chemical influence. For example, a submicron structured PLGA surface can exhibit lowered platelet adherence than the “inert” HOPG.
- (c) Tailoring surfaces with suitable topographical features in addition to a specific surface chemistry acts to reduce the platelet response.
- (d) The specific dimensions of the topographical features have a significant effect. In particular, topographical features with reduced interspacing, reduced width and increased aspect ratio were observed to significantly influence the platelet adherence and activation.

P(1,2.5,8) and pristine PLGA, two model substrates investigated in *chapter 6*, were used to elucidate the conformational changes of fibrinogen upon adsorption onto surfaces. The study demonstrated that fibrinogen interacts differently with high aspect ratio features presented on the substrate of P(1,2.5,8), as evidenced by the lower induced fibrinogen structural reorganization in comparison to the pristine surface. This finding indicates that a surface with topographical features can be design to trigger a desired fibrinogen response leading to the subsequent low surface thrombogenicity.

In conclusion, the work in this thesis has been focused in designing a material surface with low affinity for fibrinogen and its subsequent cellular events, such as the

Chapter 7: Conclusions and Perspectives

adhesion and activation of platelets. We have also presented a rationale for the observed differences in platelet adhesion/activation, and the mechanism relates not just to the amount of fibrinogen adsorbed, but also to its conformation on the substrate. The ability to regulate distinct platelet behavior by the engineering of a material's surface at nanoscale is of particular interest in regard to the potential development for blood-compatible materials. The investigations of platelet response on these topographic surfaces offer a greater understanding to the complex platelet-biomaterial interactions and will be beneficial in the development for the next generation of low-thrombotic blood contacting medical devices.

Future work can address some of the gaps in the present work or extend the scope of the work presented here. First, our studies of platelet adhesion/activation have been done under static conditions, and it is not clear if flow conditions would have any effects on the observed trends. It is recommended that future studies can be performed on the substrates with platelets under the flow (dynamic) conditions. Such a study will clearly be closer to the *in vivo* situation.

Secondly, a logical extension of this work is to validate these results *in vivo*. In general, *in vitro* and *in vivo* correlations are not linear, with many factors confounding the *in vivo* results. Thus it is important to evaluate the thrombogenicity of these engineered surfaces in an animal model: for preliminary evaluation, a rat model would suffice.

Third, our studies were performed using only platelet-rich plasma, without the influence of other cells present in blood. It would be interesting to study the

Chapter 7: Conclusions and Perspectives

competitive attachment of platelets when leukocytes are present, as well as endothelial cells to provide the physiological relevance. Or to put it in a different way, how general are the results obtained here with respect to cellular adhesion? Can these trends be sustained with other cell types, such as ECs and fibroblasts? Does fibrinogen play a similar role with attachment of other cellular types?

It is hoped that the interesting results obtained here would spur further research into understanding the material-blood interface. Such an understanding would lead to the next generation of successful blood-contacting devices.

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Appendix

Appendix

Appendix

Amplitude, z range (nm)			
a	b	c	d
22.72 ± 4.43	28.15 ± 7.37	123 ± 21.58	720.62 ± 34.84

A4.1: AFM images showing the 3D images of a) pristine PLGA, b) HOPG, c) randomly dispersed pristine PLGA-MWCNT composite and d) randomly dispersed etched PLGA-MWCNT composite. The roughness data on the amplitude (z range) is quantified in the table as shown ($n = 5$).

Appendix

A5.1 Schematic diagram illustrating the fabrication steps for a) nano porous anodized alumina (NPAA) template and b) silicon master template.

A5.2 Schematic diagram illustrating the definition of the topographical features on the substrate. (I , W , H) refers to (interspacing, width, height).