The effects and underlying mechanisms by which engineered and combustion derived nanoparticles impact platelet function.

Erica June Smyth

January 2015
Word count
56,463

A thesis submitted for the degree of
Doctor of Philosophy of Imperial College London

Platelet Biology Group
National Heart and Lung Institute
Faculty of Medicine
Imperial College London
Abstract

Combustion-derived nanoparticles (diameter ≤ 100 nm) present in air pollution are thought to be associated with the onset of platelet-driven thrombotic events such as myocardial infarction. In addition, the emergence of nanotechnology has led to increased human exposure to engineered nanoscale structures.

My first aim was to establish whether engineered nanoparticles could influence platelet function and whether this was dictated by their size and surface charge. The second aim was to evaluate if combustion-derived nanoparticles could modulate platelet aggregation in-vivo and determine the underlying mechanisms.

50 nm and 100 nm carboxyl, amine and unmodified model engineered polystyrene nanoparticles as well as diesel exhaust particles (DEP) and carbon black (CB) were intratracheal (i.t.) or intravenously (i.v.) administered into mice and in-vitro and in-vivo platelet aggregation, markers of pulmonary and systemic inflammation and plasma nitrate levels were measured.

All model engineered nanoparticles induced concentration-dependent platelet aggregation which was inhibited by conventional platelet inhibitors and endogenous vascular regulators. In contrast, amine-modified 50 nm particles enhanced agonist-induced platelet aggregation in-vitro and in-vivo. DEP increased agonist-induced platelet aggregation in-vivo that was not associated with inflammation or altered plasma nitrite levels. In contrast, CB did not increase platelet aggregation but did appear to initiate inflammation.

All nanoparticles investigated induced platelet aggregation with potencies and mechanisms that were dependent upon a distinct combination of size and surface chemistry. 50 nm cationic particles may present the largest risk to human health by exacerbating thrombotic events, as they can enhance the effects of platelet agonists at low concentrations. Additionally, exposure to DEP can enhance platelet aggregation in-vivo suggesting that the thrombotic incidents triggered by acute exposure to particulate pollutants may be platelet driven although the underlying mechanism does not appear to involve systemic inflammation or alterations in NO bioavailability.
DECLARATION OF ORIGINALITY

I, Erica June Smyth confirm that this work submitted for assessment is my own and is expressed in my own words. Any uses made within it of the works of other authors in any form (e.g. ideas, equations, figures, text, tables, programmes) are properly acknowledged at the point of their use. A full list of the references employed has been included.

Signed………………………………………………………….

Date…………………………………………………………
COPYRIGHT DECLARATION

‘The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work’
Acknowledgements

First and foremost I would like to thank my supervisor Dr Michael Emerson for his unwavering support and patience over the past four years. Few students are lucky enough to have a supervisor who devotes so much time to their development both scientifically and professionally. I couldn’t have completed this project or thesis without your help and confidence in me.

I am also thankful to my secondary supervisor Professor Terry Tetley and the Lung Cell Biology group for their help and expert advice with all the nanoparticles throughout my project.

My four years in the Platelet Biology Group (PBG) have been thoroughly enjoyable, largely due to the two best colleagues and friends a PhD student could have – Drs Antonia Solomon and Georgina Apostoli. You have both offered me a tremendous amount of support and friendship throughout the highs and lows of my PhD and I will be forever grateful to you both. Thank you to all the past and present lab members of the PBG and the wider circle of molecular medicine, you definitely made our lab a fun and exciting place to work. A massive thank you to all the blood donors who donated over the years!! I would also like to thank the British Heart Foundation and British Pharmacological Society for funding my work and the National Heart and Lung Institute for funding my studentship.

A huge thank you to all the PBG collaborators; Dr Simon Pitchford and his group (Kings College London) for all their help with the FACS experiments and Drs Pradeep Luther and Anupama Vydyanath for their assistance with the electron microscopy. The Respiratory Pharmacology group (Imperial College London) for all their help with the inflammation aspects of my project and particularly to Dr Mark Birrell for always being available and happy to dose my mice. A large part of this thesis would not be possible without you! Also, Prof. Paul Winyard and Dr Miranda Smallwood for their help with Nitrite/Nitrate measurements (University of Exeter Medical School).

I would like to thank my family for all their support throughout the last few years. I hope that this offers a tiny silver lining during these difficult times. Finally every member of the Smyth household has finished University!

Last but not least, none of this would have been possible without Sven. You have given me love and support throughout every stage of this process and have always believed in me, even when at times I didn’t believe in myself. There are no words to fully express how much this means to me.
Publications, presentations & awards

Journal articles


Conference abstracts

Poster presentations


Oral Presentations


Prizes

- Highly Commended Oral Presentation by a student of non-clinical background, National Heart and Lung Institute, Imperial College London, 2014

- Joint Best Poster Presentation for basic science, National Heart and Lung Institute, Imperial College London, 2013
Abbreviation list

ACD – Acid Citrate Dextrose
ADP – Adenosine Diphosphate
AED – Aerodynamic diameter
AM - Amine-modified
ANOVA – Analysis of Variance
ApoE - Apolipoprotein E
ATP – Adenosine Triphosphate
AUC – Area Under the Curve
CAPs – Concentrated Ambient Particles
CB – Carbon Black
CFTHB – Calcium Free Tyrode’s Hepes Buffer
cAMP- Cyclic Adenosine Monophosphate
cGMP - Cyclic Guanosine Monophosphate
CO – Carbon Monoxide
COPD – Chronic Obstructive Pulmonary Disorder
COX – Cyclooxygenase
CM – Carboxy-Modified
CNS – Central Nervous System
CV – Cardiovascular
DAG - Diacyl-glycerol
DE – Diesel Exhaust
DEP – Diesel Exhaust Particles
ELISA – Enzyme-Linked Immunosorbent Assay
EPA – Environmental Protection Agency
FAE - Follicle-Associated Epithelium
GDP - Guanosine diphosphate
GPVI – Glycoprotein VI
GTP - Guanosine-5'-Triphosphate
HR – Heart Rate
HRV- Heart rate Variability
IL – Interleukin
IP3 - Inositol Trisphosphate
K-W – Kruskal-Wallis
LDH – Lactate Dehydrogenase
LNPs – Latex Nanoparticles
L-NAME - NG-nitro-L-arginine methyl ester
MCP-1 - Monocyte Chemoattractant Protein-1
MIP-2 - Macrophage Inflammatory Protein 2
MPO - Myeloperoxidase
NP – Nanoparticle
NO – Nitric Oxide
NO2 – Nitrogen Dioxide
NOx - Nitrogen Oxides
O3 – Ozone
OR – Odd’s Ratio
PAH - Polyaromatic Hydrocarbons
PAMP - Pathogen-Associated Molecular Patterns
PBMCs - Peripheral Blood Mononuclear Cells
PEG – Polyethylene glycol
PGE1 – Prostaglandin 1
PGL2 – Prostacyclin
PKC – Protein Kinase C
PKA – Protein Kinase A
PKG – Protein Kinase G
PI3K – Phosphoinositide 3-kinase
PLC\textsubscript{2γ} – Phospholipase C
PMMA – Polymethylmethacrylate
PM – Particulate matter
PPP – Platelet Poor Plasma
PRP – Platelet Rich Plasma
PSGL1 – P-selectin glycoprotein ligand-1
ROS – Reactive Oxygen Species
RR – Rate Ratio
SEM – Standard Error of the Mean
SEM – Subendothelial Matrix
SEM – Scanning Electron Microscopy
SNARE – Soluble NSF Attachment Protein Receptor
SO\textsubscript{2} – Sulfur Dioxide
SOD – Superoxide Dismutase
TAP – Tyrode’s, ACD and PGE\textsubscript{1}
TBARS – Thiobarbituric Acid Reactive Substances
TEM – Transmission Electron Microscopy
TGFβ – Tumour Growth Factor
TLR – Toll Like Receptor
TNFα – Tumour Necrosis Factor
TRAP – Thrombin Receptor Activator Protease
TXA\textsubscript{2} – Thromboxane A\textsubscript{2}
UAP – Urban Ambient Particles
UF – Ultrafine
UM – Unmodified
VOCs – Volatile Organic Compounds
vWF – von Willebrand Factor
WHO – World Health Organisation
# Table of Contents

- **Abstract** ............................................................................................................................. 1
- **Declaration of originality** ........................................................................................................ 2
- **Copyright declaration** ............................................................................................................. 3
- **Acknowledgements** .................................................................................................................. 4
- **Publications, presentations & awards** ...................................................................................... 5
- **Abbreviation list** ...................................................................................................................... 6
- **List of Figures and Tables** ......................................................................................................... 13
- **Chapter One** ............................................................................................................................ 15
  - **Introduction** .......................................................................................................................... 16
    - **1.1.0. Platelets** ...................................................................................................................... 16
      - **1.1.1. Platelet development** ............................................................................................... 16
      - **1.1.2. Platelet adhesion to the subendothelial matrix and collagen** .................................. 17
      - **1.1.3. Calcium regulation in platelets** .................................................................................. 17
      - **1.1.4. Collagen receptors** .................................................................................................... 18
      - **1.1.5. Platelet shape change** .............................................................................................. 18
      - **1.1.6. Granule secretion** ...................................................................................................... 19
      - **1.1.7. Alpha granule release** ............................................................................................... 19
      - **1.1.8. Dense granule secretion and signal amplification** ...................................................... 20
      - **1.1.9. Inside-out signalling and integrin $\alpha_{ii}\beta_{iii}$ activation** .................................... 21
      - **1.1.10. Thrombin generation** .............................................................................................. 22
      - **1.1.11. Clot retraction and outside-in signalling** ................................................................... 22
    - **1.1.1.1. Endogenous vascular regulators of platelet aggregation** ........................................... 23
      - **1.1.1.2. Nitric Oxide** .......................................................................................................... 23
      - **1.1.1.3. Prostacyclin** .......................................................................................................... 24
    - **1.1.2. Platelets and thrombosis** ............................................................................................ 24
    - **1.1.3. Platelets and inflammation** ......................................................................................... 25
    - **1.2.0. Air pollution** .............................................................................................................. 27
      - **1.2.1. Gases** ......................................................................................................................... 27
      - **1.2.2. Particulate matter** ....................................................................................................... 27
      - **1.2.3. Composition and sources of PM10** ........................................................................... 28
      - **1.2.4. Composition and sources of PM2.5** .......................................................................... 28
      - **1.2.5. Composition and sources of PM0.1** ......................................................................... 28
      - **1.2.6. Diesel Exhaust Particles and Carbon Black** ............................................................... 29
    - **1.3.0. Air pollution and health** ............................................................................................ 31
      - **1.3.1. Particulate matter and mortality (short term)** ............................................................ 31
## Table of contents

2.2.6. Preparation and staining of nanoparticles for electron microscopy ....................... 63  
2.2.7. Preparation and staining of nanoparticle-platelet electron microscopy samples .......... 63  
2.2.8. Isolation of mouse platelets .................................................................................. 64  
2.2.9. Radiolabelling mouse platelets with Indium Oxine\textsuperscript{111} .................................................. 64  
2.2.10. Radiolabelling mouse platelets with Indium Chloride\textsuperscript{111} ......................................... 64  
2.2.11. Animals ............................................................................................................... 65  
2.2.12. Mouse model of thromboembolism ...................................................................... 65  
2.3.0. Tracheal instillation of Diesel Exhaust Particles ......................................................... 67  
2.3.1. Enzyme-Linked Immunosorbent Assay .................................................................. 67  
2.3.2. Bronchoalveolar lavage collection ......................................................................... 67  
2.3.3. Platelet Rich Plasma preparation ............................................................................. 68  
2.3.4. Total and differential cell counting .......................................................................... 68  
2.3.5. Protein quantification ............................................................................................. 69  
2.3.6. Histological analysis of nanoparticle pulmonary deposition following intratracheal instillation ...................................................................................................................... 69  
2.4.0. Statistics ................................................................................................................. 69  

## Chapter Three

3.1.0. Introduction .............................................................................................................. 71  
3.1.1. Chapter aims ............................................................................................................. 73  
3.2.0. Concentration response to platelet agonists .............................................................. 74  
3.2.1. Electron micrographs of 50 nm and 100 nm nanoparticles with different surface chemistries in modified tyrodes hepes buffer ................................................................. 74  
3.2.2. Platelet aggregation induced by a high concentration of nanoparticles ................. 77  
3.2.3. Nanoparticle induced platelet aggregation \textit{in-vitro}. ...................................................... 79  
3.2.4. The effects of nanoparticles on platelet aggregation in plasma ................................. 81  
3.2.5. Enhancement of agonist mediated platelet aggregation \textit{in-vitro} by 50 nm amine-modified nanoparticles .................................................................................................................. 81  
3.2.6. Dense granule release by nanoparticles .................................................................. 84  
3.2.7. Nanoparticle induced P-selectin expression in isolated platelets ............................. 84  
3.2.8. Thrombin induced platelet aggregation \textit{in-vivo} ............................................................. 87  
3.2.9. Enhancement of thrombin induced platelet aggregation by amine-modified 50 nm nanoparticles \textit{in-vivo}. .................................................................................................. 87  
3.2.10. Enhancement of collagen induced platelet aggregation by amine-modified 50nm nanoparticles \textit{in-vivo}. ......................................................................................... 87  
3.3.0. Discussion ............................................................................................................... 91  
3.3.1. General limitations ................................................................................................. 96  

Chapter Four .................................................................................................................... 97
Table of contents

4.1.0. Introduction ................................................................................................................................. 98

4.1.1. Aims of chapter four .................................................................................................................. 99

4.2.0. The role of the integrin α_{iiib}β_{III} and extracellular calcium in nanoparticle induced platelet aggregation ................................................................. 100

4.2.1. Nanoparticle induced lactate dehydrogenase release .......................................................... 100

4.2.2. The role of ADP, TXA_{2} and PKC in nanoparticle induced platelet aggregation .............. 103

4.2.3. Inhibition of nanoparticle induced platelet aggregation by endogenous vascular regulators is dependent on nanoparticle physicochemistry ........................................... 103

4.2.4. The physical interaction between platelets and nanoparticles ........................................... 106

4.3.0. Discussion ................................................................................................................................. 110

4.3.1 General limitations ............................................................................................................. 113

Chapter Five ........................................................................................................................................ 115

5.1.0. Introduction ............................................................................................................................... 116

5.1.1. Aims .................................................................................................................................. 118

5.2.0. Electron micrographs of DEP and CB nanoparticles suspended in sterile saline. .......... 119

5.2.1. Deposition of DEP and CB in mouse lungs following intratracheal instillation. .......... 119

5.2.2. Platelet aggregation in-vivo following intratracheal instillation of DEP, CB or saline. ... 127

5.2.3. Leukocyte and cytokine/chemokine profile in bronchoalveolar lavage fluid following intratracheal instillation of CB, DEP and saline ......................................................... 131

5.2.4. Systemic leukocyte and cytokine profile following intratracheal instillation of CB, DEP and saline. ........................................................................................................... 131

5.2.5. Platelet aggregation in-vivo in enos^{-/-} mice and nitrate levels in the blood of WT mice following intratracheal instillation of DEP, CB or saline ......................................... 136

5.2.6. Nanoparticle induced platelet aggregation sensitivity to sodium nitroprusside and iloprost. ................................................................................................................. 136

5.2.7. Enhancement of collagen induced platelet aggregation by intravenously administered diesel exhaust particles in-vivo. .................................................. 136

5.3.0. Discussion ................................................................................................................................. 141

5.3.1. General limitations ............................................................................................................ 147

Chapter Six.......................................................................................................................................... 148

General discussion .............................................................................................................................. 149

6.1.0. Implications of this work - engineered nanoparticles ....................................................... 149

6.1.1. Further work – engineered nanoparticles .............................................................................. 150

6.2.0. Implications of this work – combustion-derived nanoparticles ........................................ 151

6.2.1. Further work – combustion-derived nanoparticles ........................................................... 152

6.3.0. Final conclusions ...................................................................................................................... 154

References........................................................................................................................................... 155
Table of contents

Appendix ............................................................................................................................................. 178
List of Figures and Tables

Figure 1.1. Electron micrograph showing the structure of a platelet. ..........................................16
Figure 1.2. Diagram of the main particulates found in diesel exhaust. ........................................30
Figure 1.3. Relationship between particle size and percentage deposition with the different regions of the lung. .........................................................................................................................41
Figure 1.4. Potential mechanisms behind the effects of particulate matter on the cardiovascular system. .............................................................................................................................44
Figure 1.5. Types of common engineered nano-structures. ..........................................................49
Figure 2.1. Murine model of pulmonary thromboembolism. .......................................................66
Figure 3.1. Concentration response to platelet agonists. ..............................................................74
Figure 3.2. Electron micrographs of 50 nm and 100 nm nanoparticles with different surface chemistries in modified tyrodes hepes buffer. .................................................................76
Figure 3.3. Platelet aggregation induced by a high concentration of nanoparticles. ...............78
Figure 3.4. Nanoparticle induced platelet aggregation in-vitro. ...................................................80
Figure 3.5. Nanoparticles effects on platelet aggregation in plasma ..........................................82
Figure 3.6. Enhancement of agonist mediated platelet aggregation in-vitro by 50 nm amine-modified nanoparticles. ..........................................................83
Figure 3.7. Dense granule release by engineered nanoparticles. ..............................................85
Figure 3.8. Nanoparticle induced P-selectin expression in isolated platelets .........................86
Figure 3.9. Thrombin induced platelet aggregation in-vitro .......................................................88
Figure 3.10. Enhancement of thrombin induced platelet aggregation by amine-modified 50 nm nanoparticles in-vivo .................................................................89
Figure 3.11. Enhancement of collagen induced platelet aggregation by amine-modified 50nm nanoparticles in-vivo .................................................................90
Figure 4.1. Effects of eptifibatide and EGTA on nanoparticle induced platelet aggregation ..........101
Figure 4.2. Effect of nanoparticles on lactate dehydrogenase release by isolated platelets ..........102
Figure 4.3. The role of ADP, TXA2 and PKC in nanoparticle induced platelet aggregation ..............................104
Figure 4.4. Nanoparticle induced platelet aggregation sensitivity to sodium nitroprusside and iloprost .................................................................105
Figure 4.5. Electron micrographs displaying the physical interaction between isolated platelets and nanoparticles. .................................................................109
Figure 5.1. Electron micrographs of DEP and CB NPs suspended in sterile saline. ..................120
Figure 5.2.1. Deposition of DEP and CB (1µg per mouse) in mouse lungs following intratracheal instillation. .................................................................121
List of Figures

Figure 5.2.2. Deposition of DEP and CB (25 µg per mouse) in mouse lungs following intratracheal instillation. .................................................................122

Figure 5.2.3. Deposition of DEP and CB (50 µg per mouse) in mouse lungs following intratracheal instillation. .................................................................123

Figure 5.3. Collagen dose dependent platelet aggregation in-vivo using InCl$^{111}$ and InOx$^{111}$ labelled murine platelets. .................................................................126

Figure 5.4.1. Platelet aggregation in-vivo in mice following intratracheal instillation of DEP, CB (1 µg mouse) or saline. .................................................................128

Figure 5.4.2. Platelet aggregation in-vivo following intratracheal instillation of DEP, CB (25 µg mouse) or saline. .................................................................129

Figure 5.4.3. Platelet aggregation in-vivo following intratracheal instillation of DEP (50 µg mouse) and saline. .................................................................130

Figure 5.5. Leukocyte profile in bronchoalveolar lavage fluid following intratracheal instillation of CB, DEP and saline. .................................................................132

Figure 5.6. Cytokine profile in bronchoalveolar lavage fluid following intratracheal instillation of CB, DEP and saline. .................................................................133

Figure 5.7. Haematological profile following intratracheal instillation of CB, DEP and saline. .................................................................134

Figure 5.8. Cytokine profile in plasma following intratracheal instillation of CB, DEP and saline. .................................................................135

Figure 5.9. Platelet aggregation in-vivo in eNOS$^{-/-}$ mice following intratracheal instillation of DEP, CB or saline. .................................................................137

Figure 5.10. Plasma nitrate levels in WT mice following intratracheal instillation of DEP, CB or saline. .................................................................138

Figure 5.11. Nanoparticle induced platelet aggregation sensitivity to sodium nitroprusside and iloprost. .................................................................139

Figure 5.12. Enhancement of collagen induced platelet aggregation by diesel exhaust particles in-vivo. .................................................................140

Tables

Table 2.1. List of compounds and purchasing companies ..............................................57
Table 2.2. List of Equipment used ........................................................................59
Table 2.3. Buffer compositions .............................................................................60
Chapter One

Introduction
Introduction

1.1.0. Platelets

1.1.1. Platelet development

Platelets are anucleate discoid cells derived from pluripotent hematopoietic stem cells called megakaryocytes which are found in the bone marrow. Platelets are 2-3 µm in diameter and are present in the blood at a concentration of ~250 x 10^3 per µl where they circulate between 5 – 9 days before they are destroyed by phagocytosis in the spleen and liver. Megakaryocytes project long pseudopod-like branches (2-4 µm in diameter) called proplatelets through the endothelial cells of the blood vessels which transfuse the bone marrow. At the end of these projections, platelets buds off and enter the systemic circulation (Italiano et al., 1999).

![Electron micrograph showing the structure of a platelet.](image)

Platelets contain a cytoskeleton made up of actin microtubules which allows morphological rearrangement during activation. They contain a dense tubular system that stores internal calcium which is released upon activation. Platelets contain dense core granules which contain nucleotides (integrin receptors, 5-HT, ATP and ADP) and alpha granules which contain adhesion factors (fibrinogen, vWF, P-selectin and integrin αIIβ3). These molecules are released when platelets are activated and their contents promote further activation and aggregation of surrounding platelets by binding to cell-surface receptors and activating heterotrimeric G-protein dependent signalling cascades.
Chapter One - Introduction

1.1.2. Platelet adhesion to the subendothelial matrix and collagen

Platelets' primary role is to maintain vascular integrity and prevent excessive haemorrhage following injury. Under normal physiological conditions, platelets circulate within an intact vasculature in a quiescent state. Upon vascular injury, the subendothelial matrix (SEM) is exposed to the blood. The matrix contains many macromolecules such as collagen, fibronectin, von Willebrand factor (vWF), laminin and thrombospondin that bind to platelet cell surface receptors and mediate adhesion to the vessel wall.

Initial platelet adhesion to the SEM is greatly influenced by rheological factors. Under low shear conditions (< 1000 s\(^{-1}\)), which are found in veins and large arteries, platelets bind mainly to collagen, laminin and fibronectin. Under high shear conditions (> 1000 s\(^{-1}\)), found in small arteries, the microvasculature and diseased vessels (stenotic/atherosclerotic), platelet adhesion to the SEM is mediated via vWF. vWF is immobilised on exposed collagen by binding through its A3 domain (Hoylaerts et al., 1997, Lankhof et al., 1996). High shear rates cause the A1 domain on vWF to be exposed and subsequently bind to the cell surface receptor glycoprotein 1bα (GP1bα), which is a component of the larger GPIb/IX/V complex, on circulating platelets (Ulrichts et al., 2006). The binding of vWF to GP1bα initially tethers the platelet to the vascular wall; however, it is a reversible process and inadequate to permit firm adhesion to the SEM (Moroi et al., 1997). More stable interactions and ultimately platelet arrest are acquired through additional platelet collagen receptors and with the binding of vWF binding to the platelet surface integrin \(\alpha_{IIb}\beta_{III}\) (Savage et al., 1996).

1.1.3. Calcium regulation in platelets

Binding of platelet agonists such as collagen to cell surface platelet receptors initiates an intracellular cascade resulting in the release of Ca\(^{2+}\) from the dense tubular system (DTS) into the cytoplasm. Ca\(^{2+}\) is an essential signalling molecule involved in platelet adhesion and activation; consequently it is tightly regulated. Platelets in a quiescent state have an intracellular Ca\(^{2+}\) concentration of approximately 0.1 µM, which is 10,000 fold lower than the extracellular environment. This gradient is maintained by three calcium transporters, the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) (Bokkala et al., 1995), the plasma membrane Ca\(^{2+}\) ATPase (Rosado and Sage, 2000) and the Na/Ca\(^{2+}\) exchanger (Haynes et al., 1991). Upon platelet contact with components of the SEM or the binding of physiological agonists to cell surface receptors, Ca\(^{2+}\) is released from the DTS via receptor mediated release. Additionally, store-operated Ca\(^{2+}\) entry (SOCE) occurs following Ca\(^{2+}\) release from the intracellular stores. A transmembrane protein present in the DTS, stromal interaction molecule 1 (STIM1), detects when Ca\(^{2+}\) levels are low in the DTS and when this occurs it translocates to the plasma membrane where it associates with the Ca\(^{2+}\) channel moiety Orai1 subunit of the
Chapter One - Introduction

calcium activated channel (CRAC) (Bergmeier et al., 2009). The subsequent influx of Ca\(^{2+}\) further raises the cytosolic Ca\(^{2+}\) concentration.

1.1.4. Collagen receptors

The two primary platelet collagen receptors are the integrin \(\alpha_2\beta_1\) (Santoro et al., 1988) and glycoprotein VI (GPVI) (Clemetson et al., 1999). Although GPVI is unable to facilitate stable platelet adhesion to collagen by itself, extensive work has demonstrated its integral role in initiating intracellular platelet signalling via its association with Fc\(\gamma\) chains (Pugh et al., 2010). Platelet signalling events initiated through the activation of cell surface receptors are often referred to as “outside-in signalling”.

Intracellular signalling mediated by GPVI and/or the integrin \(\alpha_{IIb}\beta_{III}\) (Lockyer et al., 2006, Van de Walle et al., 2007) is thought to be involved in activation of the \(\alpha_2\beta_1\) integrin from its low-affinity state to its high-affinity state (Van de Walle et al., 2005) however, the signalling events leading to \(\alpha_2\beta_1\) activation have not been fully elucidated. The initial binding of \(\alpha_2\beta_1\) to collagen promotes platelet activation and stable adhesion by primarily augmenting GPVI attachment to collagen and therefore enhancing GPVI mediated signalling; Furthermore, \(\alpha_2\beta_1\) activation ultimately leads to activation of integrin \(\alpha_{IIb}\beta_{III}\) via outside-in signalling (Bernardi et al., 2006).

Murine genetic and pharmacological studies have demonstrated that GPVI has a fundamental role in platelet adhesion to exposed SEM and in release of Ca\(^{2+}\) from intracellular stores which initiates downstream signalling events leading to platelet activation (Lockyer et al., 2006, Massberg et al., 2003) (Nieswandt et al., 2000, Jandrot-Perrus et al., 1997). However, there are conflicting reports from experimental thrombosis studies about the involvement of \(\alpha_2\beta_1\) in platelet adhesion and activation; this is most likely due to variation in methodologies (Siljander et al., 2004, Gruner et al., 2003, He et al., 2003, Holtkotter et al., 2002, Kuijpers et al., 2007). It appears that both receptors work synergistically (also with GP1b) to produce firm platelet adhesion to the SEM and platelet activation, which generates an initial platelet monolayer over the site of injury (Siljander et al., 2004, Kuijpers et al., 2007, Holtkotter et al., 2002).

1.1.5. Platelet shape change

One of the primary events which occurs following the rapid increase in intracellular Ca\(^{2+}\) is platelet shape change from a discoid morphology to a spiculate sphere, with protruding filopodia. This involves cytoskeletal rearrangement and filopodia extrusion which is critical for platelet spreading, as it expands the platelet surface area, allowing maximum contact with surrounding cells and biomolecules (Misselwitz et al., 1988). The process comprises activation of the Ca\(^{2+}/\)calmodulin-
dependent myosin light chain (MLC) kinase which phosphorylates the MLC and allows direct interaction of the myosin with the actin filaments (Daniel et al., 1981, Hathaway and Adelstein, 1979).

1.1.6. Granule secretion

Upon platelet adhesion to SEM substrates and platelets agonist binding to their receptors, a series of Ca\(^{2+}\) dependent intracellular signalling events leads to exocytosis of platelet granules and release of their contents into the extracellular environment. These events are predominantly mediated via Ca\(^{2+}\) and activated protein kinase C (PKC) (Konopatskaya et al., 2009, Konopatskaya et al., 2011). The exocytosis of the granules involves their movement into close proximity of the plasma membrane which lines the open-canalicular system that is exposed to the extracellular environment. The granules fuse to the membrane via soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) (Reed, 2004). SNAREs are membrane proteins which can interact with each other and cause membrane fusion. In platelets, the protein Munc18c regulates SNARE mediated exocytosis by associating with the SNARE protein syntaxin 4 under resting conditions. Following platelet activation, Munc18c is phosphorylated by PKC which causes dissociation of Munc18c from syntaxin 4 and allows formation of SNARE complexes between the plasma membrane and the granule (Houng et al., 2003, Reed et al., 1999).

1.1.7. Alpha granule release

Platelet granules contain a plethora of bioactive protein and nucleotide modulators which bind in an auto- paracrine fashion, causing activation of proximate platelets and ultimately amplifying the initial platelet activation activity. Alpha granules have been reported to contain over 280 different biomolecules (Maynard et al., 2007) which have additional roles outside of platelet aggregation and activation, such as inflammation, coagulation, angiogenesis and wound healing. Their importance in primary haemostasis is demonstrated in patients with the congenital autosomal recessive bleeding disorder gray platelet syndrome. These patients have varying degrees of alpha granule deficiency and present with mild to moderate bleeding tendencies (Raccuglia, 1971).

vWF and fibrinogen are two important alpha granule proteins. Both promote platelet aggregation by binding to the cell surface integrin \(\alpha_{\text{IIb}}\beta_{\text{III}}\) and the GPIb/IX/V complex (vWF only) on adjacent platelets. Additionally, these molecules promote further platelet activation through outside-in signalling through these receptors.

P-selectin is another important adhesion molecule present in alpha granules. Upon platelet activation, P-selectin translocates from the alpha granule membrane to the platelet extracellular plasma membrane (Hsu-Lin et al., 1984). Approximately 10,000 P-selectin molecules are present on an
activated platelet membrane (McEver and Martin, 1984); for this reason P-selectin is commonly used as a marker of platelet activation. P-selectin’s primary function is to facilitate platelet-leukocyte and endothelial cell interactions through its ligand P-selectin glycoprotein ligand-1 (PSGL1), which is constitutively expressed on most leukocytes, endothelial cells and platelets (Spertini et al., 1996, Frenette et al., 1995). Work using P-selectin monoclonal antibodies has revealed that P-selectin has a role in platelet aggregation, through stabilisation of \( \alpha_{IIb} \beta_{IIIa} \)-fibrinogen interactions. Interestingly, this process appears to be independent of PSGL1 (Merten and Thiagarajan, 2000). Furthermore, P-selectin’s integral role in haemostasis is demonstrated in P-selectin knock-out mice, where these animals displayed a 40 % increase in bleeding time and two fold larger haemorrhagic lesions (Subramaniam et al., 1996).

1.1.8. Dense granule secretion and signal amplification

Dense granules and alpha granules are released upon platelet activation. As previously mentioned, these granules contain small molecules and nucleotides which are involved in amplification of platelet activation processes. One of the key molecules involved in this process is ADP, which is potent platelet activator. ADP initiates activation of adjacent platelets through binding to the purinergic G-coupled receptors P2Y\(_1\) and P2Y\(_{12}\). Binding of ADP to P2Y\(_1\) activates G\(_q\) and PLC-\(\beta\) signalling, leading to Ca\(^{2+}\) mobilisation, platelet shape change and weak transient platelet aggregation (Offermanns et al., 1997). However, P2Y\(_1\) signalling acts in synergy with other receptors to establish full ADP and collagen platelet responses (Mangin et al., 2004). The importance of P2Y\(_1\) signalling in haemostasis is demonstrated by defective platelet aggregation and resistance to thrombosis in P2Y\(_1\) null mice (Leon et al., 1999, Fabre et al., 1999). Binding of ADP to the P2Y\(_{12}\) receptor leads to more potent platelet activation and aggregation through G\(_{12}\) and PI3-K signalling pathways. ADP binding to this receptor also stimulates inhibition of the endogenous inhibitory pathways through adenylate cyclase (AC), reducing cytoplasmic concentrations of cyclic adenosine monophosphate (cAMP) (Zhang et al., 2001, Gachet et al., 1996).

Activation of both ADP receptors and the fibrinogen receptor \( \alpha_{IIb} \beta_{IIIa} \) is required for maximal aggregation, and for additional release of secondary agonists such as thromboxane A\(_2\) (TxA\(_2\)) from arachidonic acid (Jin et al., 2002). Increased intracellular Ca\(^{2+}\) activates phospholipase A\(_2\) (PLA2) which in turn liberates arachidonic acid from phospholipids in the plasma membrane. Cyclooxygenase (COX-1) then catalyses the formation of prostaglandin G2 (PGG2) and prostaglandin H2 (PGH2). PGH2 is transformed into TxA\(_2\) by thromboxane synthase and subsequently binds to TP receptors present on the platelet surface in an autocrine/paracrine fashion. This further amplifies the platelet aggregation signal through G\(_q\) and PLC signalling, leading to Ca\(^{2+}\) release from intracellular stores and integrin activation (Hamberg and Samuelsson, 1974, Knezevic et al., 1993, Shenker et al., 1991, Brass et al., 1987). TxA\(_2\) also initiates platelet shape change through G\(_{12/13}\) signalling which
triggers Rho/Rho-kinase mediated phosphorylation of MLC (Klages et al., 1999). In addition to being a potent (albeit short lived) agonist, TxA₂ also causes vascular smooth muscle contraction and vasoconstriction, which limits blood loss following vascular injury.

Dense granules also contain ATP which preferentially binds another subclass of P2 purinoceptors on the platelet surface, the ligand-gated cation channel P2X₁ (Vial et al., 1997). Binding of ATP leads to rapid influx of Ca²⁺ into the platelet from the extracellular environment (MacKenzie et al., 1996). Investigation into the role of the P2X₁ receptor role in platelet function has been difficult due to its desensitisation during isolated platelet preparation (Rolf et al., 2001). However, numerous studies have found that while P2X₁ signalling is unable to stimulate platelet aggregation alone, it does contribute to platelet aggregation induced by other agonists, such as collagen and TxA₂, and shear-induced platelet aggregation (Oury et al., 2003, Oury et al., 2001, Oury et al., 2004).

The neurotransmitter 5-Hydroxytryptamine (5-HT) is also contained in dense granules and binds to the 5-HT2A receptors on the platelet surface upon release. 5-HT is considered a weak platelet agonist however 5-HT2A signalling via Gq and PLCβ2 causes Ca²⁺ mobilisation, platelet shape change and reversible platelet aggregation. Similarly to TxA₂, release of 5-HT also induces vascular smooth muscle contraction, causing local vasoconstriction which limits blood loss following vascular injury (Svensson and Hamberg, 1976, Hamberg et al., 1975).

### 1.1.9. Inside-out signalling and integrin α_{IIb}β_{III} activation

The ultimate step in platelet activation is aggregation which involves cross-linking of adjacent platelets via fibrinogen binding to the α_{IIb}β_{III} integrin present on the platelet plasma membrane, to form a platelet plug. There are approximately ~ 80,000 copies of this receptor per platelet, with a reserve ‘pool’ of receptors present in the alpha granules (Wagner et al., 1996). When the platelet is in a quiescent state, this integrin has low affinity for its ligands, fibrinogen and vWF. This low affinity state is maintained by interactions between the integrin α and β subunits. Following platelet activation, resulting from activation of many adhesion and G protein-coupled receptors, this integrin adapts a high affinity confirmation, in a process known as ‘inside-out signalling’. This process is initiated following intracellular Ca²⁺ mobilisation and Diacylglycerol (DAG) formation, which activates diacylglycerol regulated guanine nucleotide exchange factor I (CalDAG-GEFI) that subsequently activates a Guanosine-5’-triphosphate (GTP) binding protein Rap1b (Dupuy et al., 2001, Kawasaki et al., 1998, Crittenden et al., 2004). Rap1b forms an ‘activation complex’ with Rap1-interacting adaptor molecule (RIAM) and a cytoskeletal protein Talin (Han et al., 2006, Lee et al., 2009). This complex relocates to the plasma membrane and Talin binds to the β3 subunit of the integrin, resulting in a confirmation change in the extracellular domain of the integrin which exposes the ligand binding site (Otey et al., 1990, Calderwood et al., 1999). It is well established that all
integrins need divalent cations to bind their ligands and Ca\(^{2+}\) is required for the binding of fibrinogen to integrin \(\alpha_{IIb}\beta_{III}(\text{Smith et al., 1994}).\)

1.1.10. Thrombin generation

Stauching unwanted bleeding following vascular injury entails a combination of primary haemostasis, resulting in platelet plug formation, and secondary haemostasis which involves activation of the coagulation cascade; this triggers further platelet activation and formation of a fibrin mesh which stabilizes the platelet plug. The interaction between these processes occurs primarily on the surface of activated platelets. An increase in cytosolic Ca\(^{2+}\) and subsequent platelet activation promotes stimulation of the ATP independent enzyme phospholipid scramblase and inhibition of aminophospholipid translocase. This leads to the transfer of negatively charged phospholipids, particularly phosphatidylserine (PS), from the inner leaflet of the plasma membrane to the outer leaflet, where it is exposed to extracellular environment (Comfurius et al., 1996, Wolfs et al., 2005). The non-enzymatic cofactor Va and the serine protease factor Xa are recruited to the membrane where they form the prothrombinase complex in a Ca\(^{2+}\) dependent manner, that binds and cleaves the substrate prothrombin yielding thrombin (Bevers et al., 1982b, Bevers et al., 1982a). One of the roles of thrombin is to convert fibrinogen into fibrin and this forms a mesh which entraps additional blood cells and ultimately stabilises the platelet plug. Additionally, thrombin induces potent platelet activation by binding to the protease activated receptor 1 (PAR1) and PAR 4 G-coupled receptors on surrounding platelets (Kahn et al., 1998). Both these receptors signal through Goq and G\(_{12/13}\) pathways. Goq activates PLC\(\beta\)2 signalling which produces a rise in cytosolic Ca\(^{2+}\) and PKC activation through IP\(_3\) and DAG (van Willigen et al., 1995). Activation of G\(_{12/13}\) causes platelet shape change through Rho/Rho kinase activation and actin remodelling (Moers et al., 2003). Both Goq and G\(_{12/13}\) pathways activate the integrin \(\alpha_{IIb}\beta_{III}\) and significantly contribute to platelet aggregation (van Willigen et al., 1995, Offermanns et al., 1997). Injection of annexin A5 or lactadherin (chelates exposed PS) into mice dramatically impaired thrombus formation \textit{in vivo}, demonstrating the functional importance of thrombin generation (Kuijpers et al., 2008, Shi et al., 2008).

1.1.11. Clot retraction and outside-in signalling

One of the final events in the haemostatic process is the constriction of the entire platelet-fibrin clot, known as clot retraction. Clot retraction enhances thrombus stability, facilitates wound repair and prevents obstruction of the damaged blood vessel. Clot retraction is platelet-dependent and mediated
by the integrin \(\alpha_{\text{IIb}}\beta_{\text{III}}\). Binding of fibrin, fibrinogen or vWF to integrin \(\alpha_{\text{IIb}}\beta_{\text{III}}\) causes phosphorylation of the \(\beta_3\) subunit and produces intracellular signalling which culminates into generation of contractile forces that pull the platelet in on itself (Law et al., 1999). This is enabled through interactions between the cytoplasmic domain of \(\alpha_{\text{IIb}}\beta_{\text{III}}\) and various signalling molecules such as calcium and integrin-binding protein 1 (CIB1) (Naik et al., 1997), talin (Calderwood et al., 1999), and the tyrosine kinases, Src (Arias-Salgado et al., 2003) and Syk (Gao et al., 1997, Woodside et al., 2001). However, an integral molecule in this process is myosin IIA (Jenkins et al., 1998, Maupin et al., 1994) which is regulated by myosin light chain kinase (MLCK) and Rho Kinase (Ono et al., 2008). The interaction between myosin IIA and the actin cytoskeleton generates the contractile force which draws the plasma membrane towards the centre of the cell (White, 1968).

1.1.1.1. Endogenous vascular regulators of platelet aggregation

1.1.1.2. Nitric Oxide

Platelets in the circulation must be kept in a quiescent state to prevent inappropriate platelet activation and platelet plug formation. This is mainly performed by two biomolecules synthesised in the surrounding endothelial cells. The first is the gaseous transmitter nitric oxide (NO), which was first identified by its ability to induce smooth muscle relaxation and ensuing dilation of the blood vessels (Ignarro et al., 1987, Palmer et al., 1987). NO is produced by the conversion of L-arginine by the enzyme nitric oxide synthase (NOS) (Palmer et al., 1988). There are three isoforms of NOS; the constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS), and inducible NOS (iNOS) (Lamas et al., 1992, Radomski et al., 1990a, Mayer et al., 1990). Endothelial derived NO regulates platelet behaviour by diffusing through the platelet membrane and binding to the receptor soluble guanylyl cyclase (sGC). NO binds to the sGC heme cofactor which causes the conversion of GTP into cyclic guanosine monophosphate (cGMP) and this subsequently activates cGMP-dependent protein kinase (PKG). PKG phosphorylates and activates many downstream proteins such as inositol-1, 4, 5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) and the vasodilator-stimulated phosphoprotein (VASP) (Antl et al., 2007, Aszodi et al., 1999, Radomski et al., 1990b) which causes inhibition of activation events such as rises in cytosolic Ca\(^{2+}\), cytoskeletal rearrangement, integrin activation and granule secretion.

The role of platelet-derived NO in inhibiting platelet activation is currently unclear and work conducted by various authors has been contradictory. Early studies suggested that the eNOS and iNOS isoforms were present in platelets (Radomski et al., 1990b, Chen and Mehta, 1996) and NO was reported to be released from activated platelets and regulate platelet recruitment in-vitro. Additionally, genetic studies reported that infusion of WT platelets into eNOS\(^{-/-}\) mice caused a significant decline in
bleeding times compared to infusion with eNOS\(^{-/-}\) platelets, suggesting a functional role for platelet-derived NO (Freedman et al., 1999, Freedman et al., 1997).

However, Gambaryan et al, found no iNOS/eNOS mRNA or protein in purified human or mouse platelets (Gambaryan et al., 2008). Moreover, further work using eNOS\(^{-/-}\) mice and pharmacological NOS inhibitors reported that the source of NO which regulate platelet aggregation was external to the platelet (Tymvios et al., 2009).

1.1.1.3. Prostacyclin

The other major endothelial-derived platelet mediator is prostacyclin (PGI\(_2\)) which is synthesised by conversion of arachidonic acid predominantly by COX-2 (McAdam et al., 1999). PGI\(_2\) binds to the IP GPCR on the platelet membrane which transduces its signal via G\(_{\alpha_s}\) causing activation of AC and increases in cAMP production. An increase in cytosolic cAMP levels causes activation of cAMP-dependent protein kinase (PKA), which inhibits platelet activation events such as secretion and aggregation (Feinstein and Fraser, 1975).

PKA phosphorylates similar protein targets to PKG such as VASP, which interferes with cytoskeletal rearrangement (Aszodi et al., 1999). Additionally, cGMP/PKG and cAMP/ PKA signalling regulates IP3 mediated Ca\(^{2+}\) mobilisation from the DTS by phosphorylation of the IP3 receptor (Quinton et al., 1996, Cavallini et al., 1996). Recent work by Aburima et al, identified RhoA as a novel substrate for PKA. Activated PKA phosphorylates RhoA, which down-regulates RhoA/ROCK signalling and subsequent MLC phosphorylation, preventing platelet shape change (Aburima et al., 2013).

1.1.2. Platelets and thrombosis

Thrombosis involves the inappropriate activation of haemostatic processes which can lead to the blockage of blood vessels and ischemic conditions such as myocardial infarction (MI) and stroke. Arterial thrombosis accounts for ~ 25 % of deaths worldwide (WHO, 2010). Many pathological conditions underlie these thrombotic events however one of the main causes of arterial thrombosis is the rupture of an atherosclerotic plaque. Rupture of an atherosclerotic plaque exposes circulating platelets to the internal components of the plaque, such as the agonist collagen, which promotes platelet activation and aggregation. Additionally, endothelial dysfunction, which has been implicated in underlying many cardiovascular conditions (Libby et al., 2002), involves disruption to the endothelium causing changes in vasomotor responses and promoting a proinflammatory and prothrombotic environment. Endothelial dysfunction is often characterised by the reduction in the synthesis of the gaseous mediator NO. This reduction in NO can promote thrombus generation as platelets are relieved of its inhibitory influence. Endothelial dysfunction is caused by traditional
cardiovascular risk factors such as smoking, hypercholesterolemia and hypertension in addition to genetic factors (Deanfield et al., 2007).

1.1.3. Platelets and inflammation

Platelets have functional roles outside of haemostasis and thrombosis, particularly in the immune response, where they couple the haemostatic and inflammatory processes to facilitate tissue repair and defence against pathogens. Secretion of platelet granules following activation releases a variety of inflammatory mediators such as cytokines (Interleukin-1β (IL-1β), Tumour Necrosis Factor α (TNFα), and Transforming Growth Factor β (TGFβ)) and platelet specific chemokines (Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES); Platelet factor-4 (PF-4); β-Thromboglobulins (β - TG)) which activate and recruit immune cells to sites of vascular injury.

Platelet activation also leads to the relocation of cell surface proteins such as P-selectin. P-selectin’s primary function is to facilitate platelet-leukocyte and platelet-endothelial cell interactions through its ligand PSGL-1, which is constitutively expressed on most leukocytes, endothelial cells and platelets (Spertini et al., 1996, Symon et al., 1996, Frenette et al., 1995). P-selectin is also present in the Weibel-Palade bodies of endothelial cells. Activation of endothelial cells through binding of thrombin or inflammatory mediators to cell surface receptors triggers relocation of P-selectin to the outer membrane, where it mediates leukocyte and platelet tethering and rolling along the endothelial surface (Lorenzon et al., 1998, Frenette et al., 1995).

Another protein which transfers to the platelet membrane upon platelet stimulation is the protein CD-40L which is a transmembrane protein also found on immune cells along with its receptor CD40. CD-40L interactions between platelets and endothelial cells can initiate inflammation by inducing cytokine release (monocyte chemoattractant protein-1, MCP-1; IL-6 and IL-8) and expression of adhesion molecules (vascular cell adhesion molecule-1, V-CAM1; intercellular adhesion molecule-1 and E-selectin) (Henn et al., 1998, Henn et al., 2001).

Both CD-40L and P-selectin have been demonstrated to be important in stabilising thrombi however they are also integral to processes such as inflammation and atherogenesis (Dong et al., 2000, Johnson-Tidey et al., 1994, Burger and Wagner, 2003, Lievens et al., 2010, Andre et al., 2002, Merten and Thiagarajan, 2000).

Platelets also have the ability to respond to inflammatory stimuli through cell surface receptors. For example, platelets contain toll-like receptors (TLR) that bind to pathogen associated molecular patterns (PAMPs) found on various prokaryotes. Platelets have been reported to contain TLR 1, 2, 4, 6, 9 (Cognasse et al., 2005, Shiraki et al., 2004) however the functional relevance of these TLRs is still ambiguous. Some authors have reported augmented responses to platelet agonists upon binding of TLR ligands, while others report reduced agonist responses or no functional effects at all (Ward et al.,
Chapter One - Introduction

2005, Sheu et al., 1998). Platelets also express the chemokine receptors CCR1, CCR3, CCR4, and CXCR4, which appear to have functional roles, as ligand binding has been demonstrated to induce increases in cytosolic Ca\(^{2+}\) and granule secretion (Clemetson et al., 2000, Abi-Younes et al., 2000). Furthermore, platelets can internalise pathogenic organisms such as bacteria and viruses however their role in the immune system as a phagocyte is still controversial (White, 2005, Youssefian et al., 2002).
Chapter One - Introduction

1.2.0. Air pollution

Air pollution is the addition of harmful substances into the atmosphere that can have adverse effects on human health and ecosystems. There are both natural and anthropologic sources of air pollution; traditionally air pollution was generated from the industrialization of developing countries, where sulphur dioxide ($SO_2$) and black smoke was produced from combustion of coal in domestic fires and industrial processes. However, in modern times air pollution is primarily generated from vehicle emissions and tyre degradation. Industrialization of urban areas over the last century has led to a rise in air pollution, which is composed of a complex mix of two primary components, gases and particulate matter (PM) (WHO, 2000).

In America, the 1970 clean air act was the first major regulation which established air quality standards in order to protect public health. The National Ambient Air Quality Standards (NAAQS) were agreed for 6 primary air pollutants: PM, lead, $SO_2$, nitrogen dioxide ($NO_2$), ozone ($O_3$) and carbon monoxide (CO) (EPA, 2011). In addition, the World Health Organization (WHO) published guidelines in 1987, which were updated in 1997, with regard to four primary pollutants: PM, $O_3$, $NO_2$ and $SO_2$ (WHO, 1987, Jantunen, 1997). This has been extended to encompass the 6 common air pollutants by the addition of lead and CO (WHO, 2000).

1.2.1. Gases

Nitrogen dioxide ($NO_2$) and nitric oxide (NO) are both oxides of nitrogen collectively called NOx. Combustion of fossil fuels from vehicle emissions is the primary source of NOx (WHO, 2000, AEA R et al., 2013). Ozone is generated through the chemical interaction between NOx and volatile organic compounds (VOCs) present in the atmosphere in a process mediated by intense sunlight. $SO_2$ is generated through the combustion of sulphur containing fuels such as coal and oil. Industrial oil processing and domestic fires were the primary sources however recent implementation of environmental regulations and legislation restricting coal burning has led to a dramatic reduction of ambient $SO_2$ levels (Passant NR, 2013). $CO_2$ has received a large amount of interest over the last decade mainly due to the concern surrounding global warming. Road transport is the largest contributor to $CO_2$ emissions, as it is produced from the incomplete combustion of carbon-containing fuels (WHO, 2000).

1.2.2. Particulate matter

PM is a complex mixture of solid and liquid particles suspended in the air. These particles are composed of inorganic and organic components such as carbon, sulphates, nitrates, metals, acids and volatile compounds (Murrells T P, 2011). Regulatory bodies such as the U.S. EPA and the Department for Environmental Food and Rural Affairs (DEFRA) in the UK, categorize PM based on
Chapter One - Introduction

it’s aerodynamic diameter. There are three main subgroups: PM10 coarse (≤ 10 µm), PM2.5 fine (≤ 2.5 µm), and PM0.1 ultrafine (UF) (≤ 0.1 µm). PM is comprised of both primary and secondary particles that are generated from different processes.

Primary particles are emitted directly into the atmosphere from combustion sources such as combustion of fossil fuels and volcanic activity. In the UK, the main source of primary particles is from stationary fuel consumption and motor vehicles, which produce primary particles through engine emissions and tyre/break wear. In London, 80% of primary PM10 particles are reported to be from road transport; Diesel engine vehicles are the main contributor to this, as diesel engines produce a larger mass of particles per vehicle kilometre compared to petrol engines (Passant NR, 2013). Secondary particles are generated through chemical reactions in the atmosphere. In the UK, these secondary particles are comprised mainly of ammonia, sulphur dioxide and nitrogen oxides as well as organic compounds (Murrells T P, 2011).

1.2.3. Composition and sources of PM10

PM10 comprises both fine and coarse particles (10 µm – 2.5 µm) which are derived from different sources. PM10 is generated from combustion processes, surface erosion, dusts and the mechanical break-up of materials, often during production processes such as construction and quarrying. PM10 particles are comprised of organic and inorganic carbon, iron, calcium, magnesium, nitrate, silicon and aluminium. They are also made up of spores, pollen and plant debris. In 2009, 23% of PM 10 in the UK was estimated to be derived from road transport, 20% from production processes and 45% from industrial, residential and energy production. However, the contribution of road transport can be dramatically different in urban centres, as this can be as high as 50 - 80% (Murrells T P, 2011, Passant NR, 2013).

1.2.4. Composition and sources of PM2.5

Secondary particles make up a substantial proportion of PM2.5 in the UK. These secondary particles consist mainly as ammonium, nitrate, sulphate, transition metals and organic and inorganic carbon. In 2011, 33.9% of PM2.5 in the UK was derived from stationary combustion sources and 34% was from transport (26% road transport). While general PM2.5 emissions have been decreasing since 1970s, the transport component has become an increasingly greater source (NAEI, 2013).

1.2.5. Composition and sources of PM0.1

PM0.1 particles consist of similar components to PM2.5 but contain large amount of inorganic ions, hydrocarbons and metals. Road transport accounted for 41% of total UK PM 0.1 emissions in 2009 (Murrells T P, 2011). While PM0.1 contributes very little to the overall mass of ambient PM, these particles contribute the most in terms of particle number (EPA, 1996).
1.2.6. Diesel Exhaust Particles and Carbon Black

Carbon black (CB) or elemental carbon is similar in structure to impure graphite and is primarily produced by combustion of wood or diesel. Combustion of diesel fuel is estimated to contribute to 70 – 90 % of total CB concentrations in western Europe (Mansfield et al., 1991). 85 % of CB produced from primary emissions has been estimated to be <120 nm in aerodynamic diameter and with a distribution bimodal size range at 50 nm and 120 nm. They are also distributed as larger agglomerated particles between 500 nm - 1µm in aerodynamic diameter (Venkataraman and Friedlander, 1994) (Fig 1.2).

Exhaust from diesel engines is a mixture of gases and particulates. The main gases present in diesel exhaust (DE) are CO, nitrogen compounds, sulphur compounds, low-molecular weight hydrocarbons (aldehydes and benzene) and polyaromatic hydrocarbons (PAH) (Wichmann, 2007, EPA, 2002). In the United States, the chemical composition of diesel exhaust from new heavy-duty diesel vehicles is 75 % elemental carbon, 19% organic carbon, 2% metals and elements, 1% sulphate and nitrate, and 3% unknown substances (EPA, 2002) (Fig 1.2).

The particulate fraction of DE or diesel exhaust particles (DEP) are also produced from incomplete combustion of diesel fuel. They contain an elemental carbon core with many adsorbed substances on the particle surface. DEP are within the PM2.5 size range and many in the PM 0.1 size category, with particles sizes between 50 and 80 nm in diameter (Huang et al., 2012). DEP can be attributable to 35 % of the PM2.5 concentration in certain urban environments however, in the United States, it is estimated to contribute to be ~ 10 % (based on mass). In Europe this is higher due to the increased use of diesel engine on-road vehicles, for example in Germany, it is estimated that 20 % of the mean PM 2.5 concentrations comes from on-road diesel vehicles (Wichmann, 2007).
Figure 1.2. Diagram of the main particulates found in diesel exhaust.

1.3.0. Air pollution and health

The detrimental effects of air pollution on human health has been recognized for decades, however the event that has received the most attention is the London smog of December 1952. London was engulfed in a SO$_2$ and particulate rich smog, that led to a dramatic rise in morbidity and mortality less than a day later; it is estimated that between 4,000-12,000 people died in the subsequent weeks and months (Schwartz, 1994, Bell et al., 2004). Similar incidents have been reported in Belgium (Firket, 1936) and the United States (H.H. Schrenk, 1949). Almost all deaths reported were from cardiovascular and respiratory diseases, and were notably higher in the elderly population.

Since then, a substantial amount of evidence from both cross-sectional and longitudinal studies has been collected, which supports the idea that there are health risks associated with both short and long term exposure to air pollution (Bell et al., 2013, Miller et al., 2007, Dockery et al., 1993). The World Health Organization (WHO) estimates that 3 million annual deaths can be attributed to air pollution (Ostro, 2004).

A large quantity of work has focused on the PM component of ambient air pollution; in particular the size ranges PM10, PM2.5 and PM0.1. PM2.5 has been specifically attributed to 800,000 premature annual deaths (WHO, 2002).

Most prospective cohort and time-series studies measure PM effects by calculating mortality rate ratios (RR) (ratios or percentages) or adjusted odds ratios (OR). The main morbidity outcomes investigated in relation to PM exposure have been; hospital admissions for cardiovascular and respiratory disease, physician and emergency room visits, incidents of lung cancer, exacerbations of respiratory conditions such as asthma and bronchitis. For investigations into specific cardiovascular conditions; blood biomarkers, incidents of cardiovascular events such as myocardial infarction (MI), parameters such as heart rate (HR) and heart rate variability (HRV) have been measured.

1.3.1. Particulate matter and mortality (short term)

The short-term effects of PM on human health have been predominantly investigated by measurement of daily changes in ambient pollution levels over an extended period of time.

One of the initial studies which paved the way for subsequent work was the Harvard six city study which followed a cohort of 8,111 patients for 16-18 years. The authors reported an increase in all-cause and cardiopulmonary mortality associated with PM2.5 and sulphate particulate pollution (Dockery et al., 1993). An extension and reanalysis of the original work further confirmed these findings (Schwartz et al., 1996). This study had advantages over previous work as their statistical analysis took into account confounding lifestyle factors such as smoking, BMI, alcohol consumption, education and occupational exposure variables. More recently, The Air Pollution and Health: a
Chapter One - Introduction

European Approach (APHEA) phase 1 (15 cities) and phase 2 (32 cities) projects found that increases in PM10 concentrations were associated with increases in all-cause mortality and death from cardiovascular disease and respiratory disease. Additionally, PM effects were more pronounced in cities with a larger proportion of elderly inhabitants, warmer climates and with higher NO2 concentrations (Katsouyanni et al., 1997, Katsouyanni et al., 1996, Katsouyanni et al., 2001). In a bid to collate data from multiple continents, the APHENA (Air Pollution and Health: A Combined European and North American Approach) study analyzed data from the APHEA 1 and 2 projects (32 cities), the US NMMAPS (National Morbidity, Mortality and Air Pollution Study) study (87 cities) and several Canadian studies (12 cities) using a common protocol, to establish whether there was consistency between each study’s findings. Additionally, the authors investigated how age and socioeconomic factors affected these associations (Peng et al., 2013). The APHENA study found that the results from the individual studies were reproducible and confirmed the main outcomes which was that there was a consistent association between PM10 or ozone and mortality (Samoli et al., 2008b).

Similar short terms results have been reported in both developed and developing cities around the world, such as London (UK) (Schwartz and Marcus, 1990), Temuco (Chile) (Díaz-Robles et al., 2014) and Beijing (China) (Zhang et al., 2013).

1.3.2. Long-term effects of air pollution and mortality

The long-term effects of air pollution on human survival have been generally investigated by calculation of an annual mean for individual air pollution components. The original Harvard six cities study found associations between long term PM exposure and mortality. Follow up studies were conducted to test the robustness of the original findings using additional covariants, and took into consideration the steady decline of PM2.5 concentrations in all 6 cities over the study period. These studies confirmed that increases in PM2.5 were associated with an increased overall risk of all-cause, cardiovascular and lung cancer mortality (Lepeule et al., 2012) (Laden et al., 2006).

Another important cohort study was The American Cancer Society (ACS) study which recruited volunteers from across 50 US states. The authors found an increase in total mortality and cardiopulmonary mortality for both men and women (Pope et al., 1995) (Pope et al., 2002) (Turner et al., 2011). Associations between long-term PM exposure and cardiopulmonary mortality are not exclusive to North America, as similar findings have been demonstrated in the Netherlands Cohort Study (NLCS-AIR) (Brunekreef et al., 2009).
Chapter One - Introduction

1.3.3. Particulate matter size and mortality

Most of the work previously mentioned has reported associations between mortality and PM10 or PM2.5. However, based on evidence from cohort studies, it appears that the 2.5 µm or 0.1 µm particles may be the most detrimental. For example, reanalysis of the Harvard six cities cohort found that mortality effects were more associated with PM2.5 than PM10 (Schwartz et al., 1996, EPA, 2002). The MED-PARTICLES project, conducted in 4 Mediterranean countries, found a positive association between PM10 and mortality however this was not significant, and was considerably more variable compared to the consistently significant associations between PM2.5 and mortality (Samoli et al., 2014, EPA, 2002).

Previously, it has been difficult to distinguish between mortality due to PM2.5 or PM0.1, as the majority of large cohort studies have not measured PM0.1 due to inadequate measurement technology. Many of these studies relied on reports of PM levels in the urban centers by the local monitoring stations, which in most cases only measured PM2.5 or PM10. In addition, PM0.1 is not homogeneously dispersed in the atmosphere because they have a tendency to concentrate around combustion sources such as large roads, making it difficult to obtain accurate measurements. Consequently, reports of associations between PM0.1 and mortality and hospital admissions have been inconsistent. An increase of PM0.1 was associated with an increase in total, cardiovascular and respiratory mortality in Germany and Finland. Further work during a 10.5 year period with declining PM levels, reported an increased RR with PM0.1 (Stölzel et al., 2007, Wichmann et al., 2000, Breitner et al., 2009, Kettunen et al., 2007). Similar positive associations with cardiovascular mortality or hospital visits and PM have been reported in Beijing (Breitner et al., 2009, Liu et al., 2013), Copenhagen (Andersen et al., 2010) and similarly for respiratory morbidity (Leitte et al., 2011).

Alternatively, Anderson et al, 2008 found that hospital admissions for cardiovascular and respiratory diseases were not associated with short-term exposure to PM0.1 (Anderson et al, 2008). Furthermore, the ULTRA study, which contained 3 panels of elderly subjects from the Netherlands, Germany and Finland reported that cardiorespiratory symptoms were constantly more associated with PM2.5 than PM0.1 (De Hartog et al, 2003). Therefore, further work is needed to elucidate the specific size of PM which is responsible for the associated mortality.

1.3.4. Particulate matter and respiratory disease

The primary route of exposure to ambient PM is inhalation via the lungs; therefore the lungs and respiratory system would be considered the primary areas to be affected. Increased mortality due to
respiratory conditions have been associated with PM exposure, for example the MED-PARTICLES project found deaths by chronic obstructive pulmonary disorder (COPD) increased following a rise in PM2.5 (Samoli et al., 2014). Long-term exposure to PM10, PM2.5 and traffic is significantly associated with increased risk and hospital visits due to pneumonia and with a decline in lung function (MacIntyre et al., 2014, Downs et al., 2007, Peel et al., 2005).

Links between asthma in adults and children and vehicle generated air pollution has received a lot of attention. One important prospective birth cohort study recruited mothers in their third trimester and monitored their children for a subsequent 8 years. This work found an association between PM2.5 and a significant increase in the incidence and prevalence of asthma (Gehring et al., 2010). Further work in California and Switzerland (SAPALDIA) with children and adults found that PM10 and PM2.5 exposure at home is associated with increased risk of new-onset asthma (McConnell et al 2010)(Künzli et al., 2009).

With regards to respiratory effects specifically due to PM0.1, Leitte et al, found PM0.1 to be adversely associated with emergency room visits for respiratory disorders and Belleudi et al found associations with hospitalisation for COPD and respiratory tract infections with PM0.1 (Leitte et al., 2011, Belleudi et al., 2010). Unsurprisingly, the risk from air pollution has been found to be higher in people with pre-existing conditions and low lung function (Laden et al., 2006).

1.3.5. Susceptible groups

Vulnerable groups within the population such as the elderly and infants, have been found to be particularly susceptible to the effects of PM. Additionally, sex has also been implicated in determining PM effects on health; however there have been inconsistent reports. The Harvard 6 cities study found male to be at higher risk from PM2.5 than females (Laden et al., 2006), while the ACS study found no differences in risk from PM2.5 between genders (Pope et al., 1995).

Age has also been consistently found to increase the risk from air pollution. The APHENA and multiple other projects have found that the effects of PM10 and PM0.1 on health were higher in the elderly (Samoli et al., 2008b) (Díaz-Robles et al., 2014) (Belleudi et al., 2010). Additionally, an education level less than high school has been reported to increase an individual’s risk from PM (Dockery et al., 1993, Brunekreef et al., 2009). Moreover, associations between the onset of nonfatal MI and traffic exposure was stronger in the unemployed than the employed (Peters et al., 2004) and the ATHENA project also found that the unemployed to be at higher risk from PM10 (Samoli et al., 2008a, Seaton et al., 1995). Individuals with preexisting morbidities such as cardiovascular disease and respiratory disorders such as asthma and COPD and with a high BMI have also been consistently reported to be at an increased risk from PM (Samoli et al., 2014, Jerrett et al., 2014).
Chapter One - Introduction

1.4.0. Particulate matter and cardiovascular disease

As previously discussed, increases in ambient PM levels are associated with increased mortality. Further work and analyses predominantly conducted by using hospital records has identified the cardiovascular conditions and the individual components of PM which are thought to underlie much of this mortality.

Reanalysis of the ACS data sets found that the increase in cardiopulmonary mortality associated with an increase of PM2.5 was due to ischemic heart disease, cardiac arrest, arrhythmia and heart failure (Pope et al., 2004). In a very recent study, the MED-PARTICLES project found an increase of PM2.5 was associated with increased cardiac deaths by 1.33% (Samoli et al., 2014). Furthermore, Dominici et al reported positive associations between day to day variation in PM2.5 and hospital admissions due to cerebrovascular events, heart failure, heart rhythm disturbances and ischemic heart disease (Dominici et al., 2006).

In 1995, Seaton et al suggested that PM related cardiovascular effects were specifically due to the PM0.1 fraction within PM10, and this has led to a focus on research into cardiovascular events associated with PM0.1 (Seaton et al., 1995).

1.4.1. Particulate matter and thrombotic events

Increasing evidence has been collected over the last decade linking transient increases in PM with thrombotic events. A study in London had initially reported associations between concentrations of five air pollutants and hospital admissions due to cardiovascular diseases the day after exposure. The most persistent association was found for acute MI and this was significantly associated with more than one pollutant (Poloniecki et al., 1997). Later work examined the effects of additional air pollution components such as PM. The case crossover study by Peters et al was one of the initial investigations which identified an associated risk of a thrombotic event with PM. They evaluated the risk of acute MIs following hourly changes in PM2.5 in the Boston area by reviewing admission logs and patients’ charts in local hospitals. The authors found significant positive associations between the onset of MI and the concentration of PM2.5 within the first 3 hours before the onset of symptoms (Peters et al., 2001a).

Another crossover study conducted in Germany assessed the association between the onset of nonfatal MI and traffic exposure. This study also focused on the effects of traffic exposure while travelling on different modes of transport. The authors reported associations between the onset of MI within one hour following exposure to traffic while travelling on public transport, in cars, buses and while riding on a motorcycle or bicycle (Peters et al., 2004). Interestingly, Andersen et al, found that PM0.1 levels
were positively associated with ischemic stroke (without AF) but not hemorrhagic stroke, and the authors speculated that the effects were probably due to thrombosis (Andersen et al., 2010).

1.4.2. Limitations to the epidemiological evidence

There are several limitations to the epidemiological work; most studies are unable to discriminate between PM0.1 and PM2.5 particle effects due to insufficiencies in PM0.1 measurements and monitoring. Additionally, variation in results between studies measuring similar parameters may be due to differences in statistical methods and confounding factors such as socioeconomic status. However, reanalysis of many large prospective cohort studies with more advanced statistical models such as Poisson models has confirmed the link between air pollution levels and adverse health effects and have also helped in distinguishing individual risk factors.

1.5.0. Mechanisms behind particulate matter effects on acute thrombotic events

Several mechanisms have been proposed for the cardiovascular events associated with ambient PM exposure; however three main hypotheses have been received the most attention. The first is that PM produces alterations in the autonomic nervous system (ANS) reflex arcs, leading to disturbances in the heart (arrhythmia, heart rate and heart rate variability) and the vasculature (vasoconstriction, endothelial dysfunction, blood pressure) (Figure 1.4). The second is that PM initiates pulmonary inflammation and subsequent release of pro-inflammatory mediators into the systemic inflammation which can promote synthesis of pro-coagulant hepatic proteins (acute-phase proteins), inflammatory cellular response (activated leukocytes and platelets), cytokine release (TNF-α, IL-6 and IL-1β) and release of vasoactive substances (histamine and ET) (Seaton et al., 1995). The third is that particles can translocate across the alveolar-blood barrier in the lung and enter the systemic circulation where they could directly interact with cells and organs (Seaton et al., 1995).

1.5.1. Particulate matter and systemic inflammation

The ability of various PM components to induce pulmonary inflammation is well established, however there is less consensus about whether this translates to a systemic inflammatory response.
One of the original cohort studies by Peters et al, found associations between PM exposure and blood levels of acute phase proteins such as C-reactive protein (CRP) in men (Peters et al., 2001b). Similarly, work conducted in London, Germany, Taiwan and Finland also reported a significant associations between PM and blood fibrinogen levels and CRP (Pekkanen et al., 2000, Peters et al., 1997, Chuang et al., 2007, Tsai et al., 2012). Positive associations between acute exposure to PM and the pro-inflammatory systemic cytokines TNF-α, IL-6 and IL-1β were found in studies conducted in Switzerland and South East Asia (Tsai et al., 2012, Ruckerl et al., 2006). Similar findings have also been reported specifically for PM0.1 in patients with coronary artery disease (Ruckerl et al., 2006). However, not all cohort or cross-sectional studies have found links between PM exposure and systemic inflammation. No association between PM2.5 and CRP levels were found in an Israeli study, The Multi-Ethnic Study of Atherosclerosis (MESA) (Diez Roux et al., 2006, Steinvil et al., 2008) or in a population of elderly individuals (Sullivan et al., 2007).

Clinical exposure studies have helped in shedding light on the effect of PM exposure on systemic inflammation however there is still much contention between studies. One of the initial human studies exposed healthy subjects to Diesel Exhaust for 1 hr and found significant increases in systemic neutrophils, platelets and lymphocytes (Salvi et al., 1999). Additionally, exposure of healthy subjects to DE has been reported to cause systemic increases in TNF-α, IL-6 levels and total leukocytes (Tornqvist et al., 2007, Brook et al., 2009). Similarly, total leukocyte and neutrophil counts increased immediately after a 2-hour exposure to concentrated ambient particles (CAPs) in healthy subjects (Brook et al., 2009).

Conversely, a lack of inflammatory response has been observed following exposure of asthmatic and healthy subjects to UF carbon particles (Xu et al., 2013). No changes in systemic inflammatory markers were detected following exposure of subjects to DE and CAPs for 1 hr or 2 hrs with intermittent exercise (Mills et al., 2005, Mills et al., 2008, Mills et al., 2007).

Animal studies have shed more light on PM-associated systemic inflammation, particularly the role of individual PM components, such as PM0.1. For example, intratracheal (i.t.) instillation of DEP into rats caused significant increases in systemic neutrophils 12 hrs and 24 hrs post i.t. instillation (Yokota et al., 2005) and a single 7 hr exposure of rats to UF CB caused increases in systemic neutrophils which interestingly were not observed following exposure to fine CB (> 100 nm) (Gilmour et al., 2004).

The hypothesis that PM mediated systemic inflammation originates from pulmonary sources has been equally inconclusive. Due to the technical and ethical issues regarding measurement of pulmonary inflammation in humans there have only been a few studies conducted, however both pulmonary and systemic inflammation has been reported in human subjects following exposure to DE (Salvi et al., 1999).
In animals, *i.t.* instillation of rats with DEP caused increases in plasma IL-6 and TNF-α 24 hrs post-exposure however, this was prior to a pulmonary inflammatory response 6 hrs post-exposure which was not evident at 24hrs, suggesting the systemic inflammation had a pulmonary origin (Robertson et al., 2012). Alternatively, *i.t.* instillation of CB, DEP and UPM into rats caused significant increases in pulmonary inflammation yet this was not associated with any alterations in plasma IL-6, TNFα or leukocytes (Kim et al., 2012, Emmerechts et al., 2010). Interestingly, systemic infusion of DEP enhanced circulating granulocytes and monocytes after 48 hrs, suggesting that carbonaceous NPs may have the potential to induce an inflammatory response should they translocate through the lung and enter the blood (Nemmar and Inuwa, 2008).

### 1.5.2. Pulmonary inflammation and particulate matter

Initiation of pulmonary inflammation and the subsequent “systemic spill-over” has been suggested as a mechanism behind the thrombotic events associated with PM. Due to the difficulty in measuring markers of pulmonary inflammation in humans, this hypothesis has been mainly supported by *in-vivo* and *in-vitro* studies. A few human studies have been conducted with contradictory findings. Exposure of healthy volunteers to DEP, DE and CAPs has been reported to cause increases in pulmonary neutrophils, B-lymphocytes, expression of adhesion factors, histamine and MPO levels (Salvi et al., 1999, Ghio et al., 2000). Conversely, exposure of asthmatic and healthy subjects to UF carbon particles and CAPs did not induce changes in airway inflammation (Pietropaoli et al., 2004, Gong et al., 2003).

The results from animal studies have been more consistent, probably due to the increased control over the experimental conditions. The majority of studies to date have investigated the effects of acute exposure following both *i.t.* administration and inhalation of rodents to various components of PM, particularly DEP, CAPs and CB. One of the original studies conducted by Nemmar et al, reported that *i.t.* instillation of DEP into hamsters induced increases in pulmonary polymononuclear cells (PMN), protein concentrations and histamine concentrations in bronchoalveolar lavage fluid (BALF) 1 hr post exposure (Nemmar et al., 2003a, Nemmar et al., 2001). This work has been extended by multiple authors by measurement of cytokine protein and mRNA levels in BALF. Elevations of MIP-2, TNFα IL-6, KC protein levels were detected in BALF following exposure to DEP, UF CB, UFP in addition to increases in macrophages, lymphocytes and neutrophils (Yokota et al., 2005, Gilmour et al., 2004, Emmerechts et al., 2010, Robertson et al., 2012, Kim et al., 2012).

PM induced pulmonary inflammation is thought to involve alveolar macrophages, as they are the primary effector cell in phagocytosis and clearance of inhaled particles. Stimulation of human and rat alveolar macrophages with diesel dust (DD), urban air dust, residual oil fly ash (ROFA) and ambient air particles (UAP) has been reported to cause significant IL-6 and TNF-α release (Becker et al., 1996, van Eeden et al., 2001).
Although PM related pulmonary inflammation is suggested to involve alveolar macrophages, exposure of a variety of cell types to numerous components of PM has indicated that most of these particles can cause cells to release pro-inflammatory cytokines and other inflammatory mediators. There has been extensive work conducted on airway epithelial cells that demonstrates the release of pro-inflammatory cytokines, reactive oxygen species (ROS) generation and cytotoxicity following exposure to PM (Becker et al., 2005, Ovrevik et al., 2011).

One of the main limitations of many of these studies is the concentrations of particles used, as many of the biological effects are often observed at high concentrations. This is partially due to the difficulty in estimating PM which an individual may be exposed to during an acute period of high ambient PM levels. Additionally, accounting for long-term exposure and a higher baseline of PM levels, such as that found in urban populations, is a challenge.

1.5.3. PM0.1 particle translocation

An alternative mechanism behind the effects of PM on the cardiovascular system is that these particles can translocate across the alveolar-epithelial barrier and enter the systemic circulation where they can directly interact with blood cells and the vasculature. This theory is supported by work in animals and humans, although limitations in the techniques used to detect particles in the blood have led to several inconclusive findings. Particles < 100 nm can be deposited within the alveolar regions of the lower airways and therefore are in close proximity to the underlying pulmonary vasculature (ICRP, 1994, Seaton et al., 1995) (Fig 1.3).

The translocation of UF particles was initially demonstrated in a hamster model, where radiolabeled nanocolloid albumin particles were introduced into the lung via i.t. instillation, and radiation was detected in the circulation 5 minutes after administration (Nemmar et al., 2001). This study was subsequently followed by work in humans where healthy subjects inhaled \(^{99m}\text{Tc}\)-labeled UF carbon and radioactivity was detected in the blood, liver and kidney 1 minute after inhalation. The authors estimated that ~8% of the UF particles had entered the circulation (Nemmar et al., 2002a). However, Brown et al, exposed healthy subjects and COPD patients to a similar technetium-\(^{99m}\text{Tc}\)-labeled UF carbon aerosol and found no traces of particles in the liver 2 hours post-exposure. The authors speculated that the results from Nemmar et al could have been due to pertechnegas contamination (Brown et al., 2002). This concern surrounding the dissolution of these particles and the leaching of the radiotracer from the particles into the surrounding tissues led to further studies with insoluble particles.

Rats exposed to insoluble \(^{13}\text{C}\) UF particles and \(^{192}\text{Ir}\) particles had these tracers present in the liver, lymph nodes and the blood following exposure (Oberdorster et al., 2002), although <1% of the total...
fraction of \(^{192}\text{Ir}\) particles appeared to enter the blood (Kreyling et al., 2002). In a similar study, exposure of rats to \(^{192}\text{Ir}\) particles of different sizes and compositions resulted in the presence of particles in extrapulmonary locations. In addition, an inverse particle-size-dependent transport into the circulation following pulmonary exposure was also reported (Kreyling et al., 2002, Kreyling et al., 2009).

Work using inorganic/organic hybrid nanoparticles (INPs) found that nanoparticles (NPs) translocate through the pulmonary epithelium into the septal interstitium, the regional draining lymph nodes and into the circulation (Choi et al., 2010). Methodological differences between studies may account for the discrepancies between their results. For example, the route of exposure is a major source of differentiation as rodents are obligatory nose breathers and humans are oral breathers. It is possible that many of the particles would be deposited in the nasopharyngeal area in inhalation studies whereas \textit{i.t.} administration ensures that particles are deposited into the lower conducting and distal airways (Schlesinger and Ultman, 1997). However interestingly, there is evidence to suggest that particles deposited in the nasopharyngeal region may translocate to areas of the CNS such as the olfactory bulb. Significant accumulation of \(^{13}\text{C}\) was detected in the olfactory bulb of rats following inhalation (Oberdörster et al., 2004).

Furthermore, one of the primary clearance pathways for particles inhaled into the lung is via mucociliary clearance into the stomach. There is increasing evidence regarding the uptake of UF particles/NPs by the gut and translocation into the blood. In rats, \text{TiO}_2\text{-NPs} and \text{Zn} particles have been detected in the regular ileum epithelium, the follicle-associated epithelium (FAE) and the blood following oral administration (Brun et al., 2014, Cho et al., 2013).

There appears to be substantial evidence that NPs can gain access to the systemic circulation following various routes of administration however, the concentration of NP that enters the blood and the ability of that concentration to cause adverse health effects is still uncertain.
Figure 1.3. Relationship between particle size and percentage deposition with the different regions of the lung.

A representative trace demonstrating percentage deposition of spherical particles in different anatomical locations of the lung in relation to their aerodynamic diameter (adapted from the annals of American Conference of Governmental Hygienists, Vol 11) (ICRP, 1994).
1.5.4. Particulate matter and oxidative stress

Oxidative stress has been implicated as the underlying cellular mechanism responsible for PM related health effects (Mills et al., 2009). Oxidative stress is considered to be a state where there is an elevation of free radical (ONOO\(^-\), H\(_2\)O\(_2\), O\(_2\)\(^-\)) levels compared to normal, which are maintained by endogenous antioxidants such as superoxide dismutase (SOD) and glutathione. Free radicals can interact with many biomolecules disrupting their function (oxidation of DNA, proteins and lipids) (Krötz et al., 2004).

Investigating the role of oxidative stress as the mechanism behind PM mediated biological effects has been difficult for numerous reasons. Firstly, oxidative stress can be difficult to detect as it may be localised to the tissue/cellular level and may not alter systemic biomarkers. In addition, oxidative stress and inflammation can mutually initiate each other which makes differentiation between the individual processes difficult.

Studies in young adults and a population of male truck workers reported that increases in the DNA oxidation biomarker 8-hydroxy-2\(^'\)-deoxyguanosine (8-OHdG), in plasma and urine, were associated with increased PM\(_{2.5}\) and PM\(_{10}\) levels (Neophytou et al., 2013).

Additionally, exposure to UFPs for 6 and 24 hours in a randomized crossover study found that these particles were associated with DNA strand breaks (SBs) and oxidized purines in peripheral blood mononuclear cells (PBMCs). These effects were strongest with the 57 nm particles derived from vehicle emissions (Brauner et al., 2007). 8-isoprostane is a marker of oxidized phospholipids and has been found in the lung condensates of healthy volunteers 6 and 24 hrs following exposure to CAPs (Mills et al., 2008). Furthermore, increased antioxidant gene expression was also reported in the liver of APOe\(^{-/-}\) mice chronically exposed to DEP (Miller et al., 2013).

Numerous in-vitro studies have identified several ROS-generating signaling pathways and molecules, mostly in the lung, which are initiated or up regulated upon PM exposure. NADPH-oxidase, mitochondrial sources, cytochrome-P450 and eNOS have all been implicated in PM mediated ROS generation (Li et al., 2006, Shukla et al., 2000, Han et al., 2001, Bai et al., 2001, Upadhyay et al., 2003).

1.5.5. Particulate matter, vascular dysfunction and NO bioavailability

Vascular dysfunction occurs when there is an imbalance between the release of vasodilating and vasoconstricting paracrine mediators by the endothelium and this can lead to thrombotic and inflammatory events (Halcox et al., 2002). Epidemiological evidence and clinical studies suggest that PM is associated with vascular dysfunction and this may be the mechanism underling PM related thrombotic events. Randomized control studies found that men exposed to diluted DE or polluted air displayed vasodilator impairment which was due to a reduction in NO signaling (Mills et al., 2005,
Brauner et al., 2007, Brook et al., 2002, Wauters et al., 2013). Similar results have been observed in other studies however importantly; the effects of DE and CAPs on vasodilation responses were abolished following filtration of the particulates (Mills et al., 2005, Brauner et al., 2007, Brook et al., 2002, Brauner et al., 2008). These studies implicate the particulate fraction of DE as the main component responsible for the effects on cardiovascular health. Furthermore, these effects do not appear to be exclusive to all particles, as exposure to carbon NPs has been demonstrated not to attenuate vascular vasodilator responses (Mills et al., 2011).

Additionally, the importance of particle composition and the role of combustion-derived particles was highlighted in a study by Mills et al, which found no changes in vascular function in volunteers with stable coronary artery disease following exposure to CAPs low in combustion-derived particles, compared to filtered air (Mills et al., 2008).

The vascular dysfunction associated with exposure to PM has been reported to be due to reduced NO bioavailability. ROS generation, mainly superoxide, has been suggested as the main mechanism behind the reduced NO bioavailability. This is because superoxide can bind to NO to produce peroxynitrite, thus sequestering it from the blood (Krötz et al., 2004). This hypothesis is supported by work *in-vitro* and *in-vivo* which found that impaired vaso-relaxation/constriction and attenuated NO production following exposure to DEP, PM2.5 and nano-TiO2 which was abolished by pretreatment with superoxide dismutase (SOD), NAC and a NADPH oxidase inhibitor (Ikeda et al., 1995, Sun et al., 2008, Nurkiewicz et al., 2009).

Moreover, work from clinical studies also support the theory the PM exposure can reduce NO bioavailability. Inhalation of UF carbon particles and DE by healthy subjects caused significant alternations in venous and plasma nitrate concentrations, which is a surrogate marker of blood NO concentrations, and blunted vaso-dilation/constriction in response to ischemia and systemic NOS inhibition (Shah et al., 2008, Langrish et al., 2013, Wauters et al., 2013). *In-vitro* experiments with HUVECs implicated superoxide generation by endothelial cells to be the primary cause of the disruption to NO signaling (Wauters et al., 2013).

An additional mechanism behind the reduced NO bioavailability which is associated with DE and DEP exposure is the uncoupling of eNOS. This mechanism was proposed following work where venous constriction was enhanced following exposure to DE but not in the presence of the eNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) (Knuckles et al., 2008). Furthermore, it has been proposed that the mechanism behind this DEP-mediated uncoupling was depletion of the NOS cofactor tetrahydrobiopterin (BH4) (Sun et al., 2008, Kahn et al., 1998).
Figure 1.4. Potential mechanisms behind the effects of particulate matter on the cardiovascular system.

Inhalation of PM has been proposed to cause cardiovascular events by interfering with the autonomic nervous system (1), inducing pulmonary and systemic inflammation (2) or by-passing the alveolar-blood barrier and entering the systemic circulation where they can interact with hematological components or the endothelium; contributing to atherogenesis, endothelial disruption and/or thrombosis (3).
1.5.6. PM0.1 responsible for thrombotic events

PM0.1 or UF particles are largely of a carbonaceous composition and are predominantly generated from traffic emissions, in particular diesel engines (Murrells T P, 2011). Work investigating PM-mediated thrombotic events has utilized both carbonaceous UF particles such as DEP or CB and more complex heterogeneous PM suspensions with a range of particle sizes. PM0.1 which have been implicated as being the fraction of PM primarily responsible for driving these thrombotic events for numerous reasons; PM0.1 contributes relatively little to the total mass of ambient PM however there are a higher number of PM0.1 particles per volume of air than fine and coarse particles. The number of PM0.1 has been demonstrated to be associated with adverse health effects (Peters et al., 1997, Krzyzanowski M, 2005) and the importance of particle number and size in inducing inflammatory and toxic effects has been observed for particles such as CB (Stoeger et al., 2006).

UF particles also have a high surface area per mass compared to larger fine and coarse particles present in air pollution (Donaldson et al., 2001). This means they have a larger surface area for toxic compounds to be adsorbed onto and they have a larger surface area to interact with the surrounding cellular milieu. In addition, several studies have reported toxicological effects associated with particle surface area rather than particle mass therefore, smaller particles would be inherently more damaging to human health due to their large surface area to volume ratio (Oberdörster et al., 1994, Sun et al., 2008).

Furthermore, PM0.1 exist as either single particles or aggregates and these particles are capable of being deposited deep within the conducting and peripheral airways (ICRP, 1994, Yeh et al., 1997). The alveoli area is primarily cleared of inhaled particles by the alveolar macrophages however UF particles are reported by several groups to evade this clearance system by disrupting macrophage phagocytosis (Renwick et al., 2004, Moller et al., 2005). This disrupted clearance means that UF particles can accumulate and interact with the alveolar epithelial cells, potentially initiating pathophysiological processes such as inflammation or translocate through the epithelial cells to extra pulmonary locations (Ferin et al., 1992b).
1.5.7. Particulate matter exposure on experimental thrombosis and platelet function \textit{in-vivo} and \textit{ex-vivo}.

As previously discussed, platelets play an integral role in thrombus generation and have therefore become a focus of research investigating the risks and mechanisms behind PM-related cardiovascular events. Most work has involved \textit{ex-vivo} human studies and \textit{in-vivo} animal studies; however one cohort study in Germany reported an immediate increase in soluble CD40L levels and platelet activation in patients with coronary artery disease following acute rises in PM0.1 (Rückerl et al., 2007).

The earliest experimental study implicating platelets as effector cells was by Berry \textit{et al.} who \textit{i.t.} dosed rats with 30 nm gold NPs and detected them in the platelets of the pulmonary capillaries (Berry \textit{et al.}, 1977). Rudez \textit{et al}, measured platelet aggregation \textit{ex-vivo} in 40 healthy volunteers and found an association between PM10 exposure and platelet aggregation (Rudez \textit{et al.}, 2009). Soluble P-selectin, sCD40L levels and leukocyte-platelet aggregates were altered in men after inhalation exposure to DE and UFP. DE exposure was also associated with increased \textit{ex-vivo} thrombus formation, suggesting that there was increased platelet activation (Stewart \textit{et al.}, 2010, Lucking \textit{et al.}, 2008).

Animal studies have helped clarify the effects of PM on experimental thrombosis \textit{in-vivo}. \textit{I.t.} instillation of UPM and DEP caused an increase in arterial and venous thrombus formation \textit{in-vivo} 10 minutes and 4hrs post-exposure (Nemmar \textit{et al.}, 2003a, Lucking \textit{et al.}, 2008, Emmerechts \textit{et al.}, 2010). \textit{I.t.} instillation of ambient PM from Dusseldorf into mice caused shortened bleeding times, reduced platelets counts and increased thrombus formation \textit{in-vivo} (Mutlu \textit{et al.}, 2006). Furthermore, \textit{i.t.} instillation of UF CB and DEP into mice has also been demonstrated to cause significant reductions in closure times (measured by PFA-100) \textit{ex-vivo} (Kim \textit{et al.}, 2012, Nemmar \textit{et al.}, 2003a). Therefore, exposure to PM and its components appear to promote thrombus generation and this may involve platelet activation.

Currently there is limited information regarding the effects of chronic exposure on thrombosis however, mice chronically exposed to CAPs demonstrated increased P-selectin expression (Wilson \textit{et al.}, 2010).

1.5.8. The effects of PM0.1 on experimental thrombosis and platelet function \textit{in-vitro}.

Platelet aggregation is an integral process in thrombosis and it has been suggested that combustion derived NPs could modulate platelet behaviour and therefore may be the underlying mechanism behind the PM-mediated thrombotic events. Most work investigating this hypothesis has involved \textit{in-}
vivo vascular injury models, which initiate multiple processes such as activation of both platelets and the coagulation pathways; therefore it has been difficult to identify the role of the individual components. Consequently, researchers have investigated the effects of carbonaceous NP/PM suspensions on platelet aggregation in-vitro. In one study, a variety of UF carbon particles including a suspension of urban PM standard reference material was demonstrated to cause concentration dependent platelet aggregation. Interestingly, this platelet aggregation was mediated by the integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$ however; there was variation between the involvement of ADP, PKC signaling and granule release in particle-induced aggregation (Radomski et al., 2005). Similarly, silica-based NPs, CB and MWCNT were reported to induce $\alpha_{\text{IIb}}\beta_{\text{III}}$-mediated platelet aggregation which appeared to involve the release of secondary agonists and activation of platelet signaling molecules such as PKC. Moreover, subthreshold concentrations of these UF particles were able to cause platelet aggregation when administered with subthreshold concentrations of the platelet agonists ADP and the TP receptor agonist U46619 (Guidetti et al., 2012). DEP also caused concentration dependent platelet aggregation and enhanced agonist induced platelet aggregation at lower concentrations than previously reported, however no effects were observed for CB (Solomon et al., 2013). Therefore, these studies support the in-vivo work previously discussed (section 1.5.7) as it appears that PM0.1 can influence platelet behaviour and this may involve the activation of platelet signaling events.

1.6.0. Nanotechnology, nanoparticles and their applications

The increasing interest in the negative effects of combustion-derived NPs on human health (described in 1.5.0) has coincided with the development of engineered nanostructures intended to benefit human health. Therefore, it is important to establish the health risks associated with nanotechnology. Nanotechnology involves the development, characterization and application of molecular structures which have at least one dimension that is ≤ 100 nm. The interest surrounding nanostructures comes from the unique properties of certain materials when they are at nano-scale size which differs from the larger counterpart. The difference in properties is firstly due to quantum effects that start to dominate the material at nano-size and this affects the conducting, electrical, optical, strength and magnetic behaviour of the material. In addition, nanostructures having a large surface area per mass unit compared to larger constructs of similar composition. Due to these properties, nanostructures have a wide range of applications including electronics, paints, optics and bionanotechnology (Dowling, 2004). Nanostructures come in a variety of shapes including spheres, rods and tubes however one nanostructure which has received a lot of attention are carbon nanotubes (CNTs). These are formed from roles of graphene sheets, nanometers in diameter but can be centimeters in length and multi-
walled or single-walled (Figure 1.5). CNTs are mechanically very strong, flexible and are exceptionally good at conducting electricity, which makes them very attractive for reinforced composites, sensors, nanoelectrics and display devices.

Other nanostructures of interest are Fullerenes (carbon60) which are spherical structures 1 nm in diameters and as their name denotes, have 60 carbon atoms arranged in a spherical structure. These have applications in lubrications and paints.

Other well-known nanostructures are dendrimers which are spherical polymers undergoing development for drug delivery and gene therapy systems (Kukowska-Latallo et al., 1996, D’Emanuele et al., 2004). Quantum dots are other nanostructures which have received attention due to the fact that they are nanosized semiconductors, which mean they can emit or absorb specific wavelengths of light depending on their size. Quantum dots are being developed for various applications such as solar cells and biological labeling (Jung and Chu, 2014).

Perhaps one of the most well-known nanostructures is the nanoparticle (NP), which is a spherical particle ≤ 100 nm in aerodynamic diameter. These particles are generated through both man-made and natural combustion processes such volcanic activity and combustion engines. Moreover, NPs are found in a many consumer products, for example zinc oxide and TiO2 are present in sun creams, as they can absorb UV light. Humans also ingest NPs, such as carbon black and TiO2, as they are present in cosmetics such as lipstick, and in processed foods such as chocolate (Dowling, 2004).
Chapter One - Introduction

Figure 1.5. Types of common engineered nano-structures

Common nanostructures which are present in commercial products or have applications in nanomedicine. All the structures have at least one dimension which is ≤ 100 nm in diameter however structures such as carbon nanotubes can be µm or m in length. Nanostructures can be synthesized from a variety of materials including biomolecules (liposomes), graphene (carbon nanotubes) and elements (gold).
1.6.1. Nanomedicine

One of the most exciting applications of NPs is their use in nanomedicine. Many endogenous molecular structures found in cells and tissues such as proteins are nanometers in size, hence there is huge potential to manipulate these systems using engineered constructs. Nano-sized structures are useful in the medical field as they can allow controlled release of drugs, target agents to specific tissues, reduce off-target side effects and evade endogenous clearance pathways which limit drug bioavailability.

Quantum dots and dendrimers both have applications in nanomedicine however elemental NPs such as silver and gold are proving to be extremely effective due to their inherent material properties. For example, gold NPs are relatively inert however they resonate when exposed to specific frequencies which can be advantageous when the NPs are accumulated in a solid tumor. Similarly, colloidal silver NPs have anti-microbial properties and are therefore infused into wound dressings, clothing and are even sold as nasal sprays for prophylactic treatment of colds and influenza (Dowling, 2004).

NPs can also be synthesized from biological materials to increase biological compatibility. Liposomes are spherical NPs composed of a phospholipid bilayer encapsulating an aqueous solution. Liposomes have proven to be successful drug-carriers as they can transport both hydrophilic and hydrophobic substances, which can either passively diffuse into cells, or the lipid bilayer can fuse with the plasma membrane of target cells. Furthermore, liposomes have been modified so they can be taken up by macrophage phagocytosis where they are dissolved, releasing their drug contents (Thorley and Tetley, 2013).

Oncology is one medical field which nano-systems and liposomes in particular have been very beneficial, mainly due to the unique vascular characteristics of certain tumors. Tumor vasculature is often unstructured and leaky, with fenestrations in the endothelial layer; this phenomenon is known as enhanced permeability and retention (EPR). NP-therapeutics administered to the systemic circulation can passively accumulate in the tumour but not in healthy tissue, limiting the side effects and increasing the drug bioavailability (Gabizon et al., 1994, Matsumura and Maeda, 1986). There are already two chemotherapy agents on the market which have been approved by the FDA: Abraxane® and Doxil®. Abraxane® is an albumin-bound paclitaxel NP ~130 nm in diameter. Abraxane® is prescribed for breast cancer as it has reduced side effects compared to conventional unbound paclitaxel and has been demonstrated to be more efficacious (Gradishar et al., 2005). Similarly, Doxil®, the liposomal encapsulated chemotherapy agent Doxorubicin, is also on the market. It is prescribed for ovarian cancer and has significantly less cardiotoxicity and increased daily compliance compared to standard care (Gabizon et al., 1994).
NPs are also being adapted for applications in bioimaging and diagnostics. For example, the MRI contrast agent gadolinium (Ge) has been conjugated onto iron oxide NPs (Babes et al., 1999) and internalised into single-walled carbon nanotubes (SWNTs) for tracking stem cells in-vivo (Tran et al., 2010).

### 1.6.2. Nanoparticle surface functionalization

The physical and chemical characteristics of NPs are incredibly diverse and these properties will vary dramatically depending on their application. One of the main reasons for the functionalization of the surface of nanostructures with charged surface groups or polymers is to prevent a major NP limitation, which is the adsorption of proteins into the NP surface, which can destabilise the particles in a suspension and cause aggregation (Lundqvist et al., 2008). Additionally, these proteins can promote NP opsonisation and clearance by the immune system, thus reducing circulation times (Chonn et al., 1992, Chonn et al., 1995). However, surface charges also are essential in dictating NP-cell interactions and depending on the NP function, can be exploited by researchers. For example, negatively and neutral charged particles can bind to complement proteins present in the blood and promote opsonisation into the RES. This phenomenon is under development for treatment of inflammatory diseases which are mediated by certain subtypes of macrophages, as uptake of highly negatively charged NPs by these macrophages causes apoptosis and sequestration into the spleen (Getts et al., 2014, Lundqvist et al., 2008).

Alternatively, many NPs are being developed with positive charges for drug delivery, as this allows the NP surface to interact with the anionic phospholipids present in the cell plasma membrane and promotes cellular uptake. Cationic polymers have also been exploited for gene transfection as they can condense nucleic acids into nano-sized packages and the cationic charge helps mask the negatively charged DNA (Anderson et al., 2005). Many cationic NPs have amine groups added to their surface so that when they are internalised into endosomes and lysosomes they can accept protons and therefore escape the endo-lysosomal degradation. This avoids the destabilisation of the nucleic acids, so they can be released into the cytosol (Panyam et al., 2002).

Many research groups have demonstrated the ability of NPs to be internalised into various cell types through both passive and energy-dependent mechanisms, however, this process appears to be dependent on surface charge. Internalisation of cationic polymers into vascular smooth muscle cells has been reported to be through clathrin-mediated endocytosis but appears not to involve caveolin (Panyam et al., 2002, Lorenz et al., 2006). Interestingly, work in immortalised alveolar type 1 cells found that a higher quantity of negatively charged 50 nm polystyrene NPs were internalised compared to 50 nm cationic NPs. This process also did not involve calveolin-mediated endocytosis but at least 50 to 70% of the total NP uptake was due to passive mechanisms (Kemp et al., 2008, He et al., 2010).
Chapter One - Introduction

One of the most well-known surface functionalizations is the hydrophilic polymer Polyethylene glycol (PEG). PEG makes nanostructures less bioreactive and more biocompatible by preventing protein opsonisation and NP agglomeration through steric hindrance (Bazile et al., 1995). This polymer has been applied to numerous nanostructures including therapeutics such as Doxil® (Gabizon et al., 1994).

Adsorption of plasma proteins such as albumin onto the surface of NPs has also been exploited for drug delivery systems. Albumin is a physiological transporter of biomolecules across the endothelial barrier via caveolin-mediated transcytosis. Its effectiveness is illustrated by Abraxane®, the albumin-bound paclitaxel (Nyman et al., 2005).

However, as certain NPs are taken up into the RES, there is concern regarding the potential accumulation of NPs in the RES organs. Development of biodegradable NPs and NPs which can be eliminated through renal filtration have helped alleviating this problem (Leroux et al., 1996, Choi et al., 2007).

1.6.3. Nanoparticles size

Other NP physicochemical properties also dictate their bioreactivity and the mechanisms involved. In particular, NP size is extremely important and varies depending on the application. Protein adsorption has been demonstrated to be dependent on NP size as well as surface hydrophobicity. For example, one study demonstrated that more albumin was adsorbed onto 200 nm NPs compared to 70 nm particles of similar composition. The authors’ explanation for this was that the curvature of the NP was greater for smaller particles and therefore prevents significant adsorption of larger proteins (Lindman et al., 2007). This has obvious consequences for circulation times and clearance by the RES, as NP size will influence adsorption of complement proteins. In addition, drugs that are targeted to tumours must be ideally < 100 nm so they can pass through the fenestrated vasculature (Roberts and Palade, 1997, Choi et al., 2007).

Furthermore, the ability and mechanisms by which NPs are internalised into cells is dictated by NP size. In general, based on internalisation of biological structures such as viruses and on cellular uptake studies, particles > 500 nm are internalised by phagocytosis whereas smaller particles are internalised via pinocytosis (Choi et al., 2010, He et al., 2010) (Roberts and Palade, 1997) (Rejman et al., 2004). NPs < 150 nm enter cells in an energy dependent process via clathrin-dependent endocytosis and 50 – 80 nm sized particles are reported to enter via a caveolae-mediated endocytosis although the exact mechanisms are still highly debated (Pelkmans et al., 2001, He et al., 2010, YAMADA, 1955). However, NPs ~ 50 nm have been demonstrated to be internalised via passive mechanisms (Kemp et al., 2008). Once again this has implications on circulation times and on targeted delivery of agents.
One final consideration on the impact of NP size is that the glomerulus pores of the nephron are ~ 6 nm in diameter, therefore NPs < 5.5 nm may be excreted through renal filtration but larger NPs will be excluded. This exclusion can be avoided by the addition of zwitterionic or neutral organic coatings as these can prevent protein adsorption which can increase the diameter of the particle. Therefore, this NP functionalization promotes efficient clearance and excretion of the particles, preventing unwanted biological accumulation (Choi et al., 2007).

1.7.0. Nanotoxicology

1.7.1. Nanoparticle physicochemistry and toxicity

As previously discussed, NPs can have their surfaces functionalised and this along with other parameters can convey different biological reactivity, depending on their application. However, these same parameters may promote undesirable interactions and consequences. There is particular growing concern regarding positively charged nanostructures, as these have been shown to be more cytotoxic in many cell types compared to negatively charged and neutral particles. Exposure of alveolar type 1 epithelial cells to cationic polystyrene NPs caused significant release of lactate dehydrogenase (LDH) and reduced mitochondrial activity, suggesting they caused membrane damage and a reduction in cell viability. This was thought to be due to the interaction between the NPs and the anionic phospholipids in the plasma membrane (Ruenraroengsak et al., 2011). Amine-modified Si and Ge NPs caused reduced cell viability, ROS generation, intracellular Ca\(^{2+}\) increases, mitochondrial disruption and initiation of apoptosis in macrophages and human colonic adenocarcinoma Caco-2 cells (Bhattacharjee et al., 2013). These effects were not observed for neutral or anionic particles. Furthermore, cationic NPs have been shown to cause more pulmonary inflammation compared to neutral and anionic particles (Nemmar et al., 2003b). Cytotoxicity has also been reported for anionic particles however this has only been reported by a few authors. For example, 20 nm and 200 nm anionic polystyrene NPs caused dose dependent decreases in cell viability and lysosome enzymatic activity in endothelial cells (Frohlich et al., 2012). Anionic NPs have also been implicated in initiating the intrinsic coagulation pathway by binding the coagulation factor FXII. Interestingly, 220 nm carboxyl-modified NPs but not 24 nm carboxyl-modified NPs had the ability to initiate this pathway. This was suggested to be because the larger particles had enough space for the assembly of the multi-molecular complexes of the intrinsic pathway (Oslakovic et al., 2011).

The hypothesis that NP size also dictates a NPs ability to cause detrimental health effects is based on experimental data using engineered and combustion-derived NPs. Numerous studies have demonstrated that smaller NPs can induce greater toxicity than larger particles. For example, UF TiO\(_2\) and polystyrene particles produced significantly more pulmonary inflammation and LDH release than
fine particles. The authors demonstrated that the inflammation was directly proportional to the surface area of the particles (Ferin et al., 1992a, Nemmar et al., 2003b, Brown et al., 2001). Furthermore, Li et al demonstrated that UF particles collected from Los Angeles were more potent at inducing oxidative stress in macrophages and bronchial epithelial cell lines than fine or coarse PM (Li et al., 2003).

1.7.2. The effects of engineered nanoparticles on platelet function *in-vitro*

The administration of nano-sized structures into the systemic circulation means that blood components such as platelets may come into direct contact with these engineered NPs. NP interactions with platelets are of particular interest, as activation of platelets may lead to thrombus generation and thrombotic events such as MI and cerebral infarctions. Similar to the work conducted with combustion-derived NPs, most studies investigating the effects of NPs on platelets have used vascular injury models, which initiate both platelet activation and the coagulation pathways, or have used *ex-vivo* approaches.

Nano-silver (Ag-NPs) induced concentration dependent platelet aggregation and significantly enhanced thrombin-induced platelet aggregation in isolated human platelet suspensions. Ag-NPs induced and enhanced typical platelet activation events such as surface expression of phosphatidylserine and P-selectin, release of the secondary agonist 5-HT and caused a significant rise in intracellular Ca\(^{2+}\). However these events were only observed following incubation with the highest concentrations. *i.t.* administration of Ag-NPs into rats caused a significant increase in platelet aggregation *ex-vivo* and *i.v.* administration caused a significant increase in venous thrombosis *in-vivo*; both effects were only observed at the highest concentrations (Jun et al., 2011).

Attempts have been made at addressing the role of NP surface chemistry on platelet function and experimental thrombosis. Systemic administration of negatively charged 60 nm carboxyl-modified (CM) polystyrene NPs caused a significant decrease in experimental thrombosis in a hamster model. Conversely, administration of 60 nm amine-modified (AM) polystyrene NPs caused a significant increase in experimental thrombosis. When the authors investigated these effects *in-vitro* using platelet rich plasma (PRP) they found that both the CM and AM NPs enhanced ADP-induced platelet aggregation (Nemmar et al., 2002b).

An *in-vitro* study by McGuinness et al, reported that AM and CM polystyrene NPs induced platelet aggregation which was assessed my measuring platelet-platelet aggregates in whole blood and isolated platelet suspensions using flow cytometry. The CM particles induced classical activation events such as phosphatidylserine and P-selectin expression whereas the AM NPs only induced phosphatidylserine expression. No effects were observed for the unmodified (UM) NPs. This work also attempted to investigate whether any of the NPs disrupted the platelet membrane by measuring hemolysis of erythrocytes following incubation with the NPs. The AM NPs caused significant hemolysis, indicative of membrane damage however this was at concentrations considerably higher
than was used for the aggregation experiments (McGuinnes et al., 2011). The mechanisms behind the reported effects of the AM and CM NPs on platelet function were not investigated.

### 1.8.0. Thesis rational and hypothesis

#### 1.8.1. The effects of engineered nanoparticles on platelet function and the role of physicochemistry

Humans may be exposed to varying concentrations of NPs either intentionally through medical procedures or unintentionally through the use of commercial products. Therefore, it is essential to understand the impact of different concentrations of NPs on biological systems, particularly low concentrations. Assessment of the effects of engineered NPs on platelet function in-vitro has to date involved using relatively high concentrations of NPs either alone or in the presence of an agonist. Additionally, the impact of NP size on platelet function has not been previously addressed. Both NP size and surface chemistry play an integral role in dictating their biological reactivity, so that distinct combinations of physicochemistries would be expected to have unique functional effects and underlying mechanisms.

Therefore, the first hypothesis of this thesis is that engineered NPs can modulate platelet behavior with potencies and mechanisms that are dependent on their size and surface chemistry.

#### 1.8.2. The effect of the combustion derived nanoparticles, diesel exhaust particles and carbon black, on platelet aggregation in vivo and the underlying mechanisms.

It is clear that acute exposure to PM0.1 and its carbonaceous components is associated with thrombotic events such as MI and stroke. These thrombotic events involve platelet activation and aggregation however to date, most work investigating the role of platelets in PM0.1 mediated effects have used either vascular injury models in-vivo or ex-vivo procedures. Furthermore, the mechanisms which have been implicated behind PM-mediated thrombotic events are inconclusive and conflicting. Therefore, there is a need to assess the ability of the major components of PM 0.1, DEP and CB, to modulate platelet behavior in-vivo, and to delineate the underlying mechanisms.

The second hypothesis of this thesis is that acute exposure to DEP or CB can enhance platelet aggregation in-vivo in a process driven by systemic inflammation or alterations in NO bioavailability.
Chapter Two
Materials and Methods
# Materials and methods

## 2.1.0. Materials

### Table 2.1. List of compounds and purchasing companies

<table>
<thead>
<tr>
<th>Compound</th>
<th>Purchased From</th>
</tr>
</thead>
<tbody>
<tr>
<td>• $[^3]$H-5-HT</td>
<td>Perkin Elmer (Cambridgeshire, UK)</td>
</tr>
<tr>
<td>• 50 nm polystyrene beads</td>
<td>Polysciences Inc (Germany)</td>
</tr>
<tr>
<td>• 100 nm amine-modified latex beads</td>
<td>Polysciences Inc (Germany)</td>
</tr>
<tr>
<td>• 100 nm carboxyl-modified latex beads</td>
<td>Polysciences Inc (Germany)</td>
</tr>
<tr>
<td>• 50 nm carboxyl-modified and unmodified polystyrene latex nanoparticles</td>
<td>Bangs laboratories (IN, USA)</td>
</tr>
<tr>
<td>• 100 nm carboxyl-modified and unmodified polystyrene latex nanoparticles</td>
<td>Bangs laboratories (IN, USA)</td>
</tr>
<tr>
<td>• Gold nanoparticles</td>
<td>Cytodiagnostics (Ontario, Canada)</td>
</tr>
<tr>
<td>• Carbon black, Printex-90</td>
<td>Degussa GmbH (Hanau, Germany)</td>
</tr>
<tr>
<td>• Diesel exhaust particles, SRM 2975</td>
<td>National Institute of Standards and Technology (Gaithersburg, MD, USA)</td>
</tr>
<tr>
<td>• Eptifibatide</td>
<td>Integrilin™; GSK Pharma (Greenford, UK)</td>
</tr>
<tr>
<td>• Bisindolylmaleimide I (BIM-1)</td>
<td>Cambridge Biosciences (Cambridge, UK)</td>
</tr>
<tr>
<td>• Acetic acid solution</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• Acid Citrate-Dextrose Solution (ACD)</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• Amine-modified latex beads</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• Apyrase</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• Bovine Serum Albumin Powder</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• Citric Acid</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• D-Glucose</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• Ethylene Glycol Tetraacetic Acid (EGTA)</td>
<td>Sigma (Poole, UK)</td>
</tr>
<tr>
<td>• Ethanol solution</td>
<td>Sigma (Poole, UK)</td>
</tr>
</tbody>
</table>
### Chapter Two – Materials and Methods

- Fibrinogen (Bovine)
- 10 % Formalin (4 % formaldehyde)
- 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid (HEPES)
- Glutaraldehyde solution
- Imipramine
- Indomethacine
- Isopropanol solution
- KCl
- Methanol solution
- MgCl₂
- Na₂H₂PO₄·12H₂O
- NaCl
- NaHCO₃
- NaOH
- PGI₂
- Roswell Park Memorial Institute 1640 medium (RPMI)
- Sodium Nitroprusside
- Thrombin (Bovine)
- Tris
- Trisodium Citrate
- Tropolone
- Urethane
- 2,5-di-(tert-butyl)-1,4-benzoquinone
- Cytospin Filters
- Sterile saline
- Collagen (Collagen fibres predominantly type I from equine tendons)
- Horseradish peroxidase-conjugated (HRP)
- Secondary antibodies (anti-rabbit IgG, anti-mouse IgG)
- Iloprost

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (Bovine)</td>
<td>Calbiochem (Darmstadt, Germany)</td>
</tr>
<tr>
<td>10 % Formalin (4 % formaldehyde)</td>
<td></td>
</tr>
<tr>
<td>4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid (HEPES)</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde solution</td>
<td></td>
</tr>
<tr>
<td>Imipramine</td>
<td></td>
</tr>
<tr>
<td>Indomethacine</td>
<td></td>
</tr>
<tr>
<td>Isopropanol solution</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td></td>
</tr>
<tr>
<td>Methanol solution</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td></td>
</tr>
<tr>
<td>Na₂H₂PO₄·12H₂O</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td></td>
</tr>
<tr>
<td>NaOH</td>
<td></td>
</tr>
<tr>
<td>PGI₂</td>
<td></td>
</tr>
<tr>
<td>Roswell Park Memorial Institute 1640 medium (RPMI)</td>
<td></td>
</tr>
<tr>
<td>Sodium Nitroprusside</td>
<td></td>
</tr>
<tr>
<td>Thrombin (Bovine)</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td></td>
</tr>
<tr>
<td>Trisodium Citrate</td>
<td></td>
</tr>
<tr>
<td>Tropolone</td>
<td></td>
</tr>
<tr>
<td>Urethane</td>
<td></td>
</tr>
<tr>
<td>2,5-di-(tert-butyl)-1,4-benzoquinone</td>
<td></td>
</tr>
<tr>
<td>Cytospin Filters</td>
<td>Thermo Scientific (Northumberland, UK)</td>
</tr>
<tr>
<td>Sterile saline</td>
<td>Harlem (Gloucester, UK)</td>
</tr>
<tr>
<td>Collagen (Collagen fibres predominantly type I from equine tendons)</td>
<td>Nycomed (Munich, Germany)</td>
</tr>
<tr>
<td>Horseradish peroxidase-conjugated (HRP)</td>
<td></td>
</tr>
<tr>
<td>Secondary antibodies (anti-rabbit IgG, anti-mouse IgG)</td>
<td>Dako (Glostrup, Denmark)</td>
</tr>
<tr>
<td>Iloprost</td>
<td>Cayman chemical (Michigan, USA)</td>
</tr>
</tbody>
</table>
### Chapter Two – Materials and Methods

- **$^{111}$Indium Oxine**
  - GE Healthcare (Buckinghamshire, UK)

- **$^{111}$Indium Chloride**
  - Mallinckrodt Radiopharmacy Services (Dublin, Ireland)

- **Scintillation Fluid**
  - National Diagnostics (Hessle, UK)

- **LDH cytotoxicity kit**
  - Roche (West Sussex, UK)

- **PE Mouse Anti-Human CD62P (555524),**
  - FITC Mouse Anti-Human CD41a (555466),
  - FITC Mouse IgG Isotype Control (555573)
  - PE Mouse IgG Isotype Control (555749)
  - BD Bioscience (Oxford, UK)

#### Table 2.2. List of Equipment used

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Purchased From</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single point extended area ratio (SPEAR) detector</td>
<td>Ev Products (Saxonburg, PA)</td>
</tr>
<tr>
<td>Sysmex F-820 Haematology Analyser</td>
<td>Sysmex (Milton Keynes, UK)</td>
</tr>
<tr>
<td>Bright-Line™ haemocytometer</td>
<td>Sigma Aldrich (Poole, UK)</td>
</tr>
<tr>
<td>Spectrum Techniques Software</td>
<td>Mumed Systems (London, UK)</td>
</tr>
<tr>
<td>Chronolog Multi Channel Aggregometer</td>
<td>Chronolog Corporation (Havertown, PA, USA)</td>
</tr>
<tr>
<td>Luminescence Spectrometer LS50B</td>
<td>Perkin Elmer (Cambridgeshire, UK)</td>
</tr>
<tr>
<td>Quartz SUPRASIL Macro/Semi-micro cell cuvette</td>
<td>Perkin Elmer Instruments (Cambridgeshire, UK)</td>
</tr>
<tr>
<td>Liquid Scintillation Counter 1414</td>
<td>WALLUC Instruments (Minnesota, USA)</td>
</tr>
<tr>
<td>Epics XL flow cytometer</td>
<td>Beckman Coulter (High Wycombe, UK)</td>
</tr>
</tbody>
</table>

#### Animals

<table>
<thead>
<tr>
<th>Animals</th>
<th>Purchased From</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enos C57/SV-129 mice (Male and Female) approximately 12 weeks old (20-30 grams)</td>
<td>Purchased from Jackson laboratories and bred at</td>
</tr>
</tbody>
</table>
Chapter Two – Materials and Methods

<table>
<thead>
<tr>
<th>Imperial College London under a heterozygous breeding program</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 male mice (20 – 25 grams)</td>
</tr>
<tr>
<td>BALB/c male mice (20 – 25 grams)</td>
</tr>
<tr>
<td>Purchased from Harlan laboratories</td>
</tr>
</tbody>
</table>

**Table 2.3. Buffer compositions**

<table>
<thead>
<tr>
<th><strong>Modified Tyrodes HEPES Buffer (Tyr)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>D-Glucose</td>
</tr>
<tr>
<td>HEPES</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>MgCl₂</td>
</tr>
<tr>
<td>Na₂H₂PO₄ 12H₂O</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>NaHCO₃</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Ca²⁺ and Mg²⁺ Free Modified Tyrodes HEPES Buffer (CMFB)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>D-Glucose</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>Na₂H₂PO₄ 12H₂O</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>NaHCO₃</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
2.2.0. Methods

2.2.1. Isolation of human platelets

Blood was taken via venepuncture from healthy consenting donors who had abstained from taking any NSAIDs or other medications for two weeks prior to the blood draw, as approved by the NHS Research Ethic Service (REC no.07/H0708/72). Donors were given an information sheet with information of the study aims and procedure and following an appropriate amount of time, participants signed a consent form and venepuncture took place.

Blood was taken into a 50 mL pull back syringe 3.2 % (w/v) sodium citrate in a 1:9 ratio using a 21 gauge butterfly cannula. Platelet rich plasma (PRP) was obtained by centrifugation at 100 g for 20 minutes. ACD (15 µl mL⁻¹) and PGE₁ (125 µg mL⁻¹) were added to the PRP and it was then centrifuged at 1400 g for 10 minutes to produce a platelet pellet. The platelet pellet was resuspended in Tyrodes HEPES buffer (THB) (134 mM NaCl, 2.9 mM KCL, 0.34 mM NaHPO₄, 20 mM HEPES, 10 mM MgCl₂, and 10 mM glucose) and centrifuged at 1400 g for 10 minutes with ACD (250 µl mL⁻¹) and PGE₁ (125 µg mL⁻¹). Platelets were counted using a F820 total cell counter (Dioner), suspended to a final concentration of 250 x 10³ cells µL⁻¹. Platelets were rested for approximately 30 minutes at 37 °C.

2.2.2. Optical platelet aggregometry

Platelet aggregation responses were investigated in vitro by measuring light transmission through a platelet suspension using an optical platelet aggregometer (Chrono-log Corporation, Havertown, PA, USA) as previously described (Born, 1962).

Isolated human platelets (section 2.1) were incubated at 37°C for 90 seconds prior to addition of an agonist. 450 µl of platelets (250 x 10³ cells µl⁻¹) were added to a siliconized glass cuvette with a Teflon magnetic stir bar and 50 µl of agonist was added. Changes in light transmission corresponding to platelet shape change and aggregation were measured for 3 minutes under stirring conditions (1200 rpm) at 37°C. For potentiation experiments, 50 µl of nanoparticle or Tyrode’s buffer was added to 400 µl of platelets immediately prior to stimulation with 50 µl of appropriate agonist.

PRP experiments were conducted with the same approach as isolated platelet experiments however platelet poor plasma (PPP) was used as a reference for 100 % aggregation instead of Tyrode’s buffer. For measurement of platelet aggregation by diesel exhaust particles (DEP) and Carbon Black (CB), the baselines were set immediately after the addition of the DEP or CB to the cuvette. This was essential as the particles interfered with the light transmission.
2.2.3. Dense granule release assay

To investigate the release of dense granules from isolated human platelets, 5-hydroxytryptamine (5-HT) was measured following stimulation with agonists and nanoparticles using a modified protocol from Poole and colleagues (Poole et al., 1997, Kemp et al., 2008).

Anti-coagulated human blood (3.2 % v/v sodium citrate) was centrifuged at 100 g for 20 minutes to obtain PRP. Platelets were loaded with $[^{3}H]$-5HT (1µCi mL$^{-1}$) by incubating the PRP for 1 hour at 37°C.

PRP was centrifuged at 1400 g for 10 minutes and the platelet pellet was resuspended in 1mL of Tyrode’s buffer supplemented with imipramine (10 µM) and cells were diluted to a density of 250 x 10$^3$ µL$^{-1}$. Platelets were rested for 30 minutes at 37°C prior to the experiment.

The isolated platelets were stimulated with appropriate agonist or nanoparticle for 3 minutes under identical conditions as the optical platelet aggregometry experiments (section 2.2.2). Aggregations were stopped by the addition of ice cold gluteraldehyde (6 % v/v) and the contents were centrifuged for 2 minutes at 1300 g to remove the platelets. 800 µl of cell free supernatant was added to 4 mL of scintillation fluid in scintillation vials and scintillation counts were measured on a WALLUC® scintillation analyser. Nanoparticle controls were run in the absence of cells to establish any interference with the assay. No interference was detected.

2.2.4. P-selectin expression in isolated human platelets

450 µl of isolated human platelets (section 2.2.1) were incubated with 50 µl of a nanoparticle or appropriate control for 10 minutes under static conditions to prevent platelet aggregation. Following this, 75 µl of platelets were added to FACS tubes and incubated with antibodies to P-selectin (anti–human CD62P conjugated to a Phycoerythrin (PE) fluorophore) and a platelet specific marker (anti–human CD41 conjugated onto a Fluorescein isothiocyanate (FITC) fluorophore) for 10 minutes. Platelets were fixed by addition of paraformaldehyde (2 % 1:1 ratio) followed by 500 µl of FACS buffer (1 % BSA in 1 x PBS). Samples were measured immediately on an Epics XL flow cytometer (Beckman Coulter, High Wycombe, UK). Live gating was used to specifically identify platelets using forward (size) and side (granularity) scatter analysis of 5000 events. Events co-staining for CD41 FITC and CD62P PE were considered to represent P-selectin expression in the platelet population.

An isotype control was used to assess non-specific antibody binding. All antibodies were from Becton and Dickinson Biosciences, NJ, USA and had been previously titrated to appropriate concentrations by our collaborator group. Data is presented as percentage CD62P positive events established using a quadrant gate. Data was analysed on a read on an Epics XL flow cytometer (Beckman Coulter High Wycombe, UK).
2.2.5. Lactate dehydrogenase assay

450 µl of isolated human platelets 250 x 10³ cells µl (section 2.2.1) were stimulated with nanoparticles, thrombin or Tyrode’s buffer in siliconized glass cuvettes under stirring conditions (1200 rpm) for 3 minutes. Platelet suspensions were transferred to polystyrene eppendorf tubes and centrifuged at 1300 g for 2 minutes to pellet the platelets. 50 µl of the supernatant was transferred to a 96 micro-titre plate.

For the longer exposure study, platelets were incubated with nanoparticles, thrombin or THB for 2 hours in polystyrene eppendorf tubes at 37°C. Platelet/nanoparticle/agonist suspensions were centrifuged at 1300 g for 2 minutes to pellet the platelets. 50 µl supernatant was transferred to a 96 micro-titre plate.

A positive control of maximum LDH release was obtained by the addition of lysis solution to control wells with platelets and incubated for 15 minutes at room temperature on a rocking plate. To measure LDH activity, reaction mixture (1:1) (Roche Solutions) was added to each well and incubated for 30 minutes in the dark. The reaction was stopped by the addition of 25 µl hydrochloric acid (Roche Solutions) and immediately read on a microtitre plate reader at 490 nm (Tecan Sunrise).

2.2.6. Preparation and staining of nanoparticles for electron microscopy

Nanoparticles were diluted in Tyrode’s buffer, sonicated for 1 minute and vortexed immediately before addition to fomvar-coated nickel grids. A drop of the suspension was placed on the grid and excess fluid was removed with filter paper. The grid was air dried for 10 minutes followed by incubation in Uranyl acetate (2:1 ethanol) for 15 minutes. The grid was left to air dry for 1 hour before visualisation.

2.2.7. Preparation and staining of nanoparticle-platelet electron microscopy samples

Isolated human platelets 250 x 10³ cells µl⁻¹ were prepared (section 2.2.1) however they were resuspended in Tyrode’s buffer supplemented with PGE₁ and ACD to prevent full platelet aggregation following addition of the nanoparticles. 4 mLs of isolated platelets were incubated with 100 µl of nanoparticles or Tyrode’s buffer (mixed) for 10 minutes under static conditions. Platelets were initially fixed in 0.1 % gluteraldehyde for 15 minutes then centrifuged at 1400 g for 10 minutes to pellet the platelets. The platelet pellet was fixed for 3 hours in 3 % gluteraldehyde followed by 3 washing steps in PBS. Platelets were stained with 1 % osmium tetroxide for 1 hour followed by 3 washing steps with dH₂O. Samples were serially dehydrated by immersion in 50, 70, 80, 90, 95 and 100 % acetone/distilled H₂O each for 5 minutes. Samples were infiltrated with acetone/araldite overnight after which they were incubated with fresh araldite at 50°C for 30 minutes with 3
repetitions. Finally, samples were placed onto acetate in araldite and incubated at 65°C for one hour then placed in capsules with fresh araldite and left to polymerize at 65°C for two days. Thin 100 nm sections were cut with a Reichert Ultracut E ultramicrotome (conducted by Dr Anupama Vydyanath). Sections were placed on copper grids and stained with 2 % Uranyl acetate for 15 minutes followed by Reynolds lead citrate for 15 minutes. Sections were left to dry for 1-2 hours before visualization.

2.2.8. Isolation of mouse platelets

C57BL/6 mice (20 - 25 g) were terminally anaesthetised with urethane (10 µl g⁻¹ 25 % w/v i.p) and bled via cardiac puncture (Figure 2.1) followed by termination using a method approved in Schedule 1 of the Animals (Scientific Procedures) Act 1986. Blood was taken into a syringe with 150 µl ACD and centrifuged at 300 g for 3 minutes to obtain PRP which was transferred to another microcentrifuge tube. 400 µl of TAP (Tyrode’s buffer, ACD and PGE₁) (Calcium Free Tyrode’s Hepes Buffer (CFTHB), ACD 3:1 v/v and PGE₁ 1 mg ml⁻¹) was added to the remaining erythrocytes and centrifuged again for 3 minutes at 300 g to obtain additional PRP. The PRP was pooled from individual mice and contaminating erythrocytes were removed by centrifugation at 200 g for 2 minutes. Platelets were isolated by centrifugation of supernatants at 1500 g for 7 minutes and platelet pellets from individual mice were resuspended in TAP and pooled together. The platelets were counted (Sysmex F-820 Haematology Analyser) and adjusted to 4 x 10⁶ cells ml⁻¹ 200 µl per experiment.

2.2.9. Radiolabelling mouse platelets with Indium Oxine¹¹¹

1.8 MBq of Indium Oxine¹¹¹ was added to isolated mouse platelets (section 2.2.8) and incubated for 10 minutes at room temperature. Excess radiation was removed by centrifugation of the platelets at 1500 g for 5 minutes and resuspended them in CFTHB, 200 µl per experiment. Scintillation counts were recorded before and after centrifugation for calculation of the percentage labelling. Platelets were allowed to rest for a minimum of 30 minutes prior to the experiment.

2.2.10. Radiolabelling mouse platelets with Indium Chloride¹¹¹

1.8 MBq of Indium Chloride¹¹¹ was added 6 µl of tropolone solution (1 µg µl⁻¹) in a microcentrifuge tube. The tropolone Indium Chloride¹¹¹ solution was added to isolated mouse platelets (section 2.2.8) and incubated at room temperature for 10 minutes. Excess radiation was removed by centrifugation at 1500 g for 7 minutes and resuspended in CFTHB, 200 µl per experiments. Platelets were allowed to rest for a minimum of 30 minutes prior to the experiment.
2.2.11. Animals

Male C57bl6 mice (or Balb/c for section 3.2.8 + 3.2.9) (Harlan, Bicester, UK) between 20 – 25 g were used. Litter mates were stored in individually ventilated cages at 22 ± 2°C and under a 12:12 light:dark cycle. Food and water was available ad libitum. Animals were allowed to acclimatize for 1 week prior to commencing experiments. All animal procedures were conducted under the Home Office project license (70/7190), with authorization from the Imperial College London Ethical Review Panel and in accordance with regulations described by The Animals (Scientific Procedures) Act (ASPA) 1986. Protocols were refined in association with the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

BALB/c mice were initially selected for experiments due to ease of administration of reagents through the tail vein however, due to an intolerance to urethane by this strain, C57BL/6 mice were selected for future experiments. Additionally, C57BL/6 mice were selected for all DEP experiments as C57BL/6 was the backgroup strain for the eNOS−/− mice.

2.2.12. Mouse model of thromboembolism

C57BL/6 mice (20 – 25 g) were terminally anaesthetised with urethane (10 µl g−1 25 % w/v i.p). The femoral vein was exposed through a minor surgical procedure. Radiolabelled mouse platelets (section 2.2.8) were injected and allowed to circulate for 15 minutes. A single point extended area ratio (SPEAR) detector (eV Products, Saxonburg, PA) probe was placed over the thorax of the mouse. Scintillation counts were recorded in the pulmonary vasculature via a UCS-20 spectrometer which was connected to a laptop (Figure 2.1D). Specialised software supplied by Mumed Systems (London, UK) was used to measure changes in counts over time. When a steady baseline had been established, a 50 µl bolus of either nanoparticles or agonist were administered and changes in scintillation counts were recorded for 10 minutes. Injections were deemed successful when the vein produced a white flush immediately after injection and there was no disturbance of the underlying tissue.
Figure 2.1. murine model of pulmonary thromboembolism

Anaesthetised donor mice were bled via cardiac puncture (A) and platelets were isolated and radiolabelled with Indium Oxine$^{111}$ or Indium Chloride$^{111}$. Radiolabelled platelets, agonists and nanoparticles were administered through the femoral vein (B) and platelets were detected in the pulmonary vasculature using a SPEAR probe placed over the thorax (C). Platelet aggregation was measured as percentage change in scintillation counts compared to the baseline (D). Image adapted from (Tymvios et al., 2008).
Chapter Two – Materials and Methods

2.3.0. Tracheal instillation of Diesel Exhaust Particles

Mice were placed into an anaesthetising chamber with isoflurane (4 %) and oxygen until they did not respond to a toe pinch and displayed no righting reflex. They were placed on a sloped surgical board suspended by their incisors. A 50 µl bolus of either saline, CB or DEP was administered through a Hamilton syringe® attached to an oral gavage steel feeding tube conducted by Dr Mark Birrell. Mice were left to recover for 4 hours prior to the experiments.

To prevent agglomeration, all nanoparticles were sonicated for 5 minutes and vortexed for 1 minute prior to administration.

2.3.1. Enzyme-Linked Immunosorbent Assay

Mouse cytokines were quantified using a standard sandwich ELISA (DuoSet, R&D Systems) as per the manufacturer’s instructions. BALF samples were diluted 1:2 (apart from IL-1β) and plasma samples were diluted 1:3 in reagent diluent (1% BSA in 1% PBS pH 7.2 - 7.4). All samples were run in triplicate with the appropriate controls and the standard curve was run in duplicate.

100 µl of capture antibody (TNF-α 0.8 µg mL⁻¹, IL-6 20 µg mL⁻¹, IL-1β 4 µg mL⁻¹, MCP-1 9 µg mL⁻¹, MIP-2 2 µg mL⁻¹ and IL-2 1 µg mL⁻¹) was added to each well of a 96-well ELISA plate and incubated at 4°C overnight. Following incubation, the wells were aspirated and washed with wash buffer (0.05% Tween20 in PBS pH 7.2 – 7.4) three times (wash step) and 300 µl of block buffer (1% BSA in 1% PBS pH 7.2 - 7.4) was added to each well and incubated for a minimum of 1 hour at room temperature. The wash steps were repeated and 100 µl of sample or standard was added to the appropriate wells and incubated for 2 hours at room temperature. Following a wash step, 100 µl of detection antibody were added (TNF-α 200 ng mL⁻¹, IL-6 150 ng mL⁻¹, IL-1β 1.5 µg mL⁻¹, MCP-1 50 ng mL⁻¹, MIP-1 75 ng mL⁻¹ and IL-2 800 ng mL⁻¹) and incubated for a further 2 hours at room temperature. After a wash step, Streptavidin-HRP (1:200 R&D systems) were added to each well and incubated for 20 minutes away from direct light. The wells were washed and 100 µl of substrate solution (1:1 H₂O₂ and Tetramethylbenzidine, R&D systems) was added for 20 minutes away from direct light. The reaction was stopped by the addition of 50 µl of sulfuric acid stop solution (R&D systems). The optical density was measured immediately on a microtiter plate reader (Tecan Sunrise) set at 540 nm with wavelength correction. Cytokine concentrations were calculated using a standard curve.

2.3.2. Bronchoalveolar lavage collection

Mice were anaesthetised with urethane (10 µl g⁻¹ 25 % w/v i.p) and placed in a supine position. The trachea was exposed via blunt dissection of the tissue and a small hole was made to facilitate
Chapter Two – Materials and Methods

cannulation with a 14 G dispensing tip (Adhesive Dispensing Ltd, UK). Bronchoalveolar lavage was performed by instilling 0.3 mL of Roswell Park Memorial Institute 1640 medium + GlutaMAX-I (RPMI, Sigma Aldrich, Poole, UK) or saline into the lungs for 30 seconds. This was repeated 3 times with fresh media/saline and samples were pooled for individual mice. Aliquots for each animal were taken for total cell counts and differential cell counts (section 2.3.3) and the remainder of the sample was centrifuged at 1900 rpm at 4°C for 10 minutes. The supernatant was removed and stored at -80°C for further analysis.

2.3.3. Platelet Rich Plasma preparation

C57BL/6 mice (20 - 25 g) were terminally anaesthetised with urethane (10 µl g⁻¹ 25 % w/v i.p) and bled via cardiac puncture (Figure 3) followed by termination using a method approved in Schedule 1 of the Animals (Scientific Procedures) Act 1986. Blood was taken into a syringe with 150 µl ACD and centrifuged at 300 g for 3 minutes to obtain PRP which was immediately frozen and stored at -80°C for quantification of cytokines/chemokines (ELISA) and nitrate/nitrite levels (gas-phase chemiluminescence) (collaboration with the University of Exeter).

2.3.4. Total and differential cell counting

Total cell counts from cells retrieved from the BALF and blood were performed manually using a Bright-Line™ haemocytometer (Sigma Aldrich, Poole, UK). Cells were incubated with Turks solution/trypan blue and five squares were counted on the haemocytometer. Differential cell counts were performed on cells recovered from the BALF by examining cytospin preparations under light microscopy (x20 and x40 objective). These were prepared by centrifuging 100 µl of cell suspensions in a cytospin (Shandon, Runcorn, UK) at 700 rpm with low acceleration for 5 minutes at room temperature. Slides were air dried, fixed in methanol and stained with modified Wright-Giemsa stain. 200 cells were counted from each slide using at least 4 different viewing fields. Cells were identified using standard morphological criteria. Percentage neutrophils, lymphocytes, eosinophils, macrophages and monocytes were calculated. Neutrophils were identified by their medium size, multi-lobed nucleus and weakly stained cytoplasm. Lymphocytes were classified by their small size, dark blue stained nucleus and no presence of cytoplasm. Eosinophils displayed a bi-lobed or figure of eight nucleus with a unique pink granular cytoplasm. Monocytes and macrophages were categorised together due to the difficulty in differentiating the two cell types. These were the largest cell type and contained a large dark nucleus with a greater amount of cytoplasm.
Chapter Two – Materials and Methods

Differential cell counts in whole blood were counted in blood smears which were prepared by smearing 10 µl of anticoagulated blood onto a microscope slide. Blood smears were fixed and stained with the cytocentrifuge preparations and cells were identified using the same criteria.

2.3.5. Protein quantification

Protein quantification was performed using a DC-protein assay (Bio-Rad) which is a modified Lowry method. BALF supernatant (5 µL) or BSA standards (5 µL of 2, 1.5, 1, 0.5, 0.25, 0.125 and 0.0625 mg mL\(^{-1}\)) were added in triplicate to a 96 well microtiter plate. An alkaline copper reagent solution was added to all wells (25 µL), followed by a Folin reagent (Bio-Rad) (200 µL). The plate was left in the dark to develop for 15 minutes at room temperature and read immediately on a microtiter plate reader at 750 nm (Tecan Sunrise).

2.3.6. Histological analysis of nanoparticle pulmonary deposition following intratracheal instillation

Immediately following i.t. instillation mice were euthanized via cervical dislocation and their lungs were dissected, briefly washed in PBS and fixed in formalin (10 %) (Sigma-Aldrich) overnight. Following this, samples were embedded in wax, sectioned (longitudinally) and stained (haematoxylin and eosin). The samples were viewed using a light microscope (x 20 and x 40 objective) and images were acquired using a ccd camera. The pulmonary locations were identified based on the cellular architecture and the DEP and CB particles were identified by their black globular physical characteristics.

2.4.0. Statistics

In results chapters three and four, data is presented as box-and-whisker plots, the horizontal lines inside the box indicate the median. The box edges extend from the 25\(^{th}\) to the 75\(^{th}\) percentiles and the whiskers represent the minimum and maximum values. In chapter five, data are presented as a scatter plot of median with minimum and maximum values and for data sets with n < 4 in chapter three. All statistical analysis was performed on raw data using GraphPad Prism version 5 software for windows (San Diego, California, USA). Paired comparisons between two groups were performed using a Wilcoxon signed rank test and between multiple groups using a Friedman test with Dunn’s comparison. All experiments involved time or day matched controls. Comparisons between two independent data sets were made using a Mann-Whitney test and multiple comparisons were made using a Kruskal-Wallis one way analysis of variance (K-W) with Dunn’s comparison. EC50 values were calculated using a non-linear regression curve fit analysis. Power calculations were conducted to
determine appropriate $n$ numbers. A $P$ value of $< 0.05$ was indicative of statistical significance.
Chapter Three

The effects of engineered nanoparticles on platelet function
Chapter Three – Results

Chapter 3

3.1.0. Introduction

There is limited information regarding the thrombotic risk posed by engineered NPs which may be introduced into the circulation during medical procedures or which translocate across the alveolar-blood barrier following inhalation. Platelet activation and subsequent aggregation is an integral process in thrombosis and combustion derived NPs such as DEP (Solomon et al., 2013) and engineered silver NPs (Jun et al., 2011) are reported to be capable of inducing platelet aggregation. Model polystyrene NPs have been used to investigate NP interactions with various cell types such as macrophages (Lunov et al., 2011), alveolar epithelial cells (Ruenraroensak et al., 2011) and platelets (McGuinnes et al., 2011) in-vitro. These NPs have also been administered in-vivo to assess tissue distribution (Sarlo et al., 2009) and NP effects on iron absorption (Mahler et al., 2012). The advantages of these particles are that they have defined sizes and surface charges, allowing the examination of the particular biological effects of NPs with distinct physicochemical properties. NP physicochemical properties have been demonstrated to be important in dictating NP modulation of platelet behaviour, such as inducing platelet-platelet aggregates and enhancing ADP-induced platelet aggregation however, this has only been established at high concentrations (McGuinnes et al., 2011, Nemmar et al., 2002b). In addition, NP size has also been demonstrated to play a crucial role in dictating NP bioreactivity, however, the significance of varying combinations of NP size and surface charge on platelet function has not been investigated.

This chapter examined the ability of model polystyrene NPs with varying sizes and surface charges to induce platelet aggregation and platelet signalling events at lower concentrations than previously published and to establish whether the effects on platelet function were influenced by NP physicochemistry.
Chapter Three – Results

3.1.1. Chapter aims

- Establish appropriate maximal, submaximal and EC\textsubscript{50} concentrations of platelet agonists \textit{in-vitro} and \textit{in-vivo} for use in functional assays.

- Evaluate the agglomeration state of NPs with defined sizes and surface chemistries in experimental buffers.

- Validate the findings from the literature regarding platelet aggregation induced by polystyrene NPs with varying surface chemistries at high concentrations, using optical platelet aggregometry.

- Assess the ability of NPs with defined sizes and surface chemistries to induce platelet aggregation in isolated platelet suspensions, PRP and whole blood and calculate potencies for each NP.

- Assess the NPs ability to enhance agonist-induced platelet aggregation in isolated platelet suspensions, PRP and \textit{in-vivo}.

- Establish whether NP-induced platelet aggregation is receptor-mediated and is associated with alpha and dense granule release.
3.2.0. Concentration response to platelet agonists

Isolated human platelet aggregation \textit{in-vitro} stimulated by collagen (0.001 – 100 µg mL\(^{-1}\)) (Fig 3.1 A) and thrombin (0.0001 – 10 U mL\(^{-1}\)) (Fig 3.1 B) was concentration dependent with a maximal aggregation occurring at 5 µg mL\(^{-1}\) for collagen and 1 U mL\(^{-1}\) for thrombin. The EC\(_{50}\) concentration for collagen and thrombin was 1.25 µg mL\(^{-1}\) and 0.06 U mL\(^{-1}\) respectively.

Platelet aggregation in response to ADP (0.1 – 100 µM) (Fig 3.1 C) in PRP was concentration dependent, with maximal aggregation occurring at 10 µM and the EC\(_{50}\) concentration was 1 µM. Platelet aggregation in response to ADP was reduced after stimulation with concentrations > 10 µM which suggests that the P2Y\(_{1/12}\) receptors became desensitised and thus less responsive to ADP.

3.2.1. Electron micrographs of 50 nm and 100 nm nanoparticles with different surface chemistries in modified tyrodes hepes buffer.

NPs with varying physicochemistries were visualised on fomvar coated grids using transmission microscopy to establish their agglomeration states in the experimental buffer. NP formation in Tyrode’s buffer was dictated by size and surface charge. Both the 100 nm and 50 nm AM NPs were present as simple agglomerates and individual particles (Fig 3.2 A and B) whereas the CM particles were predominantly present as dispersed individual particles (Fig 3.2 C and D). Conversely, the UM NPs appeared to be most prone to agglomeration as the UM 50 nm particles were found as more complex larger agglomerates (indicated by increased density) with chains and some individual particles (Fig 3.2 E) and the 100 nm UM NPs were present as large complex agglomerates and chains with no individual particles (Fig 3.2 F). Individual particles are identified by grey arrows, simple chains with black arrows and large agglomerates with white arrows on the images.
Figure 3.1. Concentration response to platelet agonists

Isolated human platelets were stimulated with increasing concentrations of collagen (0.001 – 100 µg mL\(^{-1}\)) (A) and thrombin (0.0001 – 10 U mL\(^{-1}\)) (B). Platelet rich plasma was stimulated with increasing concentrations of ADP (0.1 – 100 µM) (C). Platelet aggregation was detected as changes in light transmission and reported as maximum percentage change in light transmission. Data are presented as log concentration response curves and EC\(_{50}\) concentrations calculated using regression analysis. ADP = adenosine diphosphate. N = 3-4.
Figure 3.2. Electron micrographs of 50 nm and 100 nm nanoparticles with different surface chemistries in modified tyrodes hepes buffer.

AM, CM and UM 100 nm and 50 nm NPs were suspended in tyrode’s buffer and stained with uranyl acetate on copper fomvar grids. Images were taken by transmission electron microscopy at magnification x25000. AM 50 nm (A), 100 nm (B), and CM 50 nm (C), 100 nm (D). NPs were observed as individual particles (grey arrows), chains (black arrows) and simple agglomerates (white arrows) while the UM 50 nm (E) and 100 nm (F) NPs were observed predominantly as large complex agglomerates with some simple agglomerates and single particles. AM = amine-modified, CM = carboxy-modified and UM = unmodified.
3.2.2. Platelet aggregation induced by a high concentration of nanoparticles

Previous work which has investigated the effects of the physicochemical properties of NPs on platelet-platelet aggregates was conducted using 50 nm model polystyrene NPs, at very high concentrations (260 µg mL\(^{-1}\)) and using flow cytometry (McGuinnes et al., 2011). To replicate this earlier work using optical platelet aggregometry, isolated platelets (section 2.2.1) were stimulated with 260 µg mL\(^{-1}\) of 50 nm (Fig 3.3 A+ B) and 100 nm AM, CM and UM NPs nm (Fig 3.3 A+ C) and light transmission was measured.

The 50 nm NPs produced aggregation traces similar to the thrombin control (1 U mL\(^{-1}\)), with an initial decrease in light transmission corresponding to platelet shape change and followed by a steep increase due to platelet aggregation. The CM 50 nm NPs appeared to induce the most platelet aggregation, similar to the thrombin control whereas the UM and AM 50 nm NPs appeared to induce less platelet aggregation than the CM 50 nm NPs and thrombin.

The UM and CM 100 nm NPs appeared to interfere with the light transmission, which was signified by the sharp decrease in light transmission upon addition of the NPs (Fig 3.3 C), this was most likely due to the opaque nature of these particles at high concentrations. However, although there was clear interference with the assay by the 100 nm NPs at this concentration, these particles did appear to produce measurable aggregation. Due to the NP interference with this assay these experiments were discontinued and no statistical comparisons were performed.
Figure 3.3. Platelet aggregation induced by a high concentration of nanoparticles

Isolated human platelets were stimulated with a high concentration (260 µg mL\(^{-1}\)) of 50 nm (B), 100 nm (C) NPs, thrombin (1 U mL\(^{-1}\)) or Tyrode’s buffer and changes in light transmission were measured for 3 minutes. Data are presented as percentage maximal aggregation, median and interquartile range (A) or representative traces (B + C). AM = amine-modified, CM = carboxyl-modified, UM = unmodified, Thr = thrombin, Tyr = Tyrode’s buffer. N = 3-4.
3.2.3. Nanoparticle induced platelet aggregation in-vitro.

The ability of NPs with different physicochemical properties to induce platelet aggregation at lower concentrations than previously reported (Fig 3.2) (McGuinnes et al., 2011) was investigated. Isolated human platelets (section 2.2.1) were stimulated with increasing concentrations (0.2 – 125 µg mL\(^{-1}\)) of 50 nm (Fig 3.4 A) and 100 nm (Fig 3.4 B) UM, AM and CM NPs and changes in light transmission were measured. All NPs induced concentration dependent platelet aggregation (Fig 3.4 A+B). All aggregation, apart from the AM 50 nm (Fig 3.4 A) produced a similar level of maximal platelet aggregation to that induced by the potent platelet agonist thrombin (1 U mL\(^{-1}\)). EC\(_{50}\) values were calculated as a measure of potency (Fig 3.4 C) and identified the CM 50 nm and the UM 100 nm NPs as the most potent (18.56 and 12.76 µg mL\(^{-1}\) respectively) and the UM 50 nm and the CM 100 nm as the least potent (38.06 and 32.74 µg mL\(^{-1}\) respectively).

The influence of NP size was assessed by using two sizes of polystyrene NP (50 nm and 100 nm). Size differences were observed when platelets were stimulated with submaximal concentrations (30 µg mL\(^{-1}\)) of NP (Fig 3.4 D – F). This demonstrated that uncharged 100 nm UM NPs (Fig 3.4 E) produced more aggregation than the 50 nm smaller particles whereas the 50 nm AM (Fig 3.4 D) and the CM (Fig 3.4 F) particles were more bioreactive than their larger counterparts. Platelet aggregation induced by the AM 50 nm particles was more variable than any of the other NPs, for example platelet aggregation induced by a 60 µg mL\(^{-1}\) concentration ranged between 20 - 51 %. This may account for the discrepancies between the EC\(_{50}\) values and the size effects at 30 µg mL\(^{-1}\).

Light transmission traces produced by all NPs (Fig 3.4 D-F) were of similar appearance to light transmission traces produced by the platelet agonist thrombin (Fig 3.3 B+C), indicating that the changes in light transmission were likely due to platelet aggregation rather than interference by the NPs.

Gold NPs (60 nm) were used as a particle composition control. Isolated human platelets were stimulated with gold NPs (5 µg mL\(^{-1}\)) however no platelet aggregation was detected.
Chapter Three – Results

Figure 3.4. Nanoparticle induced platelet aggregation *in-vitro.*

Isolated platelets were stimulated with increasing concentrations (0.2 – 125 µg mL⁻¹) of 50 nm (A), 100 nm (B) NPs, gold NPs (5 µg mL⁻¹), tyrode’s buffer or thrombin (1 U mL⁻¹) and changes in light transmission was measured for 3 minutes. Data is presented as log dose response curves of maximal percentage aggregation (A + B), EC₅₀ concentrations (C) calculated by regression analysis or representative aggregation traces of AM (D), UM (E) and CM (F) NPs (30 µg mL⁻¹). AM = amine-modified, CM = carboxyl-modified, UM = unmodified. N = 6–8.
3.2.4. The effects of nanoparticles on platelet aggregation in plasma

The ability of NPs to induce platelet aggregation in the presence of plasma proteins was established by measuring changes in light transmission in plasma.
Platelet rich plasma was stimulated with 50 nm and 100 nm NPs (125 and 250 µg mL\(^{-1}\)), ADP (10 μM) or Tyrode’s buffer (Fig 3.5 C). No significant platelet aggregation was detected for any of the NPs (P > 0.05 compared to Tyrode’s buffer control).

3.2.5. Enhancement of agonist mediated platelet aggregation \textit{in-vitro} by 50 nm amine-modified nanoparticles.

Having established concentrations of NPs that could induce platelet aggregation (Fig 3.4), the ability of a sub-threshold and a potentially more relevant concentration of NP to modulate agonist responses \textit{in-vitro}, both in the presence and absence of plasma proteins was investigated.
Platelets were stimulated with EC\(_{50}\) concentrations of thrombin (0.06 U mL\(^{-1}\)) (Fig 3.6 A) and collagen (1.25 μg mL\(^{-1}\)) (Fig 3.6 B) in isolated platelet suspensions and ADP (1 μM) (Fig 3.5 C) in PRP, immediately after the addition of a sub-threshold concentration of NP (2 µg mL\(^{-1}\)) or Tyrode’s buffer. The AM 50 nm NPs significantly enhanced both the thrombin (Fig 3.6 A) and the ADP (Fig 3.6 C) induced platelet aggregation (P < 0.05 compared to Tyrode’s buffer and agonist) whereas no significant effect was detected for any other particles (P > 0.05 compared to Tyrode’s buffer and agonist) however, there was a non-significant trend of increased thrombin-induced platelet aggregation in the presence of the AM 100 nm (P > 0.05 compared to Tyrode’s buffer and agonist). No significant changes in collagen induced platelet aggregation were detected for any NP however there was a trend of reduced aggregation following incubation with the UM 50 nm NP (Fig 3.6 B). To ascertain whether the augmented light transmission in the presence of AM 50 nm NP was due to receptor mediated platelet aggregation, platelets were incubated with the \(\alpha_{IIb}\beta_{III}\) antagonist eptifibatide prior to agonist/NP stimulation. Both the control (thrombin and ADP) responses and the enhanced aggregation responses, due to the AM 50 nm NP, were abolished by eptifibatide (0.06 µg mL\(^{-1}\)). No significant differences were observed between the eptifibatide treated groups (P > 0.05 between eptifibatide/agonist and eptifibatide/ NP/ agonist).
Figure 3.5. Nanoparticles effects on platelet aggregation in plasma

Platelet rich plasma was stimulated with 50 nm and 100 nm NP (125 µg mL$^{-1}$), ADP (10 µM) or tyrodes (C) and light transmission was recorded for 3 minutes. Data are presented as median and range. Comparisons were made using a Friedman test with Dunns multiple comparisons. AM = amine-modified, CM = carboxyl-modified, UM = unmodified, NS = non-significant, Tyr = Tyrode’s buffer, TRAP = Thrombin Receptor Activator Peptide, ADP = Adenosine Diphosphate. N = 3.
Figure 3.6. Enhancement of agonist mediated platelet aggregation *in-vitro* by 50 nm amine-modified nanoparticles.

Isolated platelets were stimulated with an EC_{50} concentration of thrombin (0.06 U mL^{-1}) (A) or collagen (1.25 µg mL^{-1}) (B) and platelet rich plasma was stimulated with an EC_{50} concentration of ADP (1 µM) (C) in the presence of a subthreshold concentration of NP or gold (2 µg mL^{-1}) and changes in light transmission measured for 3 minutes. The α_{IIbβ3} antagonist Eptifibatide (0.06 µg mL^{-1}) was incubated with platelets for 5 minutes prior the addition of the AM 50 nm NP and stimulation with thrombin (A) or ADP (C). Data are presented as box-and-whisker plots. *P < 0.05 compared to Tyrode’s buffer control using a Wilcoxon signed rank test. NS = non-significant, AM = amine-modified, CM = carboxyl-modified, UM = unmodified, EPI = Eptifibatide, ADP = adenosine diphosphate, NP = nanoparticle, Coll = Collagen and Thr = Thrombin. N = 4-6.
Chapter Three – Results

3.2.6. Dense granule release by nanoparticles

The 50 nm CM NPs (60 – 125 µg mL⁻¹) and thrombin (1 U mL⁻¹) induced significant 5-HT release (P < 0.05 compared to Tyrode’s buffer control), no significant 5-HT release was detected for the 50 nm UM or 50 nm AM NPs (P > 0.05 compared to Tyrode’s buffer control) (Fig 3.7 A).

The 100 nm UM particles (60 – 125 µg mL⁻¹) and thrombin (1 U mL⁻¹) induced significant 5-HT release (P < 0.05 compared to Tyrode’s buffer control). No significant 5-HT release was detected for the 100 nm CM or AM particles (P > 0.05 compared to Tyrode’s buffer control) however there was a trend suggesting that there was 5-HT release for the highest concentration of CM particles (125 µg mL⁻¹) (Fig 3.7 B).

To confirm that the 5-HT release recorded was PKC mediated and not due to other processes such as plasma membrane disruption, platelets were stimulated with a high concentration of NP (125 µg mL⁻¹) or thrombin (1 U mL⁻¹) following pre-incubation with the broad-spectrum PKC inhibitor BIM-1 (10 µM). BIM-1 inhibited both NP and thrombin induced 5-HT release (P > 0.05 compared to vehicle control) (Fig 3.7 A and Fig 3.7 B).

3.2.7. Nanoparticle induced P-selectin expression in isolated platelets

The ability of NPs with different physicochemical characteristics to cause alpha granule release and subsequent P-selectin expression was assessed using flow cytometry.

Similar to the dense granule results, thrombin (1 U mL⁻¹), CM 50 nm (Fig 3.8 A + C) and UM 100 nm (Fig 3.8 B + D) NPs (15 - 60 µg mL⁻¹), induced concentration dependent increases in percentage platelets expressing P-selectin and mean fluorescence intensity (MFI) which was significant at the two highest concentrations (30 – 60 µg mL⁻¹) for the percentage expression and only the highest (60 µg mL⁻¹) for percentage MFI (P < 0.05 compared to the Tyrode’s buffer control). Neither of the AM particles, the CM 100 nm or the UM 50 nm NPs caused significant P-selectin expression (P > 0.05 compared to the Tyrode’s buffer control).

The results for mean fluorescence intensity (Fig 3.8 C + D) matched the percentage of platelets expressing P-selectin albeit with decreased statistical significance. Resting platelet P-selectin levels was < 15 % indicating that there was limited pre-activation during the preparation procedures.

NP interference with the assay was assessed by measuring light scattering in the absence of cells. No interference or auto fluorescence was detected.
Figure 3.7. Dense granule release by engineered nanoparticles.

Isolated human platelets were loaded with $[^3]$H-5HT and stimulated with 50 nm (A), 100 nm (B) nanoparticles (15 - 125 µg mL$^{-1}$), gold (5 µg mL$^{-1}$), thrombin (1 U mL$^{-1}$) or Tyrode’s buffer for 3 minutes. Scintillation counts were measured in cell free supernatant. Platelets were also stimulated with NPs (125 µg mL$^{-1}$), thrombin (1 U mL$^{-1}$) or vehicle (DMSO 0.1%) following incubation with Bisindolylmaleimide I (10 µM) for 5 minutes to confirm that $[^3]$H-5HT release was protein kinase C mediated. Data are presented as box-and-whisker plots, *P < 0.05, **P < 0.01, ***P < 0.001 compared to Tyrode’s buffer or vehicle control using a Friedman test and Dunn’s comparison. AM = amine-modified, CM = carboxy-modified, UM = unmodified, Thr = thrombin, Tyr = Tyrode’s buffer, Veh = vehicle, BIM – 1 = Bisindolylmaleimide I and NS = non-significant. N = 4-5.
Chapter Three – Results

Figure 3.8. Nanoparticle induced P-selectin expression in isolated platelets

Isolated human platelets were exposed to 50 nm (A + C) and 100 nm (B + D) nanoparticles (15-60 µg mL⁻¹), thrombin (1 U mL⁻¹), gold (5 µg mL⁻¹) or Tyrode’s buffer and p-selectin expression was measured using flow cytometry. Platelets were identified using an anti-CD41 antibody with a FITC fluorophore and P-selectin surface expression was detected with an anti-CD62P antibody conjugated onto a PE fluorophore. The platelet population was gated using an unstained control. Data are presented as box-and-whisker plots of percentage of platelets expressing P-selectin (A + B) or mean fluorescence intensity (C + D). * P < 0.05, ** P < 0.01 compared to Tyrode’s buffer control using a K-W test with Dunn’s comparison. AM = amine-modified, CM = carboxy-modified, UM = unmodified, NP = nanoparticle, Thr = thrombin, Tyr = Tyrode’s buffer. N = 4-5.
3.2.8. Thrombin induced platelet aggregation *in-vivo*

A submaximal concentration of thrombin was established in the mouse model of platelet aggregation as described in section (section 2.2.12), to investigate the effects of the AM 50 nm NPs on thrombin induced platelet aggregation *in-vivo*.

Isolated mouse platelets were radiolabelled with $^{111}$indium oxine and *i.v.* infused into an anesthetised recipient mouse prior to injection with increasing *i.v.* doses of thrombin (16, 32 and 64 U kg$^{-1}$). Scintillation counts were recorded for 10 minutes following each administration. Thrombin induced a typical aggregation trace, with an initial increase in scintillation counts followed by a steady decline indicating platelet aggregation and dissolution (Fig 3.9 A). Thrombin produced dose dependent platelet aggregation *in-vivo* which was evident as both percentage maximal aggregation (Fig 3.9 B) and AUC (Fig 3.9 C). Significant differences were detected between 16 and 64 U kg$^{-1}$ for both percentage maximal aggregation (P < 0.05) and AUC (P < 0.01). The dose 32 U kg$^{-1}$ was chosen as a submaximal dose for subsequent *in-vivo* studies.

3.2.9. Enhancement of thrombin induced platelet aggregation by amine-modified 50 nm nanoparticles *in-vivo.*

*i.v.* infusion of the AM 50 nm NPs (1.0 µg mL$^{-1}$) did not cause platelet aggregation (Fig 3.9 C) however following *i.v.* infusion of a submaximal concentration of thrombin (32 U kg$^{-1}$) (established in section 3.2.8), the AM 50 nm particles significantly enhanced the AUC (P < 0.05 compared to the saline control) (Fig 3.10 B). No significant changes were detected for the percentage maximal aggregation response for thrombin induced aggregation following *i.v.* infusion of the AM 50 nm particle (P > 0.05 compared to the saline control) (Fig 3.10 A). Experiments were not conducted for the UM 50 nm nanoparticle due to the high mortality rate, as the BALB/c mice did not tolerate the Urethane anaesthetic well.

3.2.10. Enhancement of collagen induced platelet aggregation by amine-modified 50nm nanoparticles *in-vivo.*

*i.v.* infusion of the AM particles or a PLNP control (UM 50 nm particles) (1.2 µg mL$^{-1}$) in C57BL/6 mice did not cause platelet aggregation (Fig 3.11 C) however following *i.v.* infusion of a submaximal concentration of collagen (50 µg kg$^{-1}$) (previously established during MRes project), the AM 50 nm particles significantly enhanced the percentage maximal aggregation response (P < 0.05 compared to the saline control) whereas no changes were detected for collagen induced aggregation following *i.v.* infusion of the UM particles (P > 0.05 compared to the saline control) (Fig 3.11 A). No significant differences in AUC were identified for either the AM or UM 50 nm particles (P > 0.05 compared to the saline control) (Fig 3.10 B).
Figure 3.9. Thrombin induced platelet aggregation in-vivo.

Isolated platelets were radiolabelled with $^{111}$indium oxine and infused into anesthetised recipient BALB/c mice prior to injection with increasing concentrations of thrombin (i.v. 16, 32 and 64 U kg$^{-1}$). Responses were recorded for 10 minutes as changes in scintillation counts over time (A). Platelet aggregation was assessed by changes in percentage maximum increase in scintillation counts (B) or area under the curve (AUC) (C). Data are presented as median ± interquartile range (B+C) or representative traces (A). *$P < 0.05$, **$P < 0.01$ for indicated comparisons using a K-W test with Dunn’s comparison test. $N = 4 - 3$. 

88
Figure 3.10. Enhancement of thrombin induced platelet aggregation by amine-modified 50 nm nanoparticles in-vivo.

Isolated platelets were radiolabelled with $^{111}$indium oxine and infused into anesthetised recipient BALB/c mice prior to injection of amine-modified NP (i.v. 1.2 µg mL$^{-1}$) or saline (0.9% NaCl). These mice were subsequently infused with thrombin (i.v. 32 U kg$^{-1}$) and responses were measured as changes in scintillation counts over time (C). Platelet aggregation was calculated as percentage maximum changes in scintillation counts (A) and area under the curve (AUC) (B). Data are presented as median±interquartile range (A+B) or representative traces (C). * P < 0.05 compared to saline control using a Mann Whitney test. NS = non-significant, AM = amine-modified, Thr = thrombin, Sal = saline. N = 4.
Figure 3.11. Enhancement of collagen induced platelet aggregation by amine-modified 50nm nanoparticles in-vivo.

Isolated platelets were radiolabelled with $^{111}$indium oxine and infused into anesthetised recipient mice prior to injection of AM, UM 50 nm nanoparticles (i.v. 1.2 µg mL$^{-1}$) or saline (0.9% NaCl). These mice were subsequently infused with collagen (i.v. 50 µg kg$^{-1}$) and responses were measured for 10 minutes and recorded as changes in scintillation counts over time (C). Platelet aggregation was calculated as percentage maximum changes in scintillation counts (A) and area under the curve (AUC) (B). Data are presented as a representative trace demonstrating changes in counts over time (C) or box-and-whisker plots, * P < 0.05 compared to saline control using a Mann Whitney test. AM = amine-modified, UM = unmodified, Coll = collagen, Sal = saline, NS = non-significant. N = 4-5.
3.3.0. Discussion

The emergence of nanomedicine means that engineered NPs will be administered into the circulation and will therefore come into direct contact with components of the blood and vasculature. Consequently, there is a need to understand how engineered NPs may interact with and influence these components. Nanostructures such as combustion-derived DEP and elemental NPs such as silver have been demonstrated to modulate platelet behaviour by inducing and enhancing platelet aggregation and therefore potentially pose a thrombotic risk (Solomon et al., 2013, Jun et al., 2011). The aim of this chapter was to assess whether engineered NPs could pose a similar risk by affecting platelet function and whether certain physical and chemical characteristics make them more thrombogenic.

This chapter determined the effects of model engineered NPs with different physicochemical characteristics upon platelet aggregation in-vitro and in-vivo and established whether this was associated with platelet activation events such as granule release. The main findings are that engineered NPs of varying surface charges and sizes can induce platelet aggregation in-vitro and subthreshold concentrations of NPs can enhance agonist-induced platelet aggregation in-vitro and in-vivo. Furthermore, the ability and the potency by which these NPs effect platelet function appears to be dependent on a distinct combination of surface charge and size.

Model polystyrene NPs have been used by many groups to investigate the fundamental impact of NPs on biological systems (Oslakovic et al., 2011, Ruenraroengsak et al., 2011, Sarlo et al., 2009, Walczak et al., 2014, Lunov et al., 2011, Lundqvist et al., 2008, Mayer et al., 2009). This is because they are homogenous particles with very distinct combinations of sizes and surface chemistries and which therefore allow the investigation of individual characteristics such as size and surface functionalization.

The NPs in the current study have been previously characterised in physiological solutions and H$_2$O (Ruenraroengsak et al., 2011) however it was important to observe the agglomeration state and to confirm the morphology and size of the particles in our experimental buffers. Predictably, the charged AM and CM particles were present as either simple complex agglomerates and individual particles respectively. This was most likely due to electrostatic repulsion between the particles. The UM particles were heavily agglomerated due to the lack of this repulsion. These results are in agreement with the zeta potentials and polydispersion indices (PDI) previously reported (Ruenraroengsak et al., 2011). This initially indicated that the more individualised NPs would display a larger surface area and therefore have the maximal amount of surface which could interact with the platelets. However, this was not the case, as the UM 100 nm NPs was largely agglomerated in the experimental buffers but had the greatest ability to induce platelet aggregation.
Chapter Three – Results

The effects of NP surface chemistry on platelet function have been previously conducted using polystyrene NPs; however, this was with a single concentration (260 µg mL\(^{-1}\)) that was estimated to be much higher than expected exposure concentrations (McGuinness et al., 2011, Gabizon et al., 1994). The work by McGuinness et al also measured platelet-platelet aggregates using flow cytometry rather than conventional born optical aggregometry. For comparative purposes and to validate the findings from this previous study, I stimulated isolated human platelets with the same concentration (260 µg mL\(^{-1}\)) and measured platelet function using optical platelet aggregometry. All of the NPs tested caused measurable platelet aggregation at this concentration, which contradicts the findings by McGuinness et al who reported that only the CM and AM 50 nm NPs caused platelet aggregation (McGuinness et al., 2011). However, at this concentration, all the 100 nm NPs interfered with light transmission following the addition of NPs to platelet suspensions, indicated by a steep increase in light transmission, which was above the amount normally observed for platelet shape change (Fig 3.3 C). This interference with the light transmission was not observed following the addition of the 50 nm NPs (Fig 3.2 B), and I speculate that the reason for this was due to the larger particles being more opaque (particles large enough to scatter light) and thus obstructing the light transmission compared to the smaller 50 nm particles. NP interference with the light transmission was not detected at lower concentrations and therefore concentrations < 260 µg mL\(^{-1}\) were selected for future experiments.

At concentrations < 260 µg mL\(^{-1}\), all the NPs investigated caused concentration-dependent platelet aggregation however their potencies, which were assessed by the calculation of EC\(_{50}\) values, varied depending on the distinct combination of NP surface chemistry and size. The concentration dependent platelet aggregation induced by the NPs was comparable to conventional platelet agonists. Additionally, NP-induced platelet aggregation was heavily influenced by biological variability, similar to the platelet aggregation induced by conventional agonists. The large error bars reflect this variability between donors (Fig 3.1 and Fig 3.4) and may have complicated the discrimination between the effects of individual NPs. The potencies for the 50 nm NPs are ranked CM > AM > UM whereas the 100 nm NPs are ranked as UM > AM > CM. The ability of NPs to induce dense and alpha granule release was also dependent on NP physicochemical characteristics and largely matched the platelet aggregation data, as only the most potent particles, the 50 nm CM and 100 nm UM NPs, induced concentration-dependent 5-HT release and P-selectin expression. Therefore, the ability of the NPs to cause platelet activation and aggregation was dependent on a distinct combination of NP size and surface chemistry.

Platelet aggregometry involves the measurement of the physical interaction between platelets which does not necessarily involve the activation of cell-signalling pathways. Alternatively, platelet granule release involves the activation of signalling pathways and rises in intracellular Ca\(^{2+}\), therefore it appears that the 50 nm CM and the 100 nm UM NPs can initiate cell signalling events and platelet activation. Due to the differences in potencies and the ability to induce granule release, there appears to be variation between the mechanisms underlying NP-induced platelet aggregation. This variation in
potencies between the particles may be due to how the NPs interact with the cell membrane and/or differences in NP internalisation into the platelets, as these processes are dictated by NP charge and size. For example, the surface functionalization of lipid NPs with anionic-groups promotes uptake into endothelial cells via calveolin-dependent mechanisms whereas cationic-groups are reported to promote clathrin-mediated endocytosis (Dwivedi et al., 2014, Lorenz et al., 2006, Voigt et al., 2014, Panyam et al., 2002). Additionally, other groups have found that NPs of the same composition but different sizes can have different biological effects (Dwivedi et al., 2014). These mechanisms warrant further investigation and visualisation of the NP-platelet interactions may shed more light on this process. Moreover, additional experiments are required to establish whether the platelet aggregation induced by the NPs is associated with other platelet-activation events such as the involvement of the integrin αIIbβIIIa and secondary agonists.

To date, there is conflicting information regarding the role of NP surface charge on platelet function. Positively charged macromolecules have been demonstrated to cause platelet aggregation and 5-HT release (Taketomi and Kuramoto, 1978). Alternatively, greater negative charges and hydrophobicity have been suggested to promote a greater amount of platelet aggregation than other surface modifications (Miyamoto et al., 1989). The current results differ from those reported by McGuiness et al, who found that the UM 50 nm did not cause platelet-platelet aggregates and that the AM and CM NPs produced similar amounts of platelet-platelet aggregates. However, similarly to our study, the work by these authors also demonstrated that the 50 nm CM NPs could induce P-selectin expression in isolated platelets and whole blood. The discrepancies between the results may be due firstly to the differences in the concentrations used as the ones in the present study are considerably lower. Exceptionally high concentrations (0.5 - 2 mg mL⁻¹) of polystyrene NPs have been used in other studies investigating the effect of NPs on haemocompatability and similarly to McGuiness et al, light transmission was not used to measure platelet aggregation (Mayer et al., 2009, Underwood et al., 1997). The current study conducted experiments using varying lower concentrations of NPs to establish a threshold concentration which effect platelets and to understand the impact of potentially more realistic NP concentrations on platelet function.

The second reason for these discrepancies may be due to the differences in the methodologies used, as the study by McGuiness et al, used flow cytometry to measure platelet aggregates compared to the current study which used optical platelet aggregometry. The authors identified cell-surface markers and so did not specifically measure platelet function (McGuiness et al., 2011).

A similar study which investigated the effect of engineered NPs on platelet function by Nemmar et al, used polystyrene NPs and reported that 60 nm AM polystyrene NPs were able to initiate platelet aggregation in PRP following 5 minute incubations (50 or 100 μg mL⁻¹) using optical platelet aggregometry. However, this was not observed for the CM and UM 50 nm NPs. This result contrasts to the current study, as none of the 50 nm or 100 nm NPs produced platelet aggregation when introduced to PRP, even at a higher concentration (125 μg mL⁻¹) (Nemmar et al., 2002b). The
Chapter Three – Results

differences between the results may be due to the pre-incubation of the platelets with the NPs for 5 minutes before measuring platelet aggregation by the previous authors. In the current study, platelet aggregation was measured immediately following the addition of the NPs, similar to when using conventional platelet agonists.

The work in chapter three is in agreement with work that demonstrated that the size and surface charge are important for dictating platelet reactivity to another nanostructure, Polyamidoamine (PAMAM) dendrimers. Although the negatively and neutral charged PAMAM dendrimers did not cause any platelet aggregation or activation events when introduced into PRP, the larger cationic PAMAM dendrimers (7.5 nm) caused concentration-dependent platelet activation and ATP release with no P-selectin expression. The authors also established that the ability for these dendrimers to cause platelet aggregation was proportional to the amount of surface amines (Dobrovolskaia et al., 2012, Mayer et al., 2009). It has been observed by other groups that NP’s of the same composition but different sizes can exert different biological effects (Dwivedi et al., 2014, Corbalan et al., 2012). Another aim of the current work was to determine the effect of a low and potentially more realistic concentrations of NP on agonist-induced platelet aggregation. The AM 50 nm NP significantly enhanced thrombin-induced platelet aggregation in an isolated platelet suspension however more importantly; this particle was able to enhance ADP-induced platelet aggregation in the presence of plasma proteins and both collagen and thrombin induced-platelet aggregation in a more complex in-vivo environment. As previously mentioned, NPs bind many plasma proteins such as albumin which creates a corona around the particle and can abolish or promote biological interactions (Lundqvist et al., 2008, Ruenraroengsak et al., 2011). However, the presence of plasma proteins and other blood cells did not appear to be an essential factor in dictating the effects of the AM 50 nm NPs on agonist-induced platelet aggregation, although it did appear to inhibit platelet aggregation when NPs were administered on their own. The combination of NP size and surface charge appeared to be a defining factor for the NPs ability to enhance platelet aggregation, as the cationic 100 nm particles has no effect on agonist-induced platelet aggregation. Furthermore, the enhanced responses did not appear to be a general NP effect, as UM 50 nm particle control, which had a similar composition and size to the 50 nm AM NP, did not alter platelet aggregation. This enhanced platelet aggregation was also confirmed to be receptor-mediated as it was inhibited by the integrin αIIbβIII antagonist eptifibatide.

This sensitivity to eptifibatide suggests that the AM 50 nm NPs promoted cell signalling events such as integrin activation which may have occurred through the physical interaction between the NPs and cell surface receptors or intracellular signalling molecules. Interestingly, NPs have also been demonstrated to inhibit integrin-mediated platelet responses in-vitro and in-vivo (Shrivastava et al., 2009).

The mechanism behind the enhanced agonist-induced platelet aggregation cannot be assumed to be same for the in-vitro or in-vivo experiments. This is due to the vastly more complex in-vivo
environment, where the NPs could interact with multiple cell types and biomolecules, including platelets.

The present in-vivo results are in agreement with Nemmar et al, who found that the i.v. administration of AM 60 nm polystyrene NPs enhanced experimental thrombosis in a FeCl₃ vascular injury hamster model. Similarly, no effects were observed following infusion of the UM 60 nm polystyrene NPs (Nemmar et al., 2002b). Additionally, Nemmar et al found that the AM 50 nm NPs dose-dependently enhanced ADP induced platelet aggregation, albeit at much higher concentrations than the present work (12.5 - 100 μg mL⁻¹) (Nemmar et al., 2002b). Therefore, the ability of positively charged NPs between 50-60 nm to modulate platelet function and promote thrombosis is supported by the current study and by work reported in the literature.

Additionally, NP physicochemical properties have been demonstrated to dictate their effects on secondary haemostasis, by activation of the coagulation cascade. Anionic NPs > 50 nm were reported to activate the intrinsic coagulation pathway and lead to thrombin generation and subsequent fibrinogen cleavage. This effect on coagulation was both charge and size dependent (Sanfins et al., 2014). Furthermore, exposure to both anionic and cationic NPs, but not uncharged NPs has been demonstrated to cause exposure of phosphatidylserine on the platelet surface, initiating platelet-mediated thrombin formation and coagulation (McGuiness et al, 2011).

NP surface charge and size heavily influences the proteins which make up the protein corona. For example, the protein corona on 100 nm AM NPs has been shown to contain certain coagulation factors whereas 50 nm AM NPs contained more lipoproteins (Lundqvist et al., 2008). The protein corona could increase the reactivity of NPs and further enhance the cardiovascular risks.

Therefore, I recommend, based on the findings from my study, that NPs which are intended to be introduced into the systemic circulation should be tested for their ability to cause platelet aggregation and coagulation, in order to establish their capacity to cause a thrombotic risk. A combination of in vitro platelet aggregometry in isolated cell suspensions and whole blood should be conducted in association with in vivo models of platelet aggregation and potentially vascular injury models. This would provide information regarding NP effects on both primary and secondary haemostasis in the presence and absence of plasma proteins and therefore determine their potential toxic effects.

Currently, there is limited information regarding the concentrations of NPs that may enter the blood either intentionally through medical procedures or unintentionally via exposure to NPs in commercial products. However, from the in-vitro experiments in the present study and the literature, it appears that NP concentrations are extremely important in dictating their biological effects. Therefore, to put the concentrations used in the current study into context we can use data available for nanomedicines used in the clinic. For example, pharmacokinetic data on the nanoparticulate cancer therapy DOXIL®, which is liposome-encapsulated doxorubicin (~80–100 nm), demonstrates plasma concentrations >10 μg ml⁻¹ (Gabizon et al., 1994). This concentration is higher than the concentration used in-vivo
Chapter Three – Results

$\sim 1 \mu g \text{ ml}^{-1}$ (assuming a circulating mouse blood volume of $1\text{–}1.5 \text{ mL}$) and the in-vitro agonist aggregation studies ($2 \mu g \text{ mL}^{-1}$).

3.3.1. General limitations

One of the main limitations to this work is that optical platelet aggregometry involves using PRP or isolated platelet suspensions which do not contain the other components of blood such as erythrocytes and plasma proteins. These components have been demonstrated to interact with NPs, therefore the absence of them may permit more NPs to interact with the platelets, exaggerating any functional effects (He et al., 2014, Lundqvist et al., 2008, McGuinnes et al., 2011). In addition, there is no vascular endothelium in these in-vitro studies and therefore no endogenous vascular inhibitors exerting their effects on the platelets which may produce an exaggerated platelet response to NPs. However, one of the main advantages of the present work is the inclusion of in-vivo studies to observe the effects of NPs in the physiologically most relevant environment. The present study has extended the current knowledge regarding NP effects on platelet function by using a range of concentrations, especially low concentrations, and investigating the role of NP size. Additionally, my work also gives us insight into the effects of engineered NPs on platelet function in-vivo. My results have implications for development of NPs for medical applications, as NPs ~ 50 nm in diameter and positively charged may potentially pose a thrombotic risk by enhancing platelet aggregation. Additionally, my study also highlights the potential difficulty in using NPs as they can interfere with certain assays and emphasises the importance of running appropriate control.
Chapter Four

The mechanisms underlying nanoparticle induced modulation of platelet function
Chapter Four

4.1.0. Introduction

I have established that engineered NPs can induce platelet aggregation and initiate signalling-mediated events such as granule secretion (chapter 3), and that these effects are dictated by a combination of NP size and surface charge. It has also been demonstrated that NPs can modulate functions in other cell types (Ruenraroengsak et al., 2011, McGuinnes et al., 2011), however there is limited information regarding the underlying mechanisms.

NPs have been demonstrated to initiate cell signalling pathways such as transcription factor Nf-κb signalling (Skuland et al., 2014), the p38 MAPK pathway (Azad et al., 2013) and PKC signalling (Lee et al., 2011), which may account for NP-induced cellular responses. Furthermore, damage to the cell lipid bilayer, leading to the leakage of cellular contents such as stimulatory nucleotides has been suggested as one mechanism of cell activation, and NPs have been reported to disrupt the plasma membrane in alveolar epithelial cells (Ruenraroengsak et al., 2011) and in erythrocytes (McGuinnes et al., 2011).

Platelet aggregation can be initiated by multiple agonists through numerous cell-surface receptors and signalling pathways. While individual platelet agonists signal through specific receptors, many share common signalling pathways leading to final signalling-mediated events such as granule secretion, release of secondary agonists and integrin-activation.

Additionally, agonist mediated platelet aggregation is sensitive to biomolecules synthesised in the surrounding vascular endothelium such as NO and PGI\textsubscript{2}. Under normal physiological circumstances platelets are kept in a quiescent state by these biomolecules to avoid inappropriate platelet aggregation and thrombus formation.

The objective of this chapter was to investigate the mechanisms underlying the modulation of platelet activation by engineered NPs and the role of physicochemistry. This was addressed by investigating the involvement of important platelet signalling molecules and secondary agonists in NP-induced platelet aggregation, and the sensitivity of aggregation to PGI\textsubscript{2} and NO. Moreover, the physical interaction between NPs and platelets was assessed by observing the NP-platelet interface by TEM and by measuring plasma membrane damage.
4.1.1. Aims of chapter four

- To establish whether the platelet aggregation induced by NPs with different physicochemistries involves the integrin $\alpha_{IIb}\beta_{IIIa}$.

- To assess the involvement of secondary agonists in platelet aggregation induced by NPs with different physicochemistries.

- To examine the sensitivity of platelet aggregation induced by NPs with different physicochemistries to the endogenous vascular regulators NO and PGI$_2$.

- To evaluate whether NPs with different physicochemistries disrupt the platelet plasma membrane following both acute and chronic exposures.

- To observe how NPs with different physicochemical properties physically interact with platelets.
Chapter Four – Results

4.2.0. The role of the integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$ and extracellular calcium in nanoparticle induced platelet aggregation.

Platelet aggregation induced by the AM, CM and UM 50 nm and 100 nm NPs (60 $\mu$g mL$^{-1}$) and the thrombin control (1 U mL$^{-1}$) was significantly inhibited by the $\alpha_{\text{IIb}}\beta_{\text{III}}$ antagonist eptifibatide (0.06 $\mu$g mL$^{-1}$) ($P < 0.05$ compared to time-matched Tyrode’s buffer control), but not the AM 50 nm particle. No significant inhibition was detected for the AM 50 nm particle ($P > 0.05$ compared to the Tyrode’s buffer time-matched control) (Fig 4.1 A). Similar results were found with the Ca$^{2+}$ chelator EGTA (2 mM) as all the platelet aggregation induced by the AM, CM and UM 50 nm and 100 nm NPs and both collagen (5 $\mu$g mL$^{-1}$) and thrombin (1 U mL$^{-1}$) controls were significantly inhibited ($P < 0.05$ compared to the Tyrode’s buffer time-matched control), excluding the AM 50 nm particle (Fig 4.1 B). Platelet aggregation for the AM 50 nm particle was significantly augmented following treatment with EGTA ($P < 0.05$ compared to the Tyrode’s buffer time-matched control) (Fig 4.1 B).

4.2.1. Nanoparticle induced lactate dehydrogenase release

To investigate whether membrane disruption occurred under the same experimental conditions that were used to measure in-vitro platelet aggregation, isolated human platelets were exposed to 50 nm (Fig 4.2 A) and 100 nm (Fig 4.2 B) NPs (2 - 125 $\mu$g mL$^{-1}$, only the highest concentration is presented) for 3 minutes and LDH was measured in cell free supernatant. None of the engineered particles caused significantly higher LDH release than either the vehicle or the thrombin control (1 U mL$^{-1}$) ($P > 0.05$ compared to the Tyrode’s buffer or thrombin control) but a significant decrease in LDH release was detected for the gold NP compared to the thrombin control ($P < 0.05$ compared to the thrombin control).

Cellular exposure to NPs, following proposed medical application or accidental exposure is expected to be both chronic and acute. Isolated human platelets were therefore exposed to 50 nm (Fig 4.2 C) and 100 nm (Fig 4.2 D) NPs (2 - 125 $\mu$g mL$^{-1}$, only the highest concentrations are presented) for 2 hours under static conditions and LDH was measured in cell free supernatant. The AM 50 nm and AM 100 nm NPs induced significant concentration dependent LDH release ($P < 0.05$ compared to the Tyrode’s buffer control) however none was detected for either of the CM, UM 50 nm or 100 nm particles ($P > 0.05$ compared to the Tyrode’s buffer control).

Cell-free NP controls were conducted to exclude any interference with the assay by the NPs, as this has been reported for other types of NPs (Han et al., 2011). No assay interference was detected for any of the NPs investigated (data not shown).
Figure 4.1. Effects of eptifibatide and EGTA on nanoparticle induced platelet aggregation

Isolated human platelets were stimulated with 50 nm and 100 nm nanoparticles (60 µg mL⁻¹), collagen (5 µg mL⁻¹) or thrombin (1 U mL⁻¹) following a 5 minute incubation with the integrin α_{IIb}β_{III} antagonist eptifibatide (0.06 µg mL⁻¹) (A) or the Ca^{2+} chelater EGTA (2 mM) (B). Time matched Tyrode’s buffer vehicle controls were included in for every NP or agonist. Changes in light transmission were measured for 3 minutes. Data are presented as box-and-whisker plots. *P < 0.05 compared to vehicle control using a Wilcoxon signed rank test. AM = amine-modified, CM = carboxyl-modified, UM = unmodified, Coll = collagen, Thr = thrombin, NP = nanoparticle, Tyr = Tyrode’s buffer, NS = non-significant. N = 6
Figure 4.2. Effect of nanoparticles on lactate dehydrogenase release by isolated platelets

Isolated human platelets were stimulated for 3 mins (Acute) with (A) 50 nm or (B) 100 nm AM, CM, UM or gold NPs (125 μg ml⁻¹), or thr (1 U ml⁻¹) or for 2 h (Chronic) with (C) 50 nm and (D) 100 nm NPs (15-125 μg mL⁻¹). Membrane integrity was assessed by measuring lactate dehydrogenase (LDH) release from cell free supernatant. LDH release is expressed as a percentage of lysed controls. AM = amine-modified, CM = carboxyl-modified, UM = unmodified, Coll = collagen, Con = control, NP = nanoparticle, Tyr = Tyrode’s buffer, NS = non-significant. Data are represented as box-and-whisker plots, for all experiments. *P < 0.05, **P < 0.01 compared to Tyrode’s buffer control (C+D) or thrombin control (A+B) using using a Friedman test with Dunn’s comparison. N = 5-4
4.2.2. The role of ADP, TXA\(_2\) and PKC in nanoparticle induced platelet aggregation

Platelet aggregation induced by the CM 50, UM 50, AM 100 and UM 100 particles (60 µg mL\(^{-1}\)), was significantly inhibited by the ADP scavenger apyrase (8 U mL\(^{-1}\)) however, of the control agonists, only ADP-induced platelet aggregation (1 µM) was significantly inhibited by apyrase (P < 0.05 compared to the Tyrode’s buffer time-match control) (Fig 4.3 A). Neither the AM 50 or CM 100 NP (60 µg mL\(^{-1}\)), induced platelet aggregation was inhibited by apyrase (P > 0.05 compared to the Tyrode’s buffer time-matched control).

CM 50, AM 100 and UM 100 NPs (60 µg mL\(^{-1}\)) stimulated platelet aggregation were inhibited by the non-selective COX inhibitor indomethacin (10 µM), as was the collagen control (5 µg mL\(^{-1}\)) (P < 0.05 compared to the Tyrode’s buffer time-matched control) (Fig 4.3 B). Platelet aggregation produced by the AM 50, UM 50 and CM 100 NPs (60 µg mL\(^{-1}\)) and thrombin (1 U mL\(^{-1}\)) was unaffected by indomethacin (P > 0.05 compared to the Tyrode’s buffer time-matched control) (Fig 4.3 B).

To investigate the involvement of PKC signalling in NP-induced platelet aggregation, isolated human platelets were incubated with either the broad spectrum PKC inhibitor BIM-1 (10µM) or DMSO (0.2 %) (Fig 4.3 C). Inhibition of PKC had no effect on platelet aggregation generated by the AM 50 nm particle (P > 0.05 compared to the DMSO (0.2 %) time-matched control). All other NP (60 µg mL\(^{-1}\)) induced platelet aggregation and both collagen (5 µg mL\(^{-1}\)) and thrombin (1 U mL\(^{-1}\)) controls were significantly inhibited by BIM-1 (10µM) (P < 0.05 compared to the DMSO (0.2 %) time-matched control).

4.2.3. Inhibition of nanoparticle induced platelet aggregation by endogenous vascular regulators is dependent on nanoparticle physicochemistry.

Platelet aggregation generated by the AM, CM and UM 50 nm and 100 nm NPs (60 µg mL\(^{-1}\)),and the collagen control (5µg mL\(^{-1}\)) was significantly inhibited by the NO donor sodium nitroprusside (SNP) (10 µM) (P < 0.05 compared to time-matched Tyrode’s buffer control). SNP had no effect on platelet aggregation generated by the AM 50 nm particle (P > 0.05 compared to time-matched Tyrode’s buffer control) (Fig 4.4 A). Similar results were found for the PGI\(_2\) mimetic iloprost (10 nM), as iloprost had no effect on platelet aggregation generated by the AM 50 nm particle (P > 0.05 compared to time-matched Tyrode’s buffer control) (Fig 4.4 B). All other NP induced platelet aggregations and the collagen control were significantly inhibited following incubation with iloprost (P < 0.05 compared to the time-matched vehicle control).
Figure 4.3. The role of ADP, TXA2 and PKC in nanoparticle induced platelet aggregation

Washed human platelets (or platelet rich plasma (A)) were stimulated with 50 nm and 100 nm NPs (60 µg mL⁻¹), collagen (5 µg mL⁻¹), thrombin (1 U mL⁻¹) or ADP (platelet rich plasma (A)) following a 5 minute incubation with the enzyme apyrase (8 U mL⁻¹) or Tyrode’s buffer (A) or the COX inhibitor indomethacine (10 µM) or Tyrode’s buffer (B), or the PKC inhibitor BIM-1 (10 µM) or DMSO (0.2 %) (C). Changes in light transmission were measured for 3 minutes. Data are presented as box-and-whisker plots. *P < 0.05 compared to time matched controls using a Wilcoxon signed rank test. AM = amine-modified, CM = carboxyl-modified, UM = unmodified, Coll = collagen, Thr = thrombin, ADP = adenine diphosphate, Indo = indomethacine, BIM-1 = Bisindolylmaleimide-1, NP = nanoparticle, NS = non-significant. N = 6.
Figure 4.4. Nanoparticle induced platelet aggregation sensitivity to sodium nitroprusside and iloprost.
Isolated human platelets were exposed to 50 nm and 100 nm AM, CM and UM NP (60 µg mL\(^{-1}\)), collagen (5 µg mL\(^{-1}\)) following a 5 minute incubation with either a NO donor SNP (10 µM) or Tyrode’s buffer (A) or the PGI\(_2\) mimetic iloprost (10 nM) or vehicle (0.1 % methyl acetate) (B). Changes in light transmission were measured for 3 minutes. Data are presented as box-and-whisker plots. *P < 0.05 compared to time-matched controls using a Wilcoxon signed rank test. AM = amine-modified, CM = carboxyl-modified, UM = unmodified, Coll = collagen, NP = nanoparticle, SNP = sodium nitroprusside, ns = non-significant. N = 6.
4.2.4. The physical interaction between platelets and nanoparticles

Platelets incubated with Tyrode’s buffer (Fig 4.5 A – C) were observed mainly in the typical resting discoid state with retained intracellular vesicles. Platelets which were incubated with AM 50 nm NPs were also observed in a relatively un-activated state, retaining their intracellular vesicles. The NPs appeared to be present in the vacuoles (Fig 4.5 F) and attached to the extracellular plasma membrane (Fig 4.5 D - F). Additionally, adjacent non-activated platelets appeared to be connected via AM 50 nm NPs (Fig 4.5 D and E).

Platelet and CM 50 nm NP interactions were via the extracellular plasma membrane. The platelets were degranulated with initial filopodia development indicting that platelet activation had occurred (Fig 4.5 G – I).

UM 50 nm NPs seemed to interact in a similar manner to the CM 50 nm NPs, they were observed in contact with the extracellular plasma membrane of degranulated, activated platelets (Fig 4.5 J – K). The 100 nm NPs had the advantage of being larger and therefore easier to identify. The AM 100 nm NPs were detected within vacuoles and attached to the platelet extracellular plasma membrane. Although these platelets displayed early signs of filopodia development, they appeared to have retained most of their granules (Fig 4.5 L – M).

Similar results were observed for the CM 100 nm NPs. These were found contained within the vacuoles (Fig 4.5 P and Q) and attached to the extracellular plasma membrane (Fig 4.5 P and R). These platelets had retained most of their granules and presented with small membrane protrusions. Conversely, the platelets incubated with the UM 100 nm NPs were found in an activated-state with few to no granules present. The NPs were only displayed on the surface of the platelet, with none detected inside (Fig 4.5 S – U).
Chapter Four – Results

[Images of microscopic sections labeled J, K, L, M, N, O, P, Q, R, with annotations and measurements]
Figure 4.5. Electron micrographs displaying the physical interaction between isolated platelets and nanoparticles

Stained sections displaying varying magnifications (5000 x, 10,000 x and 15,000 x magnification) of platelets exposed 50 nm and 100 nm NPs (50 µg ml⁻¹), imaged by transmission electron microscopy. (A - C) shows a control platelet in resting conformation. Images display interactions between platelets and amine-modified 50 nm NPs (D - F), carboxyl-modified 50 nm NPs (G – I), unmodified 50 nm NPs (J – L), amine-modified 100 nm NPs (M – O), carboxyl-modified 100 nm nanoparticles (P – R) and unmodified 100 nm NPs (S – U). NPs were observed in close proximity and in contact with the extracellular surface platelets (blue arrows) and contained in the vacuoles (green arrows).
Chapter Four – Results

4.3.0. Discussion

It has been previously established that engineered NPs could induce platelet aggregation and dense granule release and their ability and potencies were dictated by their physicochemical properties. Therefore, the aim of this chapter was to investigate the underlying mechanisms.

Platelet aggregation occurs through the activation of signalling pathways, which lead to conformational changes in the cell surface integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$. The subsequent binding of integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$ to its ligand fibrinogen connects adjacent platelets. Additionally, the integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$ requires the divalent cation $\text{Ca}^{2+}$ for correct fibrinogen binding (Smith et al., 1994). It was important to initially establish whether the platelet aggregation induced by the NPs involved the integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$, and was not due to some passive alternative process, such as agglomeration. Both the integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$ antagonist eptifibatide and the $\text{Ca}^{2+}$ chelator EGTA significantly inhibited all NP-induced platelet aggregation, apart from that induced by the AM 50 nm NP. This indicates that all NP-induced platelet aggregation (excluding the AM 50 nm) is true receptor-mediated platelet aggregation. The platelet aggregation induced by the AM 50 nm NP was not inhibited by any of the inhibitors investigated; suggesting that this was not true signalling mediated platelet aggregation. Additionally, the lack of platelet activation events by the AM 50 nm NP is supported by the reduction in platelet aggregation induced by this particle in chapter 4 compared to chapter 3. This may have been due to changes in particle stability as agglomeration of the particles may have reduced the surface area which was available to interact with the platelet.

Extracellular $\text{Ca}^{2+}$ entry, PKC activation and integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$-mediated platelet aggregation are all considered to be final common pathways in the platelet activation process. All agonist- and NP-induced platelet aggregation, was significantly inhibited by EGTA, BIM-1 and eptifibatide. However, there was variation between the involvement of secondary agonists in both NP and agonist-induced platelet aggregation. This indicates that the engineered NPs initiate different platelet-signalling pathways which are dependent on their size and surface chemistry. Interestingly, the platelet aggregation induced by the AM 100 nm NP appeared to be particularly sensitive to inhibition of COX, ADP and PKC, as the platelet aggregation was consistently low following incubation with the inhibitors. It appears that secondary agonist release is essential in the platelet aggregation induced by this particle, suggesting that although the AM 100 nm NP does not cause platelet granule release it can initiate intracellular signalling events.

Integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$ was reported to be essential in platelet aggregation induced by carbonaceous NPs such as SWCNT, MWCNT and ambient PM and similar to the present study, the involvement of PKC, TXA$_2$ and ADP was demonstrated to be particle specific (Radomski et al., 2005). Additionally, the platelet aggregation produced by these particles was also sensitive to NO and PGI$_2$ (Radomski et al.,
Furthermore, ADP but not TXA$_2$ was demonstrated to be important in platelet aggregation induced by silica-NPs and platelets (Corbalan et al., 2012). Extracellular Ca$^{2+}$ has been demonstrated to be important for platelet aggregation induced by other nanostructures, for example SWCNTs and MWCNTs produced concentration dependent platelet aggregation (in PRP) which was abolished by inhibition of SOCE channels (Semberova et al., 2009). Additionally, EGTA significantly inhibited silver NP-induced p-selectin expression (Jun et al., 2011).

In order for the NPs to cause platelet activation events such as granule release, secondary agonist release and integrin $\alpha_{IIb}\beta_{III}$ mediated platelet aggregation, they must activate cell signalling pathways. This may be due to the physical interaction between the NPs and cell surface receptors or with intracellular signalling molecules. Understanding how the NPs physically interact with the platelets, and specifically whether they adhere to the membrane or are internalised, gives insight into how the NPs cause platelet aggregation.

The 50 nm CM and UM particles and the UM 100 nm particles were observed in direct contact with the platelet extracellular membrane but none were detected inside the platelets. These platelets were displayed in a typical activated degranulated form therefore previous internalisation of these particles cannot be ruled out. The AM 50 nm particles and the CM and AM 100 nm NPs were observed both in contact with the extracellular plasma membrane and within vacuoles in the platelet cytosol.

As most platelet-agonist receptors are located on the plasma membrane, it is possible that the platelet activation induced by most of the NPs may be due to the NPs physically interacting with these receptors. Additionally, internalisation of these NPs may lead to interactions with intracellular signalling molecules, which may also lead to activation events. The presence of these NPs within vacuoles suggests that this may have been an active process, thus it appeared that the mechanism behind NP-induced platelet aggregation involves the physical interaction between the platelets and the NPs. However, although certain NPs were not detected within the platelet cytoplasm or within the vacuoles, their internalisation cannot be ruled out. As the images are representative of a specific time point, it is possible that the NPs may have either relocated out of the platelet/vacuole or subsequent activation of the platelet may have expelled the NPs via the same process of degranulation.

Additionally, due to the granular nature of platelets, it is difficult to conclusively identify NPs within the cytoplasm.

Platelets have been reported to internalise other NPs such as combustion-derived NPs such as DEP and silica NPs. These have been observed in the platelet cytosol, OCS and in contact with the extracellular plasma membrane (Solomon et al., 2013, White, 1972, Corbalan et al., 2012). Furthermore, all the NP-induced platelet aggregation studied in this chapter apart from that induced by the AM 50 NPs, was sensitive to the endogenous vascular regulators NO and PGI$_2$. NO and PGI$_2$ exert their inhibitory effects by causing increases in intracellular cGMP and cAMP respectively which inhibits many signalling pathways and events stimulated by agonists, such as intracellular Ca$^{2+}$ rises, granule secretion, cytoskeleton rearrangement and integrin activation (Feinstein and Fraser, 1975,
Antl et al., 2007). Therefore sensitivity to NO and PGI$_2$ reinforces the conclusion that all the NPs, could cause platelet aggregation through activation of canonical signalling pathways. Additionally, this means that healthy people with an intact vascular endothelium may have some protection from the influence of engineered NPs.

It has been demonstrated in various cell types (Ruenraroengsak et al., 2011, McGuinnes et al., 2011) that NPs with specific physicochemical properties can disrupt plasma membrane integrity, leading to release of cytoplasmic contents. However, none of the NPs caused significant LDH release above that released by the platelet agonist thrombin under acute experimental conditions, suggesting that the NP-induced platelet aggregation was not caused by loss of membrane integrity and by release of stimulatory molecules from platelet granules. The significant decrease in LDH release following acute exposure to the gold NP was most likely due to the lack of platelet aggregation induced by this particle. Platelet aggregation in response to thrombin and the NPs caused a baseline LDH release, potentially due to platelet membrane rearrangement which occurs during the platelet activation process. However, this membrane rearrangement may not have been present following exposure to the gold NP and therefore resulted in little LDH release.

In contrast, both AM NPs produced significant LDH release following a 2 hour incubation. As this was observed for both sizes of NP it most likely is due to the interaction between the positively charged particles and the negatively charged platelet membrane. Indeed, AM NPs were observed by ion-conductance microscopy to put holes in the plasma membrane of alveolar epithelial cells and to cause LDH release in erythrocytes (Ruenraroengsak et al., 2011, McGuinnes et al., 2011). Furthermore, cationic NPs have been demonstrated to cause LDH release and decrease cell viability in macrophages and bronchial epithelial cells (Xia et al., 2008). Following internalisations, cationic NPs have also been suggested to induce cytotoxicity by disturbing the mitochondrial membrane and disrupting the electron transport chain (Bhattacharjee et al., 2010, Xia et al., 2004).

Platelet aggregation induced by AM 50 nm NP could not be attributed to release of internal stimuli due to disturbances in the platelet membrane as there was no LDH release (acute exposure). It also does not appear to be attributable to activation of canonical platelet signalling pathways, as none of the inhibitors of such pathways inhibited the AM 50 nm NP– induced platelet aggregation. Interestingly, eptifibatide did inhibit the enhanced agonist-induced platelet aggregation by the AM 50 nm particle, suggesting that although the particle may not initiate signalling events, it can promote them.

The platelet aggregation induced by the AM 50 nm NPs can potentially be explained by the EM images which showed the AM 50 NPs in physical contact with the platelet membrane and attaching adjacent resting platelets. The platelet aggregation induced by the AM 50 nm NP may therefore be due to the physical bridging of non-activated platelets by these NPs. As this process would be highly dependent on the state of NP agglomeration, it would also account for the increased aggregation detected in the presence of EGTA, since Ca$^{2+}$ chelation would reduce the ionic content of the buffer.
Chapter Four – Results

High ionic solutes are known to increase NP agglomeration by screening the repulsive forces between them (Pavlin and Bregar, 2012). The reduction in NP agglomeration would therefore provide a larger surface area for the platelets to interact with, leading to increased platelet aggregation. In addition, bringing platelets into close proximity to each other may promote contact-dependent pathways through Eph and Ephrins. Activation of this process may explain how the AM 50 nm NP enhanced agonist-induced platelet aggregation as these pathways are shared with conventional platelet agonists such as thrombin and collagen (Prevost et al., 2004, Prevost et al., 2002). The current data which demonstrates that the platelet aggregation induced by the AM 50 nm NP is insensitive to inhibitors of conventional platelet signalling pathways are in general agreement with work using dendrimers. This work found that cationic dendrimers (7.5 nm) caused concentration-dependent platelet aggregation which was unaffected by inhibition of COX, intracellular Ca\textsuperscript{2+}, P2Y1/12, P2X, integrin \( \alpha_{\text{IIB}}\beta_{\text{III}} \), PKC and SERCA. Additionally, similar to the present study, scanning electron microscopy images displayed significant membrane damage caused by the cationic dendrimers (Dobrovolskaia et al., 2012, Xia et al., 2008).

Taken together, the data in this chapter and the data in the literature suggest that both acute and chronic exposure to cationic NPs may potentially have detrimental health effects by enhancing platelet responses to stimuli or by cytotoxicity, respectively. This is compounded by the fact that the mechanism behind these adverse effects appears to be due to passive agglomeration which may not respond to conventional antiplatelet therapies such as COX and integrin inhibitors. My findings also indicate that the AM 50 nm NP-induced platelet aggregation is insensitive to endogenous protective mechanisms.

In conclusion, the present work indicates that the platelet aggregation enhanced and induced by engineered NPs involved activation of classical signalling pathways and passive agglomeration, which was dependent on NP physicochemistry. This activation of signalling pathways and agglomeration appears to be achieved by the NPs physically interacting with the platelet membrane and by internalisation into the platelet.

4.3.1 General limitations

Platelet aggregation is the final event in the platelet activation process and involves a multitude of different signalling molecules and pathways which can make identification of individual signalling molecules difficult, as their influence on platelet aggregation can be masked or compensated for by other signalling pathways. Limitations to using platelet aggregation to assess the involvement of certain signalling molecules are demonstrated by the lack of inhibition for collagen and thrombin induced platelet aggregation following incubation with apyrase. In addition, the aggregation produced by the AM 50 nm NP was highly variable making it difficult to accurately observe inhibition.
Non-specific effects of the inhibitors must also be taken into account when interpreting the results. BIM-1, the PKC inhibitor, antagonises the 5-HT3 receptor at the concentration used in the present study. Platelets express the 5-HT3 receptor however its role in platelet function is still uncertain (Stratz et al., 2008, Liu et al., 2012). Furthermore, apyrase catalyses the breakdown of both ATP and ADP into ADP+P and AMP+P respectively. The concentration of apyrase was selected based on a concentration response and the concentration was similar to other concentrations used in the literature (Cifuni et al., 2008) however, as platelets release both ATP and ADP, we cannot rule out the possibility that ADP may have been generated. This may have complicated the interpretation of the findings. Further work with receptor antagonists would confirm the involvement of ADP signalling.
Chapter Five
The effects and underlying mechanisms of combustion derived nanoparticles on platelet function \emph{in-vivo}
Chapter Five – Results

Chapter 5

5.1.0. Introduction

Exposure to ambient PM is associated with thrombotic events such as MI and stroke (Peters et al., 2001a). The PM 0.1 or UF fraction of air pollution which is principally generated from vehicle emissions, is suggested to be primarily responsible for these thrombotic events (Andersen et al., 2010). Platelet activation and aggregation are important processes involved in the thrombotic events associated with exposure to UF PM and previous work has found that DEP, one of the major components of urban UF PM, can induce and enhance platelet aggregation and thrombus formation in-vitro and ex-vivo (Nemmar et al., 2003a, Lucking et al., 2008). Additionally, exposure of animals and humans to DEP and DE has been demonstrated to enhance thrombus formation (Mills et al., 2007, Lucking et al., 2011, Nemmar et al., 2003a); however this work was conducted in vascular injury models and ex-vivo preparations which involve multifactorial processes that do not distinguish the specific effect on platelet function. Therefore, the effects of UF PM on platelet aggregation and the underlying mechanisms remain poorly understood (Solomon et al., 2013, Gilmour et al., 2004, Nemmar et al., 2003c, Lucking et al., 2008, Mills et al., 2011).

Initiation of pulmonary and systemic inflammation following inhalation of UF particles has been proposed as one mechanism by which UF particles can promote platelet aggregation and thrombosis. Inflammatory mediators generated in the lung may enter the circulation and produce systemic inflammation, which is recognised as a promoter of thrombosis (section 1.1.3). Numerous authors have reported the release of pro-inflammatory cytokines and leukocyte infiltration in the lungs following exposure to UF particles however, there are conflicting reports regarding the presence of a subsequent systemic inflammatory response (Oberdorster et al., 1992, Mills et al., 2005, Xu et al., 2013, Frampton et al., 2006, Nemmar et al., 2003c, Mills et al., 2011). In addition, it has been reported by numerous authors that nanoscale materials can transverse the pulmonary epithelial barrier and enter the circulation in humans and animals models, where these particles can interact with various blood components including platelets (Kreyling et al., 2009, Nemmar et al., 2002a, Nemmar et al., 2001, Oberdorster et al., 2002). This suggests that UF particles may influence platelet behaviour through direct contact with platelets themselves or indirectly through contact with other haematological components.

Additionally, it is reported that DEP can reduce the bioavailability of NO and thereby reduce the inhibitory influence NO has on platelets, promoting platelet activation events. The uncoupling of eNOS has been proposed as one mechanism behind this reduced NO bioavailability (Knuckles et al., 2008, Wauters et al., 2013, Langrish et al., 2013).
Chapter Five – Results

The aim of this chapter was to establish whether introduction of DEP or CB into the lungs of mice could modulate platelet aggregation *in-vivo*, using a mouse model of pulmonary thromboembolism; and to evaluate whether the effects on platelet aggregation are associated with pulmonary and/or systemic inflammation, or related to NO and eNOS activity.
In addition, DEP were administered systemically to investigate the impact of UF particles on platelet aggregation, should they translocate the alveolar-blood barrier and be introduced into the systemic circulation.
5.1.1. Aims

- Determine the site of pulmonary deposition of DEP and CB following \textit{i.t.} administration.

- Determine the effect of DEP and CB on platelet aggregation \textit{in-vivo} following a pulmonary route of exposure.

- Establish whether pulmonary exposure to DEP or CB causes pulmonary and/or systemic inflammation.

- Assess the influence of NO and PGI$_2$ on DEP induced platelet aggregation \textit{in-vitro} and \textit{in-vivo}.

- Investigate the effect of systemic administration of DEP on platelet aggregation \textit{in-vivo}.
5.2.0. Electron micrographs of DEP and CB nanoparticles suspended in sterile saline.

DEP and CB NPs were visualised on fomvar coated grids using transmission electron microscopy to establish their agglomeration states in saline (NaCl 0.9 %). DEP formations in saline (NaCl 0.9 %) were present as chains of individual particles, simple and complex agglomerates (indicated by increased density) (Fig 5.1 A, C, E and G) whereas the CB particles were predominantly present as simple agglomerates (Fig 5.1 B) and complex agglomerates (indicated by increased density) (Fig 5.1 B, D, F and H). Simple chains were indicated with black arrows, simple agglomerates with white arrows and large agglomerates with grey arrows on the images.

5.2.1. Deposition of DEP and CB in mouse lungs following intratracheal instillation.

Following i.t. instillation of a low dose of DEP or CB (1 µg mouse) histological analysis of the mouse lungs was conducted (section 2.3.6). No aggregates of DEP (Fig 5.2.1 A + B) or CB (Fig 5.2.1 C + D) were observed in either the conducting airways or the alveolar regions. No difference was observed between either of the sections of the particle exposed lungs and the saline controls (Fig 5.2.1 E + F). Following i.t. instillation of an intermediate dose of DEP (Fig 5.2.2 A + B) or CB (Fig 5.2.2 C + D) (25 µg mouse), aggregates of DEP were observed in the alveolar spaces and in contact with the pulmonary epithelial cells (Fig 5.2.2 B). Aggregates of CB were detected in the conducting airways (Fig 5.2.2 C) and in the alveoli (Fig 5.2.2 D). All CB agglomerates appeared to be in contact with the epithelial cells and within the lung parenchyma.

Following i.t. instillation of a high dose of DEP (50 µg mouse) (Fig 5.2.3 A + B) or CB (Fig 5.2.3 C + D), were observed in the alveolar spaces and in contact with the pulmonary epithelial cells (Fig 5.2.3 A + B). Large aggregates of CB were detected throughout the lung parenchyma in the conducting airways and in the alveoli (Fig 5.2.3 C). All CB agglomerates appeared to be in contact with the epithelial cells and in some areas, covering large portions of individual alveoli (Fig 5.2.3 D).
Figure 5.1. Electron micrographs of DEP and CB NPs suspended in sterile saline.

DEP and CB NPs were suspended in sterile saline (NaCl 0.9 %) and this solution was placed onto nickel formvar grids. Images were taken by transmission electron microscopy at magnification x10,000 and x15,000. DEP (A - D) and CB (E - H) nanoparticles were observed as chains (black arrows) and simple agglomerates (white arrows) and large complex agglomerates (grey arrows).
Figure 5.2.1. Deposition of DEP and CB (1µg per mouse) in mouse lungs following intratracheal instillation.

Anaesthetised mice (5 % isoflourane) were i.t. instilled with DEP (A + B) and CB (C + D) NPs (1 µg mouse) or saline (NaCl 0.9 %) (E + F). Mice were killed via cervical dislocation immediately after i.t. instillation and their lungs were removed and fixed in formalin. Samples were embedded, sectioned and stained with hematoxylin and eosin. Slides were visualised on a light microscope at X 20, 000 and X 40, 000. Sections from 3 mice exposed to each treatment were observed and representative images are shown. DEP = diesel exhaust particles, CB = carbon black.
Figure 5.2.2. Deposition of DEP and CB (25 µg per mouse) in mouse lungs following intratracheal instillation.

Anaesthetised mice (5 % isoflourane) were i.t. instilled with DEP (A + B) and CB (C + D) NPs (25 µg mouse) or saline (NaCl 0.9 %) (E + F). Mice were killed via cervical dislocation immediately after i.t. instillation and their lungs were removed and fixed in formalin. Samples were embedded, sectioned and stained with hematoxylin and eosin. Slides were visualised on a light microscope at X 20,000 and X 40,000. Sections from 3 mice exposed to each treatment were observed and representative images are shown. Black arrows indicate agglomerates of DEP or CB. DEP = diesel exhaust particles, CB = carbon black.
Figure 5.2.3. Deposition of DEP and CB (50 µg per mouse) in mouse lungs following intratracheal instillation.

Anaesthetised mice (5 % isoflurane) were i.t. instilled with DEP (A + B) and CB (C + D) NPs (50 µg mouse) or saline (NaCl 0.9 %) (E + F). Mice were killed via cervical dislocation immediately after i.t. instillation and their lungs were removed and fixed in formalin. Samples were embedded, sectioned and stained with hematoxylin and eosin. Slides were visualised on a light microscope at X 20,000 and X 40,000. Sections from 3 mice exposed to each treatment were observed and representative images are shown. Black arrows indicate agglomerates of DEP or CB. DEP = diesel exhaust particles, CB = carbon black.
5.2.2. Collagen dose dependent platelet aggregation in-vivo using InCl\textsuperscript{111} and InOx\textsuperscript{111} labelled murine platelets.

Due to the cessation of InOx\textsuperscript{111} production by the manufacturer, InCl\textsuperscript{111} was employed as a replacement. A dose response to collagen was conducted to establish that platelets labelled with InCl\textsuperscript{111} could respond to collagen in a similar manner to platelets labelled with InOx\textsuperscript{111}, in a model of pulmonary thromboembolism (Tymvios et al., 2008). Donor mouse platelets were labelled with 1.8 MBq of InCl\textsuperscript{111} or InOx\textsuperscript{111} and infused into recipient mice. Increasing doses of collagen (25, 50, 75 and 100 µg kg\textsuperscript{-1}) were subsequently administered through the femoral vein. Administration of collagen at all doses, excluding the highest (100 µg kg\textsuperscript{-1}), caused a typical initial increase in platelet aggregation which was followed by dissolution to baseline for both InCl\textsuperscript{111} (Fig 5.3 A) and InOx\textsuperscript{111} labelled platelets (Fig 5.3 B). After administration of 100 µg kg\textsuperscript{-1} collagen, death occurred in one mouse after 3 minutes following infusion of InCl\textsuperscript{111} labelled platelets (Fig 5.3 C + E). Dose dependent increases in maximum percentage platelet aggregation (Fig 5.3 C + D) and AUC (Fig 5.3 E + F) were observed for both InCl\textsuperscript{111} and InOx\textsuperscript{111} labelled platelets. Comparable labelling efficiency was observed for both isotopes (Fig 5.3 G).
No statistics were performed on the InCl\textsuperscript{111} data due to the low number of replicates and the labelling efficiency as these were calculated as percentages.
Chapter Five – Results

[A] InCl$^{111}$

[B] InOx$^{111}$

[C] Max % Aggregation vs. $\mu$g kg$^{-1}$

[D] Max % Aggregation vs. $\mu$g kg$^{-1}$

[E] AUC vs. $\mu$g kg$^{-1}$

[F] AUC vs. $\mu$g kg$^{-1}$
Figure 5.3. Collagen dose dependent platelet aggregation *in-vivo* using InCl$^{111}$ and InOx$^{111}$ labelled murine platelets.

Isolated platelets were radiolabelled with InOx$^{111}$ or InCl$^{111}$ and infused into anesthetised recipient mice prior to injection with increasing doses of collagen (i.v 25, 50, 75 and 100 µg kg$^{-1}$). Responses were recorded as changes in counts over time for InCl$^{111}$ (A) or InOx$^{111}$ (B) (representative trace) and platelet aggregation was assessed by changes in maximum percentage increase in scintillation counts for InCl$^{111}$ (C) or InCl$^{111}$ (D) and area under the curve (AUC) for InCl$^{111}$ (E) or InOx$^{111}$ labelled platelets (F). $\pm$ one data point due to mortality following administration. Labelling efficiency was calculated as percentage labelling (G). Data are presented as median ± interquartile range. InCl$^{111}$ = Indium Chloride$^{111}$, InOx$^{111}$ = Indium Oxine$^{111}$. * = p < 0.05. N = 2 for InCl$^{111}$, N = 4 for InOx$^{111}$. 
5.2.3. Platelet aggregation *in-vivo* following intratracheal instillation of DEP, CB or saline.

No significant differences were observed for maximum percentage platelet aggregation (Fig 5.4.1 A) or AUC (Fig 5.4.1 B) in response to collagen (50 µg kg\(^{-1}\)) 4-5 hours following *i.t.* instillation of DEP or CB (1 µg mouse) (P > 0.05 compared to saline control). Collagen induced platelet aggregation following *i.t.* instillation of DEP, CB or saline caused a typical initial increase in platelet aggregation which was followed by dissolution to baseline (Fig 5.4.1 C).

There was a significant enhancement of the AUC (P < 0.05 compared to saline control) (Fig 5.4.2 C) in response to collagen (50 µg kg\(^{-1}\)) and a non-significant trend towards enhanced maximum platelet aggregation (P > 0.05 compared to saline control) 4-5 hours following *i.t.* instillation of DEP (25 µg mouse) (Fig 5.4.2 A). Unlike the saline control, platelet aggregation did not return to baseline following *i.t.* instillation of DEP (Fig 5.4.2 E).

There was no significant enhancement of the AUC (Fig 5.4.2 D) or maximum platelet aggregation (Fig 5.4.2 B) in response to collagen (50 µg kg\(^{-1}\)) (P > 0.05 compared to saline control) 4-5 hours following *i.t.* instillation of CB (25 µg mouse). Collagen induced platelet aggregation following *i.t.* instillation of CB or saline caused a typical initial increase in platelet aggregation which was followed by dissolution to baseline (Fig 5.4.2 F).

No significant differences were observed for maximum percentage platelet aggregation (Fig 5.4.3 A) or AUC (Fig 5.4.3 B) in response to collagen (50 µg kg\(^{-1}\)) 4-5 hours following *i.t.* instillation of DEP (50 µg mouse) (P > 0.05 compared to saline control). Collagen induced platelet aggregation following *i.t.* instillation of DEP or saline caused a typical initial increase in platelet aggregation which was followed by dissolution to baseline (Fig 5.4.3 C).
Chapter Five – Results

Figure 5.4.1. Platelet aggregation *in-vivo* in mice following intratracheal instillation of DEP, CB (1 µg mouse) or saline.

Mice were *i.t.* instilled with DEP, CB (1 µg mouse) or sterile saline (0.9 %). Isolated platelets were radiolabelled with $^{111}$indium chloride and infused into mice 4-5 hours following *i.t.* instillation, prior to injection with collagen (*i.v.* 50 µg kg$^{-1}$). Responses were recorded for 10 minutes as changes in counts over time. Platelet aggregation was assessed by changes in percentage maximum increase in scintillation counts (A) or area under the curve (AUC) (B). Data are presented as median ± interquartile range (A-B) or representative traces (C). NS = non-significant, compared using a K-W test with Dunn’s comparison. N = 5.
Figure 5.4.2. Platelet aggregation in-vivo following intratracheal instillation of DEP, CB (25 µg mouse) or saline.

Mice were i.t. instilled with DEP, CB (25 µg mouse) or sterile saline (0.9 %). Isolated platelets were radiolabelled with $^{111}$indium oxine and infused into mice 4-5 hours following i.t. instillation, prior to injection with collagen (i.v 50 µg kg$^{-1}$). Responses were recorded as changes in counts for 10 minutes. Platelet aggregation was assessed by changes in percentage maximum increase in scintillation counts (A+B) or area under the curve (AUC) (C+D). Data are presented as median ± interquartile range (A-D) or representative traces (E + F). *P < 0.05 compared using a K-W test with Dunn’s comparison. NS = non-significant, DEP = diesel exhaust particles, CB = carbon black, SAL = saline. N = 5-8.
Figure 5.4.3. Platelet aggregation in-vivo following intratracheal instillation of DEP (50 µg mouse) and saline.

Mice were i.t. instilled with DEP (50 µg mouse) or sterile saline (0.9 % NaCl). Isolated platelets were radiolabelled with $^{111}\text{In}$ indium oxine and infused into mice 4-5 hours following i.t. instillation, prior to injection with collagen (i.v. 50 µg kg $^{-1}$). Responses were recorded as changes in counts for 10 minutes. Platelet aggregation was assessed by changes in percentage maximum increase in scintillation counts (A) or area under the curve (AUC) (B). Data are presented as median ± interquartile range (A-B) or representative traces (C). Data is compared using a Mann Whitney U test. NS = non-significant, DEP = diesel exhaust particles, SAL = saline. N = 6.
Chapter Five – Results

5.2.4. Leukocyte and cytokine/chemokine profile in bronchoalveolar lavage fluid following intratracheal instillation of CB, DEP and saline.

Total and differential leukocyte counts and cytokine/chemokine protein levels were quantified in the BALF 4-5 hours after i.t. instillation of mice with DEP, CB or saline. A significant increase in total leukocytes (P < 0.05 compared to saline) was observed following i.t. instillation of CB (25 µg mouse) and a non-significant trend of increased total leukocytes (P > 0.05 compared to saline) was observed following i.t. instillation of DEP (25 µg mouse) (Fig 5.5 A). A significant increase in neutrophils (p < 0.05 compared to saline) (Fig 5.5 B) and a non-significant trend (P > 0.05 compared to saline) of increased macrophages was detected (Fig 5.5 C) following i.t. instillation of CB. A significant increase in macrophages (P < 0.05 compared to saline) (Fig 5.5 C) but no significant changes were observed for neutrophils (P > 0.05 compared to saline) (Fig 5.5 B) following i.t. instillation of DEP. Neither CB nor DEP caused any significant changes (P > 0.05 compared to saline) in eosinophils (Fig 5.5 D) or lymphocytes (Fig 5.4 E).

No significant increases in IL-6 (Fig 5.6 A) or TNF-α (Fig 5.6 B) (P < 0.05 compared to saline) were observed in BALF 4-5 hours following i.t. instillation of DEP (25 µg mouse) which was measured in cell free supernatant by ELISA. A significant increase in IL-6 (Fig 5.6 A) (P > 0.05 compared to saline) but no significant changes in TNF-α (Fig 5.6 B) (P < 0.05 compared to saline) were detected 4-5 hours following i.t. instillation of CB (25 µg mouse). No IL-1β was detected in the BALF following exposure to DEP, CB or saline (data not shown).

5.2.5. Systemic leukocyte and cytokine profile following intratracheal instillation of CB, DEP and saline.

Total and differential cell counts were conducted in whole blood and cytokine/chemokine protein levels were quantified in the plasma 4-5 hours after i.t. instillation of mice with DEP, CB or saline. No significant differences (P > 0.05 compared to saline) in total systemic leukocytes (Fig 5.7 A), neutrophils (Fig 5.7 B), lymphocytes (Fig 5.7 C), monocytes (Fig 5.7 D), erythrocytes (Fig 5.7 E) or platelets (Fig 5.7 A) were detected in mice 3-4 hours following i.t. instillation of DEP, CB (25 µg mouse) or saline.

A significant increase in cytokine IL-6 (P < 0.05 compared to saline) (Fig 5.8 A) and a non-significant trend of increased MCP-1 (P > 0.05 compared to saline) (Fig 5.8 B) was detected following i.t. instillation of CB, however no significant differences (P > 0.05 compared to saline) were detected for MIP-1 (Fig 5.8 C) or IL-2 (Fig 5.8 D) following i.t. instillation of CB. I.t. instillation of DEP (Fig 5.8 A – D) did not cause significant differences (P > 0.05 compared to saline) in any of the plasma cytokines/chemokine’s measured.
Figure 5.5. Leukocyte profile in bronchoalveolar lavage fluid following intratracheal instillation of CB, DEP and saline.

Mice were i.t. instilled with DEP, CB (25 µg mouse) or sterile saline (0.9 % NaCl). Bronchoalveolar lavage fluid (BALF) was obtained 4-5 hours following i.t. instillation via catheterisation of the trachea and instillation of 400 µl of sterile saline (NaCl 0.9 %) for 30 s (x 3). Total leukocytes (A) and differential leukocyte counts were measured. Neutrophils (B), macrophages (C), eosinophils (D) and lymphocytes (E). Data are presented as median and interquartile range. *P < 0.05, **P < 0.01, ***P < 0.001 compared using a K-W test with Dunn’s comparison. NS = non-significant, SAL = saline, DEP = diesel exhaust particles and CB = carbon black. N = 5-6.
Figure 5.6. Cytokine profile in bronchoalveolar lavage fluid following intratracheal instillation of CB, DEP and saline.

Mice were *i.t.* instilled with DEP, CB (25 µg mouse) or sterile saline (0.9 % NaCl). Bronchoalveolar lavage fluid (BALF) was obtained 4-5 hours following *i.t.* instillation via catheterisation of the trachea and instillation of 400 µl of sterile saline (NaCl 0.9 %) for 30 s (x 3). Protein concentrations of cytokines IL-6 (A) and TNFα (B) were measured. Data are presented as median and interquartile range. *P < 0.05 using a K-W test with Dunn’s comparison. NS = non-significant, SAL = saline, DEP = diesel exhaust particles and CB = carbon black. N = 5-6.
Figure 5.7. Haematological profile following intratracheal instillation of CB, DEP and saline.

Mice were i.t. instilled with DEP, CB (25 µg mouse) or sterile saline (0.9 % NaCl). Blood was obtained 4-5 hours following i.t. instillation via cardiac puncture. Total leukocytes (A) and differential blood cell counts were measured. Neutrophils (B), lymphocytes (C), monocytes (D), erythrocytes (E) and platelets (F). Data are presented as median and interquartile range. NS = non-significant, SAL = saline, DEP = diesel exhaust particles and CB = carbon black. N = 6-8.
Figure 5.8. Cytokine profile in plasma following intratracheal instillation of CB, DEP and saline.

Mice were i.t. instilled with DEP, CB (25 µg mouse) or sterile saline (0.9 % NaCl). Bronchoalveolar lavage fluid (BALF) was obtained 4-5 hours following i.t. instillation via catheterisation of the trachea and instillation of 400 µl of sterile saline (0.9 %) for 30 s (x 3). Protein concentration of cytokines IL-6 (A), MCP-1 (B), MIP-1 (C) and IL-2 (D) were measured. Data are presented as median and interquartile range. *P < 0.05 using a K-W test with Dunn’s comparison. NS = non-significant, SAL = saline, DEP = diesel exhaust particles and CB = carbon black. N = 5-6.
5.2.6. Platelet aggregation \textit{in-vivo} in eNOS $^{-/-}$ mice and nitrate levels in the blood of WT mice following intratracheal instillation of DEP, CB or saline.

4-5 hours following \textit{i.t.} instillation of DEP (25 µg mouse) into eNOS $^{-/-}$ mice, a significant enhancement of the AUC (P < 0.05 compared to saline control) (Fig 5.9 B) in response to collagen (50 µg kg$^{-1}$) and a non-significant trend towards enhanced maximum platelet aggregation (P > 0.05 compared to saline control) was observed (Fig 5.9 A). Unlike the saline control and CB, platelet aggregation did not return to baseline following \textit{i.t.} instillation of DEP (Fig 5.9 C). No significant changes in AUC or maximum platelet aggregation were detected for CB (25 µg mouse) treated eNOS $^{-/-}$ mice (P > 0.05 compared to saline control) (Fig 5.9 A+B).

Comparisons between the response to collagen (50 µg kg$^{-1}$) following \textit{i.t.} instillation of DEP and CB in WT and eNOS $^{-/-}$ mice found that eNOS $^{-/-}$ mice had a borderline significant higher AUC compared to the WT mice (Fig 5.9 D) however no significant differences were detected between WT or eNOS $^{-/-}$ mice for maximal percentage increase in platelet aggregation (Fig 5.9 E).

\textit{i.t.} instillation of DEP or CB (25 µg mouse) in WT mice did not cause any significant alterations in plasma nitrate levels (P > 0.05 compared to saline control) (Fig 5.10). Nitrate levels were below the detection limit of the assay.

5.2.7. Nanoparticle induced platelet aggregation sensitivity to sodium nitroprusside and iloprost.

Platelet aggregation produced by all concentrations of DEP (12 – 50 µg mL$^{-1}$) and the collagen control (5 µg mL$^{-1}$) was significantly inhibited by the NO donor SNP (10 µM) (P < 0.05 compared to time-matched Tyrode’s buffer control) (Fig 5.11 A). Similar results were found for the PGI$_2$ mimetic iloprost (10 nM) as all concentrations of DEP (12 – 50 µg mL$^{-1}$) and the collagen control (5 µg mL$^{-1}$) were significantly inhibited (P < 0.05 compared to the vehicle time-matched control) (Fig 5.11 B).

5.2.8. Enhancement of collagen induced platelet aggregation by intravenously administered diesel exhaust particles \textit{in-vivo}.

\textit{i.v.} infusion of DEP (1.0 µg mL$^{-1}$) did not cause platelet aggregation per se within an initial 10 minute monitoring period (Fig 5.12 C) however following an \textit{i.v.} infusion of a submaximal concentration of collagen (50 µg kg$^{-1}$), DEP infusion significantly enhanced the AUC (P < 0.05 compared to the saline control) (Fig 5.12 B) and a non-significant trend towards enhanced maximum percentage aggregation was detected (P > 0.05 compared to the saline control) (Fig 5.12 A).
Figure 5.9. Platelet aggregation \textit{in-vivo} in eNOS\textsuperscript{-/-} mice following intratracheal instillation of DEP, CB or saline.

Mice were \textit{i.t} instilled with DEP, CB (25 µg mouse) or sterile saline (0.9 %). Isolated platelets were radiolabelled with \textsuperscript{111}indium chloride and infused into mice 4-5 hours following \textit{i.t.} instillation, prior to injection with collagen (50 µg kg\textsuperscript{-1}). Responses were recorded for 10 minutes as changes in counts over time. Platelet aggregation was assessed by changes in percentage maximum increase in scintillation counts (A) or area under the curve (AUC) (B). Fold change from the saline control in percentage maximum increase in scintillation counts (D) and AUC (E) following \textit{i.t} instillation of DEP and CB in WT and eNOS\textsuperscript{-/-} mice. Data are presented as median ± interquartile range (A-B, D-E) or representative traces (C). NS = non-significant, *\(P < 0.05\) compared using a K-W test with Dunn’s comparison or Mann-Whitney signed rank test, N = 8-4.
Figure 5.10. Plasma nitrate levels in WT mice following intratracheal instillation of DEP, CB or saline.

Mice were *i.t* instilled with DEP, CB (25 µg mouse) or sterile saline (0.9 %). Blood was taken *via* cardiac puncture 4-5 hours following exposure and nitrate and nitrite levels were measured in plasma using gas-phase chemiluminescence. Data are presented as median ± interquartile range, NS = non-significant compared using a K-W test with Dunn’s comparison, N = 6.
Figure 5.11. Nanoparticle induced platelet aggregation sensitivity to sodium nitroprusside and iloprost.

Isolated human platelets were exposed to DEP (50-12 µg mL⁻¹), collagen (5 µg mL⁻¹) following a 5 minute incubation with either a NO donor SNP (10 µM) or Tyrode’s buffer (A) or the PGI₂ mimic iloprost (10 nM) or Tyrode’s buffer (B). Changes in light transmission were measured for 3 minutes. Data are presented as box-and-whisker plots. *P < 0.05 compared against time matched controls using a Wilcoxon signed rank test. DEP = diesel exhaust particles, Coll = collagen, NP = nanoparticle, SNP = sodium nitroprusside, Tyr = Tyrode’s buffer. N = 5.
Figure 5.12. Enhancement of collagen induced platelet aggregation by diesel exhaust particles in-vivo.

Isolated platelets were radiolabelled with $^{111}$indium oxine and infused into anesthetised recipient mice prior to injection of DEP (i.v. 1.0 µg mL$^{-1}$) or saline control (0.9% NaCl). These mice were subsequently infused with collagen (i.v. 50 µg kg$^{-1}$) and responses were measured for 10 minutes and recorded as changes in scintillation counts over time. Platelet aggregation was calculated as percentage maximum changes in scintillation counts (A) and area under the curve (AUC) (B). Data are presented as a typical trace demonstrating changes in counts over time (C) or median±interquartile range, * P < 0.05 compared to saline control with Mann-Whitney test. DEP = Diesel exhaust particles, Sal = saline, NS = non-significant. N = 5.
5.3.0. Discussion

Acute exposure to ambient PM is associated with thrombotic events such as MI and stroke (Peters et al., 2001a). PM 0.1 has been implicated in being the fraction of PM responsible for these events and the majority of these particles are carbonaceous products of the combustion process (WHO, 1987). To date, most work investigating PM effects on platelet aggregation has utilised vascular injury thrombosis models *in-vivo* or *ex-vivo* approaches making it difficult to understand the role of platelets in PM mediated thrombosis. This chapter aimed to establish whether pulmonary exposure to the carbonaceous NPs DEP and CB has an effect on platelet aggregation using a mouse model of pulmonary thromboembolism. A secondary aim was to determine the underlying mechanisms behind these effects.

It was important to initially take into consideration the method of NP delivery. Many authors use inhalation as this is more representative of human exposure however, this can also promote ingestion from grooming and skin exposures, which can affect the final administered dose. Alternatively, *i.t.* dosing can ensure accurate dosing into the conducting and distal airways. Nonetheless, this can also lead to uneven distribution and potential for residual damage to the trachea from the procedure (Driscoll et al., 2000). Due to the technical and dosing considerations, it was decided that *i.t.* instillation would be the most practical and suitable method of administering DEP and CB in this study.

DEP and CB formation in experimental buffers was initially established by observing the particles at high resolution using TEM. DEP formations in saline were present as chains of individual particles, simple and complex agglomerates whereas the CB particles were predominantly present as simple and complex agglomerates. These formations largely resemble DEP present in ambient air pollution (Park et al., 2003). Heavy particle agglomeration suggests that these NPs may have a decreased surface area, which would be available for interaction with biological elements.

One of the principle reasons why PM 0.1 is considered to be responsible for the harmful effects of ambient PM is due its region of deposition within the airways. Nano-sized particles have a higher deposition efficiency and are deposited within the lower airways including the alveoli (ICRP, 1994). Deposition of DEP in the (inter)alveolar spaces of mice lungs has also been observed following *i.t.* instillation by other authors (Nemmar et al., 2009a). It was therefore essential to initially establish that the DEP and CB were deposited in these pulmonary regions following *i.t.* administration in my experiments, to confirm that it was representative of the human situation. No DEP or CB was observed in any of the pulmonary regions at the lowest dose. This may be due to several factors; limitations in the sensitivity of light microscopy may mean that non-agglomerated or small agglomerated particles were not detectable. Alternatively these particles may have translocated directly into the circulation or have been removed by the clearance pathways in the lung such as
mucociliary clearance or alveolar macrophages. DEP and CB were observed both in the conducting airways and the distal alveoli following administration of higher doses. These particles appeared to be in both small and large agglomerates, which were comparable to the NP formations that were observed in experimental buffers by TEM. Therefore, *i.t.* administration of the combustion derived NPs in the present study adequately represented the human situation.

The thrombotic events associated with inhalation of PM0.1 involve platelet activation and aggregation however, currently there is limited information regarding the influence of DEP and CB on platelet function *in-vivo*. 25 μg of DEP administered to the lung of mice caused a significant increase in agonist-induced platelet aggregation *in-vivo* 4-5 hrs post-exposure. These results potentially indicate that the thrombotic events associated with acute exposure to PM0.1 may be driven by a direct effect on platelets.

The effects of DEP on thrombosis have been observed in vascular injury models and *ex-vivo* clinical studies. Similar results have also been demonstrated following a longer 24 hr exposure period. Nemmar et al reported that there was an increase in platelet aggregation *in-vivo* following *i.t.* administration of DEP however; this was assessed by measuring a change in platelet numbers, which may have been influenced by multiple factors. Furthermore, *i.t.* instillation of DEP caused a significant decrease in occlusion time in mice cerebral venules (Nemmar et al., 2009a). Similar findings by other groups in venous and arterial occlusion times have been demonstrated *in-vivo* following a 24hr exposure to DEP (Emmerechts et al., 2010, Mutlu et al., 2007, Nemmar et al., 2009a). Similar to the present study, acute exposure to DEP has also been demonstrated to affect thrombosis. DEP caused significant increases in venous and arterial thrombus formation in a hamster after only 10 minutes. Additionally, this group also reported that a 30 and 60 minute exposure to DEP caused an increase in closure time *ex-vivo* (Nemmar et al., 2003a, Nemmar et al., 2009a). Although there are limited clinical studies, increases thrombosis has been reported in human subjects’ *ex-vivo* following an acute exposure (2-4 hrs) to DE (Lucking et al., 2008). Most work to date has involved using vascular injury models and *ex-vivo* approaches which have made it difficult to delineate the specific role of platelets in DEP-mediated thrombosis. This study is the first time that DEP has been demonstrated to affect platelet aggregation *in-vivo*, in the presence of an intact endothelium, which is important as the endothelium plays a crucial role in platelet regulation.

Interestingly, CB had no effect on platelet aggregation *in-vivo* at any dose. This finding is similar to other groups who have demonstrated that *i.t.* instillation of CB did not cause any significant changes in closure times *in-vivo* after 24 hours (Kim et al., 2012, Nemmar et al., 2009a). Both CB and DEP particles have a carbonaceous core but the DEP has many adsorbed compounds on its surface, such as PAHs and transition metals (Wichmann, 2007). Both PAH and transition metals can induce ROS which is thought to be involved in platelet activation (Begonja et al., 2005). The current data suggests that the effect of DEP on platelet aggregation may be due to the presence of these compounds.
Interestingly, no effect on platelet aggregation was observed at the highest DEP concentration (50 μg per mouse). The images acquired after 50 μg of DEP were i.t. instilled into mouse lungs suggests that the DEP is deposited as large agglomerates which are micrometer in diameter. The large diameter of these agglomerates would potentially make these particles easier to be cleared by alveolar macrophages and mucociliary clearance and therefore promote the removal of the DEP from the lungs. This rapid removal of DEP from the lungs may have reduced the effects of DEP on platelet aggregation, as the particles may not have been present long enough to initiate adverse events such as pulmonary inflammation or translocate through the epithelial layer.

The secondary aim of this research was to determine the underlying mechanism behind DEP-enhanced platelet aggregation. One hypothesis is that PM0.1 can induce pulmonary inflammation and that various inflammatory mediators such as cytokines, spill over into the systemic circulation (Seaton et al., 1995). Systemic inflammation can promote thrombosis through activation of the acute phase response and initiation of the coagulation cascade. Platelets have the capacity to bridge the inflammatory and thrombotic responses by having the ability to respond to and release pro-inflammatory mediators and stimuli. Leukocytes and pro-inflammatory cytokines were measured in BALF 4-5 hours after i.t. administration of DEP and CB. Exposure to DEP caused a significant increase in total cell counts in the BALF and the differential cell count revealed that this was due to an increase in macrophages and there was a non-significant trend of increased lymphocyte numbers. No increases were observed for other leukocytes, cytokine levels or protein levels. Bacteria and particulates which enter the lung are cleared primarily by alveolar macrophages via phagocytosis (Green and Kass, 1964, Goldstein et al., 1974). The increase in macrophages following DEP exposure may have been due to particle overload, as the alveolar macrophages present in the lungs may have been insufficient to handle the number of particles, hence additional macrophages were recruited into the alveolar spaces (Oberdorster, 1995). Additionally, UF particles have been demonstrated to impair alveolar macrophages phagocytic ability and this may have reduced the clearance of the particles and led to the accumulation of macrophages in the lung (Renwick et al., 2004). Alveolar macrophages play a central role in pulmonary inflammation by releasing cytokines such as IL-1β and TNF-α which can recruit neutrophils and lymphocytes to the site of infection. DEP did not appear to induce an inflammatory response as no hallmarks of pulmonary inflammation, such as cytokine release or neutrophil influx, which are mediated by conserved signalling pathways were detected. However, the increased macrophages and non-significant trend of increased lymphocytes does suggest the possibility that the acute timing may have been just on the threshold for an inflammatory response. Large increases in macrophages and dendritic cells with only small increases in neutrophils have been reported in the BALF of mice following exposure to engineered nanoparticles (Hardy et al., 2013). However, the lack of PMNs present in the BALF following DEP exposure disagrees with some of the current literature, as DEP has been reported to cause increases in pulmonary PMNs, protein and
Chapter Five – Results

histamine concentrations 1 hour following i.t. instillation in hamsters (Nemmar et al., 2003a). Furthermore, a 6 hour exposure caused increases in PMNs, protein and IL-6 levels in rats (Robertson et al., 2012). There is evidence that the route of administration can have a significant impact on the inflammatory cell burden following exposure of DEP. i.t. dosing has been shown to significantly increase these parameters compared to inhalation (Larcombe et al., 2014). However, there was no evidence that this occurred in the current model. This may be due to experimental differences, particularly in operator competency. Interestingly, the acute exposure to the CB particles caused significant increases in neutrophil numbers in the BALF. This was also associated with significant increases in IL-6 and a non-significant increase in TNF-α.

The IL-1β levels for the saline control and NPs were below the limit of detection for the assay. These results contradict studies in the literature which found that pulmonary exposure to CB and DEP can activate the inflammasome (Reisetter et al., 2011, Bourdon et al., 2012, Provoost et al., 2011). CB has been demonstrated to cause NFκB gene expression and subsequent cytokine release 1 – 4 hrs post exposure. Oxidative stress was established as the underlying mechanism (Shukla et al., 2000). However, most inflammasome activation has been reported following a 24 hr exposure to CB or DEP therefore the discrepancies between the current results and the literature may be due to differences in exposure times.

No increases in cytokine levels or leukocyte numbers were detected in the blood 4-5 hours following exposure to DEP therefore the enhanced platelet aggregation induced by exposure to DEP (25 μg mouse) was not associated with a systemic inflammatory response. As previously discussed, the evidence regarding the association between systemic inflammation and DEP exposure has been conflicting. Similar to the findings of the current study, acute exposure of human subjects to DE caused changes in thrombosis but no alterations in systemic cytokines or acute phase proteins (Lucking et al., 2011). Additionally, no changes in systemic inflammatory markers were detected in healthy men and men with coronary artery disease exposed to DE for 1 hr with intermittent exercise (Mills et al., 2005, Mills et al., 2007) or 2 hrs after exposure to CAPs (Mills et al., 2008).

Additionally, a study by Emmerechts et al, found no changes in systemic leukocytes or cytokines following a 4 hrs exposure to DEP (Emmerechts et al., 2010). However, increases in systemic IL-6 and TNF-α 24 hrs following exposure to DEP have been reported in humans (Tornqvist et al., 2007), animals (Robertson et al., 2012) and at longer > 24 hr time intervals (Nemmar and Inuwa, 2008). Based on the current data and the ambiguous data available in the literature, it appears that DEP does not induce systemic inflammation following acute exposure to DEP and therefore does not appear to be the underlying mechanism behind the enhanced platelet aggregation observed in the present work. Exposure of mice to CB (25 μg mouse) caused significant increases in blood IL-6 and a non-significant increase in MCP-1 however no changes were detected in systemic leukocytes.
Systemic inflammation has been reported following a single 7 hr exposure of rats to UF CB although this was with increases in circulating leukocytes (Gilmour et al., 2004). UFP collected under urban driving conditions have been demonstrated to cause MCP-1 release in endothelial cells which was abolished by inhibition of NFκB (Li et al., 2010). Activation of NFκB by CB in the current study would account for the release of pro-inflammatory cytokines both into the circulation and the lung. The release of pro-inflammatory cytokines by CB further supports the conclusion that systemic inflammation was not the underlying mechanism behind the DEP-induced enhanced platelet response, as CB did not alter platelet aggregation in-vivo but was associated with systemic inflammation. One potential reason for the lack of effect on platelet aggregation by CB may be due to the immune system effectively clearing the particles through an inflammatory response. Alternatively, the DEP may evade the immune system and thus have the ability to influence various systemic cell types including platelets.

A mechanism behind the DEP-enhanced platelet aggregation which was explored in this chapter was that DEP can reduce the bioavailability of NO and thereby reduce the inhibitory influence NO has on platelets. Acute exposure to DE has been reported to cause increases in plasma nitrite levels, suggesting an increase in basal generation of NO. The authors speculated that this increase in nitrite was due to compensatory mechanisms causing the over production of NO (Langrish et al., 2013). Additionally, DEP has been demonstrated to enhance vasoconstriction in humans and mice and it is thought that this is due to a reduction in NO bioavailability. The uncoupling of eNOS and the production of NO-scavenging superoxide has been proposed as one mechanism behind the reduced NO bioavailability (Sun et al., 2008, Knuckles et al., 2011, Knuckles et al., 2008). Superoxide is suggested to be released by xanthine dehydrogenase which leads to the formation of peroxynitrite (Manzo et al., 2012). If i.t. instillation of DEP caused a reduction of NO levels in the present study then in the absence of eNOS derived NO, DEP would be expected to have little effect on agonist-induced platelet aggregation compared to a vehicle control, as both groups would already be relieved of this inhibition. However, eNOS^-/- mice had a borderline significant fold increase in agonist-induced platelet aggregation following DEP exposure compared to the WT control. Additionally, DEP did not appear to influence NO blood concentrations as plasma nitrate and nitrate levels were unaltered. Therefore, a reduction in NO levels did not appear to be the underlying mechanism behind the effect of DEP on platelet aggregation, moreover, NO appeared to be protective against DEP-mediated effects on platelet function in-vivo. The present work is the first to use eNOS^-/- mice to identify the role of eNOS derived NO on DEP-mediated effects on platelet function in-vivo however these results contradict the findings reported by other groups.

To confirm that the DEP-mediated effects on platelet aggregation were sensitive to vascular regulators such as NO, the effects of NO and PG1_2 on DEP-induced aggregation of human platelets in-vitro was established. The DEP-induced platelet aggregation was significantly inhibited by both a NO donor and a PG1_2 mimetic. Thus it appears that DEP-mediated effects on platelet aggregation are sensitive to
endogenous vascular regulators which may mean that healthy individuals, with a functional vascular endothelium may be protected from the cardiovascular effects of DEP.

The enhanced platelet aggregation induced by DEP did not appear to involve systemic inflammation or alterations in NO levels, therefore an alternative mechanism was investigated. The translocation of particles < 100 nm from the lung to the blood has been suggested to occur within minutes of pulmonary exposure (Kreyling et al., 2002, Nemmar et al., 2002a, Nemmar et al., 2001, Choi et al., 2010, Manzo et al., 2012). Particles present in the blood could disrupt platelet function through direct interactions with platelets or other blood components. In the present study, the effect of a low concentration of DEP on platelet function was investigated by infusing DEP into the circulation. The i.v. administration of DEP caused significant increases in agonist-induced platelet aggregation therefore, the effect of DEP on platelet aggregation following i.t. instillation may have been due to the particles evading the pulmonary clearance pathways, translocating across the lung-blood barrier and interacting with the biological components within the vasculature. I speculate that the enhanced platelet aggregation may be due to ROS generation due to the compounds present on the DEP surface. Exogenous ROS has been demonstrated to promote collagen-induced platelet aggregation (Pratico et al., 1991).

The increase in platelet aggregation following i.v. infusion of DEP agrees with work reported by other groups. Systemic administration of DEP caused prolonged bleeding, increased HR and blood pressure in SHR rats (Nemmar et al., 2009b) and carbonaceous NPs injected i.v. caused significant increases in arteriole thrombosis in the microcirculation of a mouse, and increases in circulating P-selectin (Holzer et al., 2014).

The doses of combustion derived NPs used in the current study are similar (Nemmar et al., 2003a, Robertson et al., 2012) and considerably lower than studies in the literature (Acciani et al., 2013, Yang et al., 2003). Mouse doses in this study were roughly based on the inhalation rate of 1.2 m$^3$ h$^{-1}$ for an average person (ICRP, 1994) and peak concentrations of PM in the urban centres in developed countries, 480 µg m$^{-3}$ (PM$_{2.5}$ concentration in underground tube platform 2011) (Seaton et al., 2005) and in developing countries such as China 1000 µg m$^{-3}$ (Krzyzanowski and Cohen, 2008). For a 60 kg adult the dose of PM over 5 hours would be ~ 0.05 mg kg$^{-1}$ and ~ 0.1 mg kg$^{-1}$ respectively. The doses used in the current study 1, 25 and 50 µg per mouse (~ 30 g) would be 0.03, 0.8 mg kg$^{-1}$ and 1.6 mg kg$^{-1}$ respectively. One factor which influenced the decision regarding the doses to be investigated in this study was the method of administration. I.t. instillation dosing required the combustion derived NPs to be suspended in an aqueous solution and this promoted particle agglomeration. While PM is found in the air as agglomerates and single particles (EPA, 2002), the presence of large complex agglomerates in this study may have affected the deposition and final dose of these particles within
the distal airways. Therefore, higher doses of DEP and CB were administered to account for DEP loss by clearance mechanisms such as mucociliary evacuation. Additionally, another consideration with regards to the concentrations used in this study is the inability to replicate the background pollution burden of human subjects living in urban centres. The mice used for measuring platelet aggregation following *i.t.* instillation of DEP and CB are housed in individually ventilated cages and therefore would have a very low/negligible background level of combustion derived PM. Thus, administration of higher doses of DEP and CB helped to account for this. A final note concerning particle concentration is that the final systemic concentration of DEP in the current study following *i.v.* administration for a 25 g mouse was 0.05 mg kg\(^{-1}\). This dose was lower than doses used in the literature and therefore potentially more representative of human exposure (Nemmar and Inuwa, 2008, Kim et al., 2012).

In conclusion, the work in this chapter demonstrates that pulmonary exposure to a high dose of DEP can modulate platelet function *in-vivo* however this is not associated with an inflammatory response or disruption to NO levels. Furthermore, it appears that DEP-mediated effects on platelet aggregation are sensitive to endogenous platelet regulators, suggesting that healthy individuals may be protected from PM-mediated cardiovascular events. Similarly, administration of a low dose of DEP into the circulation of a mouse also enhanced agonist induced platelet aggregation. Thus, the direct effect of DEP on platelets or other haematological components following particle translocation may be a potential mechanism behind the enhanced-platelet aggregation following pulmonary exposure to DEP. Conversely, CB did not alter platelet function following *i.t.* administration but initiated pulmonary and systemic inflammation. Therefore, the effects on platelet aggregation did not appear to be a general carbonaceous particle effect and potentially involves the adsorbed compounds present on the DEP surface. Additionally, the opposing biological effects of CB and DEP may be due to the interactions that these particles have with the immune system and their ability to be effectively cleared from the lungs and circulation.

### 5.3.1. General limitations

When assessing the effects of DEP and CB on systemic and pulmonary inflammation, classic pro-inflammatory cytokines and chemokine’s were measured. However, other inflammatory mediators may have been released. Therefore the measurement of additional cytokines such as IL-8/KC may have provided more insight into the effects of DEP and CB on the inflammatory response. Additionally, due to resource constraints the effects of a mid-dose between 1 and 25 µg per mouse on platelet aggregation was not investigated. This dose would provide greater insight into a threshold dose of DEP which can affect platelet function.
Chapter Six

General Discussion
General discussion

6.1.0. Implications of this work - engineered nanoparticles

The objective of the first two chapters of this thesis was to evaluate the effects of engineered NPs on platelet function and establish whether these effects and the underlying mechanisms were dictated by NP size and surface charge. This resulted in the first publication which has investigated the effect of both size and surface charge parameters of engineered NPs on platelet function \textit{in-vitro} and \textit{in-vivo} (Smyth et al., 2014). The results of my study suggest that subtle changes in these parameters can have profound effects on platelet behaviour. This has implications for NPs which are being developed for drug delivery systems, since minor changes to the NP to improve efficacy may lead to dramatic changes in its safety profile.

My work has expanded on work by McGuinnes et al and Nemmar et al, by assessing the impact of NP size on platelet function and by measuring the effects of engineered NPs on platelet function \textit{in-vivo} (Nemmar et al., 2002b, McGuinnes et al., 2011). In addition, my work used NP concentrations which were markedly lower than ones which have been previously investigated and are therefore more representative of real exposure levels.

Furthermore, the identification of the involvement of signaling molecules in NP-induced platelet aggregation, such as COX, has implications for the development of strategies to protect against side effects associated with exposure to engineered NPs. Due to the role COX can play in the platelet aggregation caused by engineered NPs, conventional therapeutics such as aspirin may be suitable for preventing thrombosis following exposure to the engineered NPs with similar physical and chemical characteristics to the ones in the present study.

My work indicates that cationic 50 nm NPs present the greatest concern to cardiovascular health compared to the other particles used in my study, as these particles can uniquely enhance agonist–induced platelet aggregation at low concentrations both in the presence of plasma proteins and in a complex \textit{in-vivo} environment. Therefore, it is plausible that exposure to cationic NPs ~ 50 nm may exacerbate thrombotic processes involving platelet activation in patients with underlying cardiovascular conditions, such as at rupture sites on atherosclerotic plaques. Moreover, platelet aggregation induced by the cationic 50 nm NP is unresponsive to endogenous vascular platelet regulators and anti-platelet therapies such as COX inhibitors. This indicates that these NPs may also pose a thrombotic threat to the healthy population with a healthy vascular endothelium and that alternative strategies to prevent unwanted thrombotic side-effects following exposure to these NPs would have to be sought.
Chapter six – General Discussion

My study also confirmed that a positively charged NP surface could lead to disturbances in the membrane integrity of the platelet plasma membrane. This finding, that positively charged NPs can disrupt the plasma membrane, contributes to the growing body of evidence that finds cationic surface charges can promote cytotoxic processes in multiple cell types (Ruenraroengsak et al., 2011, McGuinnes et al., 2011). The cytotoxicity associated with positively charged surfaces may have implications for the development of cationic NPs for therapeutics or diagnostics. Many positively charged NPs are developed for applications such as gene therapy (Liang et al., 2012, Wang et al., 2006); however, my study suggests that the potential for adverse side effects may potentially outweigh the potential benefits.

6.1.1. Further work – engineered nanoparticles

Further work to investigate the mechanisms behind the membrane damage and cytotoxicity induced by the cationic NPs would be beneficial as this may give further mechanistic insight into how the AM NPs enhance platelet aggregation. Cationic NPs have been demonstrated to cause the formation of holes in the lipid plasma membrane of alveolar cells. Experiments using ion-conductance microscopy and platelets could shed light on whether the AM NPs in this study caused LDH release via this mechanism. ROS generation, oxidative stress and reduction in mitochondrial activity have been implicated as one mechanism behind the toxicity associated with cationic NPs (Bhattacharjee et al., 2010). Measurement of ROS in platelets can be difficult as ROS are produced during normal platelet processes (Caccese et al., 2000). Additionally, NPs themselves have been reported to interfere with some techniques used to measure ROS, for example they can oxidize ROS sensitive dyes in the absence of cells (Brown et al., 2001). However, measurement of platelet viability using a MTT assay and measurement of ROS release in-vivo by measurement of the byproducts of lipid peroxidation such as Thiobarbituric Acid Reactive Substances (TBARS) may shed more light on the role of ROS in NP-induced cytotoxicity and enhanced platelet aggregation.

Understanding the mechanism behind the internalization of the NPs into the platelets would further help in understanding how NPs affect platelet function. An initial step would be to identify whether NP internalization is through a passive or energy dependent mechanism. A basic experiment which would confirm whether NPs were internalized via active mechanisms is to image isolated platelets which have been exposed to NPs at 4°C using TEM. Moreover, there are multiple modes of endocytosis such as clathrin- and calveolin – dependent endocytosis which may be involved in the internalization of NPs. Pre-incubation of platelets with inhibitors of these pathways would provide further understanding of how NPs with different physicochemical characteristics interact with platelets.

Much of the investigation into the mechanisms behind NP-induced platelet aggregation in this thesis has involved inhibiting key signaling pathways. Many of the inhibitors employed targeted pathways
activated by multiple receptors and agonists. Activation of many platelet signaling pathways results in tyrosine kinase phosphorylation and activation of key signaling molecules (Brass, 2010). Evaluation of the phosphorylation states of many of these signaling proteins by western blotting following exposure of platelets to NPs with different physicochemistries would help distinguish the involvement of specific signaling pathways. Furthermore, NP-induced platelet aggregation measured in the presence of specific receptor inhibitors would also provide further information regarding the contribution of individual platelet receptors in NP-induced platelet aggregation.

Finally, many NPs under development or in the clinic are functionalized with various polymers, most notably the polymer PEG (Park et al., 2009). PEGylation of gold NPs has been suggested to reduce their reactivity with platelets (Santos-Martinez et al., 2014), therefore it would be interesting to investigate in future studies whether the functionalization of the engineered NPs used in the present study with PEG would reduce their effects on platelet function.

6.2.0. Implications of this work – combustion-derived nanoparticles

The aim of the final results chapter of this thesis was to establish the effect of combustion derived NPs, DEP and CB, on platelet aggregation *in-vivo* and to determine the underlying mechanisms. The work presented in chapter 5 has expanded on previous clinical and animals studies reported in the literature, by measuring the effects of exposure to DEP and CB on platelet aggregation *in-vivo*, in the presence of an intact vascular endothelium. This is important because platelets are regulated by molecules synthesized in the endothelium and therefore, by employing this model I have been able to establish the effects of DEP on platelet function under normal physiological conditions, which would be expected to be present in the healthy human population. Additionally, this model has allowed me to delineate the effect DEP and CB have on platelets *in-vivo*, as there is no direct activation of the coagulation cascade per se (Nemmar et al., 2003c, Mills et al., 2008, Mills et al., 2011). DEP caused a significant increase in agonist-induced platelet aggregation *in-vivo* however no effect was detected following exposure to CB. This enhanced platelet aggregation by DEP provides a potential mechanism behind the reported thrombotic events which are associated with acute increases in ambient PM reported in epidemiological studies (Peters et al., 2001a). Furthermore, the lack of effect on platelet aggregation by the CB also highlights the influence that the adsorbed compounds on the DEP surface have on cell function and that these compounds may underlie many of the negative health effects associated with PM exposure.

The secondary aim of the work in chapter 5 was to establish the mechanism by which DEP enhances platelet aggregation. One of the main hypotheses regarding the thrombotic events associated with PM is that induction of pulmonary and systemic inflammation by PM promotes a pro-thrombotic
environment; however the findings from the current study do not support this hypothesis as the combustion derived CB induced pulmonary and systemic inflammation, but was not associated with alterations in platelet aggregation. Furthermore, the enhanced platelet aggregation induced by DEP did not appear to involve disruption to NO blood levels or eNOS activity, which has been suggested by other authors (Langrish et al., 2013, Manzo et al., 2012).

However, although the current study cannot confirm that the combustion derived NPs can translocate the alveolar-epithelial blood barrier and enter the circulation, it does suggest that should DEP enter the blood, they can modulate platelet behaviour and potentially contribute to thrombotic events such as MI.

Moreover, the effects of DEP on platelet function appear to be sensitive to endogenous vascular regulators. Therefore, it is possible that a healthy population may be protected from the effects of DEP and ambient PM upon the cardiovascular system and those susceptible populations such as patients with cardiovascular dysfunction are at risk of PM-induced thrombotic events. This is due to the dysfunction of their vascular endothelium which leads to reduce synthesis of NO and subsequent inhibitory effects on platelets. This theory is supported by the multiple epidemiological studies which have found susceptible groups to be at increased risk from MI following acute increases in PM (Peters et al., 2001a, Schwartz, 1999).

The influence DEP has on platelet aggregation which has been observed in this thesis suggests that a reduction in exposure to diesel emissions would have considerable benefits for public health. Furthermore, the health effects associated with exposure to vehicle emissions should be taken into consideration when developing government policies on vehicle and fuel use.

6.2.1. Further work – combustion-derived nanoparticles

The movement of a small concentration of DEP from the lung into the blood may account for the enhanced platelet aggregation induced by DEP in this study. While the translocation of NPs through the lung and into the blood has been previously reported (Nemmar et al., 2001, Nemmar et al., 2002a, Choi et al., 2010) we cannot presume that the DEP administered into the mice lungs in the present work did enter the blood stream. Further work with labelled NPs, that could be identified in the circulation, would help establish whether DEP administered into the lungs of mice could enter the blood stream, and would help substantiate this theory.

The enhanced platelet aggregation induced by DEP did not appear to be associated with pulmonary or systemic inflammation however this conclusion was centered primarily on the absence of typical pro-inflammatory cytokines. Measurement of additional cytokines such as IL-8/KC may shed more light on the effects of DEP on the inflammatory response.

ROS generation has been described as one of the primary mechanisms underlying the detrimental health effects of ambient PM (Mills et al., 2009, Mutlu et al., 2006, Rhoden et al., 2005). The role of
ROS in DEP enhanced platelet aggregation could be investigated by the assessment of ROS release in-vivo. This could be achieved by the measurement of lipid peroxidation by-products, such as Thiobarbituric Acid Reactive Substances (TBARS) in the plasma of mice exposed to DEP and CB. Additionally, pre-treatment of mice with anti-oxidants such as ascorbic acid would also provide information regarding the involvement of ROS in DEP-mediated effects on platelet aggregation.

Soluble P-selectin (sP-selectin) is considered to be a biomarker for platelet activation (Chung et al., 2009) and has been reported to be associated with increased risk of MI and stroke (Ridker et al., 2001). Elevated levels have also been demonstrated to intensify the impact of experimental stroke (Kisucka et al., 2009). It is plausible that DEP may have caused enhanced platelet aggregation through elevations in blood levels of sP-selectin therefore measurement of sP-selectin using flow cytometry would confirm this. Additionally, P-selectin expression is associated with systemic inflammation; therefore it would be interesting to establish whether CB exposure was also associated with elevated blood levels of sP-selectin. This may give insight into whether exposure to CB could contribute to inflammatory conditions such as atherosclerosis and other cardiovascular conditions.

The data from the current study suggests that chemicals and compounds present on the surface of the carbon core of DEP may be responsible for the enhanced platelet aggregation, as no effects were observed for the CB which does not contain these adsorbed components (EPA, 2002). Further work investigating the effects of these adsorbed chemicals on agonist-induced platelet aggregation in-vitro and in-vivo would explore this theory and may help identify specific compounds which are responsible for the effects of DEP on platelet aggregation.
6.3.0. Final conclusions

Engineered NPs which may be introduced into the circulation through medical procedures or indirectly through exposure to commercial products may present a thrombotic risk since they can modulate platelet function. I have shown that subtle changes in individual physical or chemical parameters can have profound effects on how NPs interact with and affect platelet function. Additionally, positively charged NPs ~ 50 nm in diameter may present the greatest concern as they have a unique ability to promote platelet aggregation at low concentrations and in the presence of plasma proteins.

Exposure to DEP can enhance platelet aggregation responses and this may provide a mechanism behind the thrombotic effects which are associated with acute exposure to PM. Additionally, the mechanisms underlying this enhanced platelet response do not appear to involve the initiation of systemic inflammation or alterations in NO bioavailability. However, this study does provide evidence that the presence of a low concentration of DEP in the blood could cause the thrombotic events associated with exposure to PM.
References


References


REFERENCES


References


References


References


particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A*, 65, 1513-30.


References


References


References


References


References


systemic inflammation in rats without impairing endothelial function ex vivo or in vivo. *Part Fibre Toxicol.*, 9, 9.


References


References


References


References


Appendix
Induction and enhancement of platelet aggregation in vitro and in vivo by model polystyrene nanoparticles

Erica Smyth¹, Antonia Solomon¹, Anupama Vydyanath², Pradeep K. Luther², Simon Pitchford³, Teresa D. Tetley⁴, and Michael Emerson¹

¹Platelet Biology Group and ²Electron Tomography of Muscle Sarcomere, National Heart and Lung Institute, Imperial College London, London, UK, ³Institute of Pharmaceutical Science, King’s College London, London, UK, and ⁴Lung Cell Biology Group, Section of Pharmacology and Toxicology, National Heart and Lung Institute, Imperial College London, London, UK

Abstract

Nanoparticles (NPs) may come into contact with circulating blood elements including platelets following inhalation and translocation from the airways to the bloodstream or during proposed medical applications. Studies with model polystyrene latex nanoparticles (PLNPs) have shown that NPs are able to induce platelet aggregation in vitro suggesting a poorly defined potential mechanism of increased cardiovascular risk upon NP exposure. We aimed to provide insight into the mechanisms by which NPs may increase cardiovascular risk by determining the impact of a range of concentrations of PLNPs on platelet activation in vitro and in vivo and identifying the signaling events driving NP-induced aggregation. Model PLNPs of varying nano-size (50 and 100 nm) and surface chemistry [unmodified (uPLNP), amine-modified (aPLNP) and carboxyl-modified (cPLNP)] were therefore examined using in vitro platelet aggregometry and an established mouse model of platelet thromboembolism. Most PLNPs tested induced GPIIb/IIIa-mediated platelet aggregation with potencies that varied with both surface chemistry and nano-size. Aggregation was associated with signaling events, such as granule secretion and release of secondary agonists, indicative of conventional agonist-mediated aggregation. Platelet aggregation was associated with the physical interaction of PLNPs with the platelet membrane or internalization. 50 nm aPLNPs acted through a distinct mechanism involving the physical bridging of adjacent non-activated platelets leading to enhanced agonist-induced aggregation in vitro and in vivo. Our study suggests that should they translocate the pulmonary epithelium, or be introduced into the blood, NPs may increase the risk of platelet-driven events by inducing or enhancing platelet aggregation via mechanisms that are determined by their distinct combination of nano-size and surface chemistry.

Introduction

Engineered NPs have a wide range of applications and are manufactured for industrial use in electronics, paints, lubricants and batteries as well as in medical diagnostics and drug delivery systems (Godin et al., 2010). NPs include a wide range of inorganic (e.g. metals, metal oxides) and organic (e.g. polymer–drug conjugates and non-viral vectors for gene therapy) materials with widely varying physicochemical properties. The properties of NPs which are being exploited in the medical and industrial fields such as high surface reactivity could also lead to unwanted, and potentially harmful, biological effects. The importance of relating NP physicochemistry to biological impact has been highlighted (Oberdörster, 2010; Oberdörster et al., 2005; Shatkin et al., 2010) and studies using model PLNPs with defined and varying physicochemistry have shown that the effects of engineered NPs on cells and tissues are critically dependent on their nano-size, chemical composition and the surface functionality/reactivity of the particle (McGuinnes et al., 2011; Ruennaroengsak et al., 2012).

When inhaled, NPs travel deep into the respiratory region where they reportedly induce inflammation and oxidative stress (BeruBe et al., 2007; Donaldson et al., 2005; Oberdörster et al., 1992). Following inhalation, there is also evidence of translocation of NPs across the pulmonary epithelial barrier to the blood and internal organs (Kreyling et al., 2009). NPs could also potentially be deliberately introduced to circulating blood during proposed medical applications. Circulating blood elements, including platelets, may therefore be exposed to engineered NPs. Platelets are central mediators of thrombotic events such as myocardial infarction. Exposure of platelets to engineered carbon NPs (Radomski et al., 2005) and diesel exhaust particles (Solomon et al., 2013) has been shown to induce platelet aggregation in vitro, and delivery of combustion-derived NPs to the lungs of experimental animals enhanced thrombosis, which was associated with both airways inflammation (Nemmar et al., 2001) and direct translocation of nano-sized particles to the bloodstream (Nemmar et al., 2004).
thrombus formation in vivo with potencies that vary with physicochemistry (McGuinnes et al., 2011; Nemmar et al., 2002). Depending upon physicochemistry, platelet activation may be mediated either via expression of classical mediators of activation on the platelet surface or disruption of the platelet membrane (McGuinnes et al., 2011). In the current study, we have explored the ability of concentrations of model PLNPs lower than those previously reported to induce platelet aggregation and enhance aggregation induced by conventional agonists. The signaling events and secondary agonists driving NP-induced aggregation were defined and the relevance of in vitro studies to the whole organism explored using a mouse model of platelet thromboembolism.

Materials and methods

Materials

Materials used: collagen (equine tendon, Nycomed Pharma, Sinshelm, Germany); 111indium oxine (GE Healthcare, Amersham, UK); amine-modified polystyrene nanoparticles [Sigma–Aldrich (Poole, UK); carboxyl-modified and unmodified polystyrene latex nanoparticles (Bangs laboratories, IN); gold nanoparticles (cytodiagnostics, Ontario, Canada); epifluorotetrazolium (IntegrinTM, GSK, Middlesex, UK); LDH cytotoxicity kit+ (Roche, West Sussex, UK); 1H-5HT (Perkin Elmer UK, Cambridge, UK), bisindolylmaleimide I (BIM-I; Cambridge Biosciences, Cambridge, UK)]. Flow cytometry antibodies: PE Mouse Anti-Human CD62P (555524), FITC Mouse Anti-Human CD41a (555466), FITC Mouse IgG Isotype Control (555573) and PE Mouse IgG Isotype Control (555749) (BD Bioscience, Oxford, UK), all other reagents were purchased from Sigma–Aldrich (Poole, UK) and were of analytical grade. Tyrodes–HEPES buffer (THB) contained 134 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 0.05 mM Na2HPO4, 20 mM HEPES, 5 mM glucose and 1 mM MgCl2 (pH 7.4).

Nanoparticle preparation

Polystyrene latex nanoparticles at (100 nm diameter) and below (50 nm diameter, the upper limits of nanoscale, with a range of surface chemistries including cationic (amine-modified, aPLNP), anionic (carboxyl-modified, cPLNP) and unmodified (uPLNP) were used; gold NPs were used as a relatively inert nanoparticulate control of similar aerodynamic diameter to test NPs (60 nm). NPs were diluted in THB (in vitro assays) or sterile saline (0.9% w/v NaCl; in vivo experiments). To reduce agglomeration, particles were sonicated for 1 min (Clifton Ultrasonic Bath, Clifton, NJ) and then vortexed for 1 min prior to dilution and just before use.

Preparation of washed human platelets

Washed platelet suspensions were prepared from blood, collected in citrate, from consenting healthy aspirin-free, male and female human donors (23–55 years) as previously described (Jones et al., 2010). Platelet preparations were maintained in a resting state by addition of citrate and PGE1. Informed consent was obtained from all donors and the procedures were approved by the NHS National Research Ethics Service.

Transmission electron microscopy of nanoparticles

A drop of NPs suspended in experimental buffer was placed on formvar-coated nickel grids for 10 min. Excess fluid was removed with a filter paper and the grid was further air dried for 10 min. Samples were visualized after heavy metal staining with 2% uranyl acetate. A JEOL 1200 EX electron microscope (JEOL (UK) Ltd, Tokyo, Japan) operated at 100 kV was used to examine the grids. Images were recorded with a Tietz Fastscan CCD digital camera (Gauting, Germany) and Tietz EMMenu 4.0 software (Gauting, Germany) was used to capture and display images.

Light transmission aggregometry

Platelets were stimulated with either NPs, platelet agonists or THB and aggregation was measured for 3 min immediately following the addition of stimuli at 37 °C under stirring conditions (1200 rpm) in an optical aggregometer (Chrono-log Corporation, Haventown, PA) (Born, 1962). In some experiments, NPs were added to platelet suspensions immediately prior to stimulation with thrombin (0.06 U ml−1) or ADP (1 μM). Aggregation studies were conducted in THB containing 1 mM CaCl2. NPs themselves did not affect baseline light transmission so that resetting of baselines was not required following their addition.

LDH assay

Membrane disruption was assessed by measuring lactate dehydrogenase (LDH) release from washed platelets. Supernatant from platelets exposed to NPs, thrombin or THB for 3 min or 2 h (to assess cytotoxicity associated with platelet aggregation assays and more prolonged exposure, respectively) was incubated at 37 °C with a commercial reaction mixture for 30 min at 20 °C in a 96-multi-well plate. LDH activity was assessed through formazan dye formation where absorbance was read at 490 nm. Data were expressed as a % of positive controls (lysed platelets). Appropriate NP controls were run to establish interference with the assay, none was detected.

Dense granule secretion

Platelet-rich plasma (PRP) was incubated with 1 μCi ml−1 [3H]-5HT and platelets isolated as previously described (Jones et al., 2010) before re-suspension in THB containing 1 μM imipramine to a density of ~4 × 108 cells ml−1. Platelets were stimulated with NPs and/or thrombin for 3 min under stirring conditions. [3H]-5HT release was measured using a WALLAC® scintillation analyser (Perkin Elmer, Waltham, MA). [3H]-5HT release was expressed as decays per min.

Flow cytometry

Isolated platelets were incubated with NPs (0.2–60 μg ml−1), thrombin (1 μl ml−1) or vehicle control for 10 min and read on an Epics XL flow cytometer (Beckman Coulter High Wycombe, UK). Platelets were positively identified via the CD41-FITC antigen and the surface expression of P-selectin was quantified using an antibody against CD62P-PE. NP interference with the assay was assessed by measuring light scattering in the absence of cells. No interference or autofluorescence was detected.

Transmission electron microscopy of platelets

Washed platelets were exposed to NPs or THB for 10 min then fixed in 3% glutaraldehyde (1 h), followed by incubation with 1% osmium tetroxide (1 h) and further serial dehydration using acetonemostillized water. Samples were infiltrated with acetone/ araldite overnight and then placed into araldite-containing capsules and left to harden over 2 days. Thin ~100 nm sections were cut, stained with 2% uranyl acetate followed by Reynolds lead citrate and visualized as described above.

Animals

Male C57BL/6 mice (Harlan, Bicester, UK) between 20 and 25 g were used. Food and water were available ad libitum.
Animal procedures were performed in accordance with our Home Office licence and with authorization from the Imperial College London Ethical Review Panel. Protocols were refined in association with the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (Tymvios et al., 2008) and are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010).

**In vivo platelet aggregation**

Citrated blood was collected via cardiac puncture from mice anesthetized with urethane (2 g kg⁻¹ i.p.). Platelets were isolated as previously described (Tymvios et al., 2008) and incubated with 1.8 MBq ¹¹¹indium oxine for 10 min. A previously established mouse model of *in vivo* platelet aggregation was employed. Anesthetized mice (1.5 g kg⁻¹ urethane i.p.) were intravenously (i.v.) infused (200 µl in 5 s) via an exposed femoral vein with radiolabeled platelets prior to an i.v. bolus of NPs or vehicle control, which was followed by a bolus infusion of collagen. Entrapment of platelet aggregates in the pulmonary vasculature following aggregation in the systemic circulation was quantified by recording changes in indium counts with a Single Point Extended Area Ratio (SPEAR) probe (eV products, Saxonburg, PA) positioned over the pulmonary vasculature and custom software (Mumed systems, London, UK) as previously described (Tymvios et al., 2008). It has been previously characterized that increases in platelet counts in the pulmonary vasculature correspond to platelet aggregation which is independent of vascular tone (Moore et al., 2010). The model allows direct measurement of platelet activity in contrast to conventional vascular injury models which measure the broader process of thrombus formation (Tymvios et al., 2008).

**Data and statistical analysis**

Unless otherwise stated, data are presented as box-and-whisker plots, the horizontal lines inside the boxes represent the median, the box edges extends from the 25th to 75th percentiles and the whiskers represent the minimum and maximum values. All statistical analyses were performed on raw data using Prism 5 Graphpad software (GraphPad Software Inc., La Jolla, CA). Paired comparisons between two groups were performed using a Wilcoxon signed rank test and between multiple groups using a Friedman test with Dunn’s comparison. All experiments involved time-matched controls. Comparisons between two independent data sets were made using a Mann–Whitney test and multiple comparisons were conducted using a Kruskal–Wallis one-way analysis of variance test with Dunn’s comparison. Summary data (EC₅₀ values) were determined by regression analysis (non-linear curve-fit). Power calculations were conducted to determine appropriate n numbers. A *p* value <0.05 was indicative of statistical significance.

**Results**

**Visualization of nanoparticle formations**

Transmission electron microscopy revealed NPs to be present in experimental buffers as a mixture of individual particles, chains of particles and agglomerates (Figure 1). Particle diameters were confirmed as 50 and 100 nm. Surface modification led to changes in the extent and nature of agglomeration so that aPLNPs and cPLNPs were more likely to occur as individual particles as well as simple chains of two to three particles and simple agglomerates of three to five particles (Figure 1A, B, D and E). In contrast, uPLNPs were predominantly present as more complex, overlapping (indicated by increased density) agglomerates although...
individual particles and simple chains were also detected (Figure 1C and F). Figure 1 shows typical electron micrographs for 100 (A–C) and 50 nm (D–F) PLNPs with arrows to indicate the different particle formations (individual particles, chains and agglomerates). We have previously reported the surface charge densities (zeta potentials) of the PNLPS used in this study (Ruenraroengsak et al., 2012).

Nanoparticles induce platelet aggregation

All PLNPs induced apparent concentration-dependent (15–60 μg ml$^{-1}$) platelet aggregation indicated by increases in light transmission in isolated platelet suspensions (Figure 2A and B). Gold NPs of similar aerodynamic size (50 nm) did not induce aggregation (data not shown). The potencies of the various PLNPs, defined by their EC$_{50}$ values, did not induce aggregation (data not shown). The potencies of the various PLNPs, defined by their EC$_{50}$ values, were ranked in order of potency which varied according to their physicochemical properties (Figure 2A and B). Of the 50 nm PLNPs tested, cPLNPs were the most potent, and uPLNPs the least (Figure 2A). In contrast, comparison of 100 nm PLNPs showed uPLNPs to be the most potent whereas cPLNPs were the least (Figure 2B).

A subthreshold concentration (2 μg ml$^{-1}$) of 50 nm aPLNPs significantly ($p<0.05$) increased the response to an EC$_{50}$ concentration of thrombin (0.06 U ml$^{-1}$) and this effect was abolished by the GPIIb/IIIa antagonist eptifibatide (Figure 2C). None of the other PLNPs had a significant ($p>0.05$) effect on thrombin-induced platelet aggregation (Figure 2C). Similar data were obtained in the presence of plasma proteins where the aggregation response to ADP (1 μM) in platelet-rich plasma (PRP) was uniquely enhanced by 50 nm aPLNPs and this effect abolished by eptifibatide (Figure 2D).

Nanoparticle-induced aggregation is not associated with loss of membrane integrity

Very low, but significant ($p<0.05$ compared to THB control) and concentration-dependent LDH release was detected in supernatants following 3 min of PLNP-induced platelet aggregation. However, the levels detected following exposure to excess concentrations (125 μg ml$^{-1}$) of all 50 nm (Figure 3A) and 100 nm (Figure 3B) PLNPs were not significantly different ($p>0.05$) from those occurring following exposure to 1 U ml$^{-1}$ thrombin.

We also measured LDH release following a longer exposure (2 h) and found a concentration-dependent (15–125 μg ml$^{-1}$) release in the presence of 50 nm (Figure 3C) and 100 nm (Figure 3D) aPLNPs that was significantly greater than a 2-h exposure to thrombin. cPLNPs and uPLNPs had no significant effect under these conditions irrespective of particle size (Figure 3C and D).

Nanoparticle-induced aggregation is driven by varying signaling events depending upon particle physicochemistry

The ability of NPs to induce concentration-dependent, PKC-mediated dense granule secretion, measured as $^3$H-5HT secretion, corresponded with their ability to induce aggregation so that greatest secretion was observed with 50 nm cPLNPs and...
Figure 3. Effect of nanoparticles on lactate dehydrogenase release by isolated platelets. Isolated human platelets were stimulated for 3 min with (A) 50 nm or (B) 100 nm PLNPs (125 mg ml⁻¹) or for 2 h with (C) 50 nm and (D) 100 nm PLNPs (15–60 µg ml⁻¹). Gold NP (125 µg ml⁻¹), or thrombin (Thr, 1 U ml⁻¹) controls were included. Membrane integrity was assessed by measuring lactate dehydrogenase (LDH) release from cell free supernatant. LDH release is expressed as a percentage of lysed controls (Con). Data are represented as box-and-whisker plots, n = 4 for all experiments. *p < 0.05, **p < 0.01 compared to vehicle control using a Friedman test with Dunn’s comparison.

Figure 4. Effect of nanoparticles on dense granule release and P-selectin expression in isolated platelets. Dense granule secretion was assessed as [³H]-5HT release by isolated platelets following exposure to (A) 50 nm or (B) 100 nm PLNPs (15–125 µg ml⁻¹), gold or thrombin (Thr, 1 U ml⁻¹) for 3 min. No significant (p > 0.05) 5-HT release was detected for maximal concentrations of NPs and thrombin following incubation with the PKC antagonist BIM-I (10 µM). Flow cytometry was used to measure the percentage of platelets expressing P-selectin following exposure to 50 nm (C) and 100 nm (D) PLNPs (15–60 µg ml⁻¹), thrombin (1 U ml⁻¹) or gold. Data are represented as box-and-whisker plots, n = 4 for all experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared to vehicle control using a Friedman test with Dunn’s comparison (A and B) and Kruskal–Wallis test with Dunn’s multiple comparison test (C and D).

100 nm uPLNPs (Figure 4A and B). Dense granule secretion was inhibited by the PKC antagonist BIM-I and was not detected in the presence of 50 nm aPLNPs, even at supramaximal concentrations (Figure 4A and B). Surface P-selectin expression mirrored dense granule secretion and aggregometry data in terms of % platelets expressing P-selectin (Figure 4C and D) and mean fluorescence intensity (data not shown). Once again, 50 nm aPLNPs and gold NPs had no effect.

With the exception of 50 nm aPLNPs, the GPIIb/IIIa antagonist eptifibatide significantly inhibited PLNP-induced aggregation indicating a GPIIb/IIIa-mediated aggregation response similar to that occurring with a conventional platelet agonist such as thrombin (Figure 5A). In the case of 50 nm aPLNPs, the lack of an effect of eptifibatide indicated a fall in light transmission independent of GPIIb/IIIa (Figure 5A). Similarly, all PLNP-mediated aggregation was driven by extracellular Ca²⁺.
Figure 5. Role of signaling events in mediating nanoparticle-induced platelet aggregation. Maximal platelet aggregation responses of PLNPs (60 μg ml⁻¹) or thrombin (1 U ml⁻¹) in the presence of (A) the GPIIb/IIIa antagonist epifibatide (EPI, 0.06 μg ml⁻¹), (B) the ADP scavenger apyrase (8 U ml⁻¹), (C) the cyclooxygenase antagonist indomethacin (10 nM), (D) Ca²⁺ free media and EGTA (2 mM) or (E) the protein kinase C antagonist BIM-I (10 μM). Data are represented as box-and-whisker plots, n = 6 for all experiments. *p < 0.05 compared to time matched controls using a Wilcoxon signed rank test, ns = non-significant (p > 0.05).

(Figure 5D) and PKC (Figure 5E) with the exception of 50 nm aPLNPs which induced apparent platelet aggregation independent of these signaling events.

Major differences were observed with regards to the role of secondary agonists. 50 nm cPLNP and 100 nm aPLNP-induced aggregation was impaired following inhibition of ADP activity by apyrase (Figure 5B) and inhibition of thromboxane A₂ generation from cyclooxygenase by indomethacin (Figure 5C). In contrast, 100 nm cPLNP-induced platelet aggregation was not significantly affected by these reagents. Similar signaling events drove aggregation induced by 50 and 100 nm uPLNPs since both involved ADP but not thromboxane A₂ (Figure 5B and C). It was notable that 50 nm aPLNP-induced aggregation was not dependent upon any of the signaling processes tested but that removal of extracellular Ca²⁺ led to a significant increase in light transmission, in contrast to all other PLNPs where light transmission was reduced to varying extents (Figure 5D).

Mechanism of physical interaction of nanoparticles with platelets

Transmission electron microscopy revealed 50 nm cPLNPs (Figure 6B) and uPLNPs (Figure 6C) in close proximity to or in physical contact with the extracellular surface of the platelet membrane. NPs were present as individual particles or short chains. The more complex formations present in isolated uPLNP suspensions (Figure 1C and F) were not observed in contact with platelets. Platelets in proximity or in physical contact with 50 nm cPLNPs and uPLNPs appeared to be more degranulated and activated, shown by the loss of intracellular granules and loss of the discoid shape of non-activated platelets (Figure 6A shows control resting platelet with intact intracellular organelles; Figure 6B and C show activated, degranulated platelets in physical contact with cPLNPs and uPLNPs, respectively). In contrast, platelets in contact with 50 nm aPLNPs contained intact intracellular organelles and appeared in a resting conformation (Figure 6D). Discoid platelets were frequently adhered to adjacent platelets by chains of 50 nm aPLNPs (Figure 6E). In addition, 50 nm aPLNPs were found both in physical contact with the extracellular surface of platelet membranes (arrows, Figure 6D and E) and internalized within tightly fitting intracellular vacuoles (arrowheads, Figure 6D). Such internalization was not apparent with 50 nm cPLNPs and 100 nm uPLNPs (Figure 6B and H).

Investigations with 100 nm PLNPs showed similar associations with the platelet membrane (arrows, Figure 6G and H) as well as internalization within platelet vacuoles (arrowheads, Figure 6F and G). Linkage of non-activated platelets by chains of 100 nm PLNPs was not observed and therefore occurred uniquely with 50 nm aPLNPs.

In vivo platelet aggregation

Infusion of relatively low concentrations (1.2 μg/mouse) of 50 nm aPLNPs or uPLNPs into mice did not induce measurable platelet aggregation per se (Figure 7A). However, subsequent administration of collagen (50 μg kg⁻¹) induced platelet aggregation that was significantly greater in terms of amplitude of response (Figure 7B) in the presence of aPLNPs compared with saline pre-treated collagen controls. In contrast, the same dose of uPLNPs had no effect on subsequent collagen-induced platelet aggregation in vivo (Figure 7B).
Increased use of NPs inevitably leads to increased human exposure so that evaluation of their functional and toxicological impact becomes essential. Cationic NPs are currently being developed for drug delivery systems, targeting tumors and facilitating gene therapy (Wang et al., 2006) due to their efficacious uptake by, and interaction with, cells. Similarly, anionic and neutral NPs have potential clinical applications in enhancing drug circulation times (Gabizon et al., 1994). Many NP applications involve their introduction to the systemic circulation leading to interactions with blood components, including platelets. This inescapably necessitates comprehensive investigations of NP–platelet interactions.

We found NPs in our study to be present in the form of individual particles as well as agglomerates which, to some extent, resembles the mixed effective densities of NPs in ambient air (Park et al., 2003) and correlates with their reported zeta potentials predicting the presence of repulsive forces to encourage the presence of individual particles (Ruenraroengsak et al., 2012). Relatively high concentrations [260 μg ml⁻¹ (McGuinnes et al., 2011) and 50–100 μg ml⁻¹ (Nemmar et al., 2002)] of 50 nm surface-modified PLNPs have previously been shown to induce platelet activation. In contrast, equivalent concentration of uPLNPs did not induce aggregation (McGuinnes et al., 2011; Nemmar et al., 2002). We now show that all of the 50 nm PLNPs tested, as well as larger 100 nm PLNPs, are able to induce platelet aggregation if applied in appropriate amounts (15–60 μg ml⁻¹).

Application of lower concentrations of NPs in our study revealed that NP reactivity at lower concentrations is not determined by size or surface modification alone but by a combination of these factors. Thus, the ranking of potency between NPs of differing surface chemistry varies with nano-size. This observation can be explained by a recent study demonstrating that NPs of identical composition and surface charge but differing size can exert different, even opposing, biological behaviors (Dwivedi et al., 2014). These behaviors result from changes in the mechanism of interaction or disruption between NPs and biological membranes.

Figure 6. Electron micrographs of isolated human platelets following exposure to nanoparticles. Stained sections displaying varying magnifications of platelets exposed to 50 μg ml⁻¹ of PLNPs. (A) shows a control platelet in resting conformation. 50 nm cPLNPs (B) and uPLNPs (C) are seen in close proximity and in contact with (arrows) the extracellular surface of activated, degranulated platelets. Non-activated, discoid platelets with 50 nm aPLNPs in contact with the platelet surface (arrows, D) and enclosed within intracellular vacuoles (arrowheads, D). Platelets are also shown tethered to each other by chains of aPLNPs (E). 100 nm aPLNPs and cPLNPs enclosed within intracellular vacuoles (arrowheads, F and G) and in association with the extracellular surface of the platelet membrane (arrows, G and H).

Figure 7. Effect of nanoparticles on collagen-induced platelet aggregation in vivo. Mouse platelets radiolabeled with ¹¹¹Indium oxine were infused into an anaesthetized recipient mouse prior to injection with 50 nm aPLNPs, 50 nm uPLNPs (1.2 μg/mouse i.v.) or saline (0.9% NaCl) followed by collagen (50 μg kg⁻¹ i.v.). Platelet aggregation was measured by changes in platelet-associated radioactivity. Data are presented as (A) representative traces of percentage change in ¹¹¹Indium counts over time and (B) box-and-whisker plots of maximum percentage increase in ¹¹¹Indium counts in response to collagen (50 μg kg⁻¹ i.v.). n = 5, *p < 0.05 using a Mann–Whitney test.
or surfaces (Dwivedi et al., 2014). In addition, the larger surface area of equivalent masses of smaller NPs will lead to greater adsorption of surface molecules which can also determine their mechanism of interaction with cell membranes (Fissan et al., 2012). In platelets, different mechanisms of interaction with the surface membrane would be expected to define the interaction of NPs with platelet surface receptors and intracellular signaling molecules, and ultimately impact the potency and mechanism by which NPs induce platelet activation and aggregation.

It was also noted that the state of agglomeration of particles did not appear to be an over-riding factor in determining reactivity since both predominantly agglomerated (e.g. 100 nm uPLNPs) and predominantly individualized (50 nm cPLNPs) particles had relatively high potencies. Both of these NPs were observed in close proximity to the plasma membrane and, in contrast to other NPs investigated in our study, there was no indication of internalization (although this cannot be entirely ruled out). We speculate that the negative and neutral charges prevented internalization but may have promoted size-specific interactions with cell surface receptors leading to initiation of intracellular signaling events such as platelet granule release and activation.

Membrane disruption by engineered NPs could potentially provide a triggering mechanism in platelets by eliciting release of granules and internal stimuli (McGuinnes et al., 2011; Ruenraaroengsak et al., 2012). We found that in the time frame of aggregation assays, LDH release was not greater than that occurring following an exposure to thrombin. Therefore, NP-induced aggregation occurs concurrently with cell signaling events associated with conventional aggregation (degranulation and P-selectin expression) rather than a causative cytotoxic response. Since inhalation of NPs by humans could involve longer exposure periods, we also measured LDH release following a more prolonged exposure. uPLNPs caused significantly more LDH release than that observed following a 2-h exposure to thrombin, indicating membrane damage and potential release of intracellular stimuli. Erythrocyte membrane disruption upon exposure to high concentrations of 50 nm aPLNPs has previously been described (McGuinnes et al., 2011) and is now shown to occur in platelets upon exposure to the lower concentrations used in the current study.

Changes in light transmission, upon exposure of platelets to NPs cannot be assumed to be driven by conventional GPIIb/IIIa-mediated aggregation since passive or non-specific processes such as agglomeration of particles with cells could also affect functional assays. Studies with the GPIIb/IIIa antagonist eptifibatide showed that all the NPs tested, excluding 50 nm aPLNPs, induced GPIIb/IIIa-mediated aggregation which was also dependent on PKC signaling and Ca\textsuperscript{2+} entry. These are considered to be final common pathways of platelet activation. Further mechanistic analyses demonstrated the differential involvement of the secondary agonists ADP and thromboxane A\textsubscript{2}. The signaling events driving NP-induced activation therefore vary between NPs of differing physicochemistry and from those induced by conventional platelet stimuli but merge at common final activation pathways. Since anti-thrombotics target secondary signaling events, strategies for reducing the impact of NPs on platelets will vary with physicochemistry and may differ from those used to combat conventional platelet-driven disorders.

In the case of 50 nm aPLNPs, the recorded fall in light transmission occurred independently of GPIIb/IIIa, cyclooxygenase, ADP and PKC suggesting a process other than conventional aggregation such as non-specific platelet agglomeration. 50 nm aPLNP-induced light transmission was also shown to be enhanced in the absence of extracellular Ca\textsuperscript{2+}. This supports our proposal of an agglomeration rather than a signaling process since high ionic content of solutes has been shown to promote agglomeration of NPs by screening the repulsive forces between them (Pavlin & Bregar, 2012). Reduced ionic content in the presence of EGTA may lead to agglomeration of NPs and increase surface area leading to the enhanced response observed. In addition, electron microscopy suggested that aPLNPs cause agglomeration of platelets by physically bridging platelets to form "aggregates" of non-activated platelets. This contrasts with images of other NPs used in our study which show a more conventional activation process involving degranulation associated with GPIIb/IIIa activation, surface P-selectin expression and other signaling events.

Recent evidence from other cell types shows that NPs can activate a range of molecular targets including protein kinases and redox-sensitive transcription factors (Marano et al., 2011). NPs are also reported to cause aggregation of membrane receptors and subsequent modulation of signaling (Huang et al., 2009). Similar processes may occur in platelets following the observed physical association of PLNPs with the external surface of the platelet membrane. Additional processes may arise from the observed internalization of aPLNP within platelet vacuoles. Further study of these mechanisms is warranted.

aPLNPs of 50 nm diameter enhanced agonist-mediated, GPIIb/IIIa-driven platelet aggregation at a concentration of 2 μg ml\textsuperscript{-1} in our study, which is in accordance with their previously reported ability to enhance ADP-induced aggregation at higher concentrations [12.5–100 μg ml\textsuperscript{-1} (Nemmar et al., 2002)]. This effect was evident, not only in isolated platelet preparations but also in the presence of plasma proteins (Figure 2D; Nemmar et al., 2002). Our images indicating linkage of platelets by chains of aPLNPs may provide a partial explanation for this effect since presumably physical tethering of platelets in close physical proximity to one another will facilitate their subsequent aggregation via well described contact activation processes. Additionally, the internalization of these particles or NP-induced membrane perturbation may be associated with the induction of signaling processes to prime platelets for subsequent activation. Our data suggest that exposure to NPs with the same or similar physicochemistry to 50 nm aPLNPs could propagate conventional pathophysiological triggers of platelet activation at relatively low concentrations.

Data acquired with isolated platelets cannot be assumed to reflect a physiological setting. It has been reported previously that PLNPs can both enhance and inhibit experimental thrombosis \textit{in vivo} (Nemmar et al., 2002). These effects may involve direct platelet activation but this is difficult to delineate in multi-factorial models involving vascular injury. We therefore conducted \textit{in vivo} experiments using a mouse model which directly measures platelet aggregation and introduced 50 nm aPLNPs, to circulating blood by intravenous infusion. We showed enhanced platelet aggregation within the vasculature \textit{in vivo} in the presence of sub-threshold concentrations of NPs suggesting an increased thrombotic risk \textit{in vivo} that is platelet-driven. NPs which were unable to enhance aggregation \textit{in vitro} (e.g. 50 nm uPLNPs) were similarly unable to enhance aggregation \textit{in vivo}, strengthening the validity of both the present, and previously published (McGuinnes et al., 2011; Nemmar et al., 2002), \textit{in vitro} data. There remains uncertainty over the quantities of NPs which translocate from the lung to the circulation, however insight into the potential levels of exposure to nanomedicines during medical procedures means that we can place the concentrations used in our study into a relevant context. For example, pharmacokinetic data on the nanoparticle-late cancer therapy DOXL\texttextsuperscript{®}, which is liposome-encapsulated doxorubicin (∼80–100 nm), demonstrates plasma concentrations >10 μg ml\textsuperscript{-1} (Gabizon et al., 1994) considerably higher than that used in the current \textit{in vivo} study (∼1 μg ml\textsuperscript{-1} assuming a circulating mouse blood volume of 1–1.5 ml).
Conclusion

In summary, the potencies and mechanisms by which NPs initiate platelet activation and aggregation are dependent upon a combination of their aerodynamic size, surface chemistry and the concentrations at which they are applied. When assessing the impact of engineered NPs upon human health, careful consideration should be given to their distinct physicochemical properties. NPs of similar size, surface chemistry and states of agglomeration should not be assumed to behave in a similar manner to NPs which differ in one or more of these characteristics. Furthermore, NPs that do not induce platelet aggregation per se may still act in other ways and at relatively low concentrations to enhance agonist-mediated platelet aggregation in vivo and so may be expected to increase the risk of platelet-driven thrombotic events, should they translocate the respiratory epithelium or otherwise be introduced to the blood environment.

Declaration of interest

The authors declare that they have no competing interests. This work was supported by a Project Grant from the British Heart Foundation (PG/10/80/28605).

References


